Niger. J. Physiol. Sci. 39 (June 2024): 1 – 8
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

The Role of Artificial Intelligence in Health Care

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Summary: Artificial intelligence (AI) plays a leading role in transmuting the field of healthcare. Numerous aspects of AI have been incorporated into the healthcare delivery system. For instance, in disease diagnosis, the practice of personalised treatment plans and precision medicine are AI-dependent. This review gives a widespread role of AI in healthcare, with a focus on applications, and challenges. Deep brain stimulation, statistical analysis, machine learning, and deep learning are a few examples of AI-powered technologies that have contributed immensely to biomedical research and medical imaging advancement. Moreover, AI algorithms are pivotal in genomics research, easing the identification of genetic markers related to disease vulnerability and treatment reaction, thereby aiding the practice of precision medicine. Apart from diagnosis and treatment strategies, AI assists in healthcare management and resource optimization, along with the discovery and therapy of drugs. Forecasting of disease outbreaks, effective allocation of hospital resources, and management of patient traffic rely mostly on predictive analytics driven by AI. Again, AI-powered virtual health assistance, telemedicine has aided patient appointments and support, giving real-time support and health recommendations. Although AI algorithms provide outstanding breakthroughs in healthcare, AI adoption is cumbered by numerous dares such as monetary concerns, regulatory hurdles, data privacy fears, and ethical considerations associated with AI applications, such as algorithm bias and transparency. Futuristically, AI application in healthcare holds vast potential, such as early disease detection, drug discovery, and optimization of treatment. Concerted efforts targeted at tackling the prevailing challenges and creating holistic control would be important to tie together the full potential of AI in rejuvenating the healthcare delivery system.

Keywords*: Artificial Intelligence, Healthcare, Personalized medicine, Precision medicine, Disease Diagnosis, Treatment Plans*

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Manuscript received- April 2024; Accepted- May 2024

DOI: https://doi.org/10.54548/njps.v39i1.1

INTRODUCTION

According to the Merriam-Webster dictionary, healthcare refers to “efforts made to maintain, restore, or promote someone's physical, mental, or emotional well-being especially when performed by trained and licensed professionals” (Merriam-Webster, 2023). Healthcare involves making sure that we are kept running as a singular being, with each organ and system working the way they are expected to because if there is a problem with one, there is a problem with all. Due to how broad the human body is, healthcare can be divided into various sectors, each focusing on specific areas. Such include pharma/biotech, health tech/medical devices, providers (doctors, nurses, physical therapists), and so on (Ridley, 2022). Each sector and others make sure that an individual is provided the best care possible but as time goes on, innovations have to be made and we have to conform to bigger changes. That is where Artificial Intelligence comes in.

Artificial intelligence (AI), which can also be known as machine intelligence, refers to a computer system’s ability to learn from given data and convert it (all on its own) into comprehensible information (Laskowski and Tucci, 2022). The term AI is often used to describe a computer that mimics cognitive processes that occur in the brains of humans throughout problem-solving and learning (Lund *et al*., 2020). It explains that AI has the ability or the potential to act in the way a human would when trying to complete basic tasks, calculate mathematical formulations, and even provide new information not known to man. Current use of this is in the healthcare delivery system, as well as, the discovery and design of drugs (Harrer *et al*., 2019). An excellent example of this is the computational modelling based on AI and machine learning (ML) principles and it can be a good tool for identifying and validating chemical compounds, identifying targets, synthesizing peptides, evaluating drug toxicity and physiochemical properties, monitoring drug efficacy and effectiveness, and drug repositioning (Zhong *et al*., 2018).

With the introduction of AI concepts, ML, and deep learning (DL) algorithms, virtual screening (VS) of compounds from chemical libraries containing over 106 million compounds has become simple and time-saving. Furthermore, AI models reduce toxicity issues that come from off-target interactions (Gupta *et al*., 2021).

Narrow Artificial Intelligence

A type of AI, known as artificial narrow intelligence (ANI) has been known to aim to improve the performance of a specific job, be it following weather updates, creating data science reports, or even planning games like poker or chess, its main function or ability is specific to a particle task, hence the name. As would be expected, it lacks the true ability to "think" like a human in terms of full consciousness and an awareness of oneself and various emotions attached to the decisions we make in our everyday lives. It cannot make a choice that involves placing one's emotions and feelings over logical reasoning and this could be counted as one of its flaws (Spiceworks, 2022). They might come off as complex and intelligent, but they are lacking in the core presets and characters that make human intelligence superior to any other kind. Examples include Google Translate, Google Assistant, Siri, and other natural language processing software. While these tools can communicate with us and process and grasp the language we use, they are classified as weak AI because they lack the fluidity and flexibility required to think for themselves like humans do. In other words, this AI system can't function on its own. The benefits of this form of AI include faster decision-making because they process information as well as complete tasks much faster than humans, relieving humans of many tedious, routine, and mundane tasks, and serving as a basis for the eventual creation of further intelligent AI variations such as general AI as well as super AI (Spiceworks, 2022).

Artificial General Intelligence

A more complex form of AI is artificial general intelligence (AGI), which, unlike ANI, enables a computer to perceive, learn, and execute intellectual activities similar to humans (McLean *et al*., 2021). This makes it more advanced in the sense that it can allow machines to simulate the way humans think and act and can also tackle any type of complicated issue thrown at it. These machines are hypothesized to function identically to humans since they are meant to have complete understanding as well as cognitive computing skills (Fjelland, 2020).

AGI is built upon the theory of mind AI paradigm and the hypothesis of this mind-level AI is concerned with teaching robots how to understand and comprehend human behavior and the fundamental features of consciousness. With such a solid AI basis, AGI can plan, make judgments, have cognitive abilities, deal with uncertain situations, and use past information in decision-making to increase accuracy. AGI enables robots to do novel and inventive tasks (Kanade, 2022a).

As of 2024, the AGI system is merely a product of science fiction as no form of this system exists but we believe that if they were ever to be created, they could function almost identically to humans, or even better than it would have a larger capacity to acquire and analyse massive data sets. Its capabilities could span from innovation, and perception of stimuli, all the way to natural language understanding and even mobility. Researchers also have predicted that AGI systems would have a higher level in terms of capabilities that involve the ability to handle multiple kinds of learning as well as algorithms, create rigid frameworks for all tasks, understand symbol systems, use different types of knowledge, understand belief systems, engage in metacognition, and apply metacognitive knowledge (Lutkevich, 2022).

Artificial Super Intelligence

Another hypothetical AI is known as the artificial super intelligence (ASI) which can potentially outperform even the capabilities of AGI by demonstrating cognitive capabilities and developing its thinking abilities. Often known as super AI, it is regarded as the most sophisticated, powerful, and intelligent sort of AI, surpassing the intellect of some of the world's finest brains (Kanade, 2022b).

These machines are supposedly self-aware and are capable of abstracting and interpreting concepts that humans cannot. What makes them distinct is the fact that they could perceive and comprehend human experiences and feelings which cannot be said of the two other types. Depending on the AI's cognitive capabilities, it can develop its sense of emotions, beliefs, and wants. Due to these unique skills, it could potentially have applications in almost all areas of human interest and can execute any things that humans can. They have been projected to make more precise decisions and solve problems than humans (Kanade, 2022b). At present, superintelligence is a hypothetical potential rather than a practical reality, as most computer science research is focused on ANI but it is sure that the world would definitively benefit from the implementation of such a powerful AI technology (Vishaal, 2023).

Deep Learning Models

Deep learning models (DLMs) employ what is known as artificial neural networks (ANN) to conduct complex computations on massive volumes of data. The reason they do this is that ANNs can imitate how the brain computes information. This sort of machine learning greatly relies on the functionality and operation of the human brain and its algorithms instruct computers to gain from examples fed into them. This makes DLMs a perfect fit for sectors such as healthcare, e-commerce, entertainment, and advertising. During training, algorithms extract features, organize objects, and uncover relevant data patterns by utilizing unknown components in the input distribution (Biswal, 2024). Deep Learning methods include convolutional neural networks (CNNs), recurrent neural networks (RNNs), generative adversarial networks (GANs), multilayer perceptrons (MLPs), deep belief networks (DBNs) and can deal with nearly any type of data and require a lot of processing power and knowledge to tackle complex problems (Mohapatra, 2022).

Applications of AI in healthcare

As mentioned previously, certain aspects of AI play a leading role in transforming the field of healthcare and its delivery system. Its models and algorithms help in areas such as disease diagnosis, personalised treatment places, health and genetics research (Figure 1), and much more as will be discussed (Davenport and Kalakota, 2019).

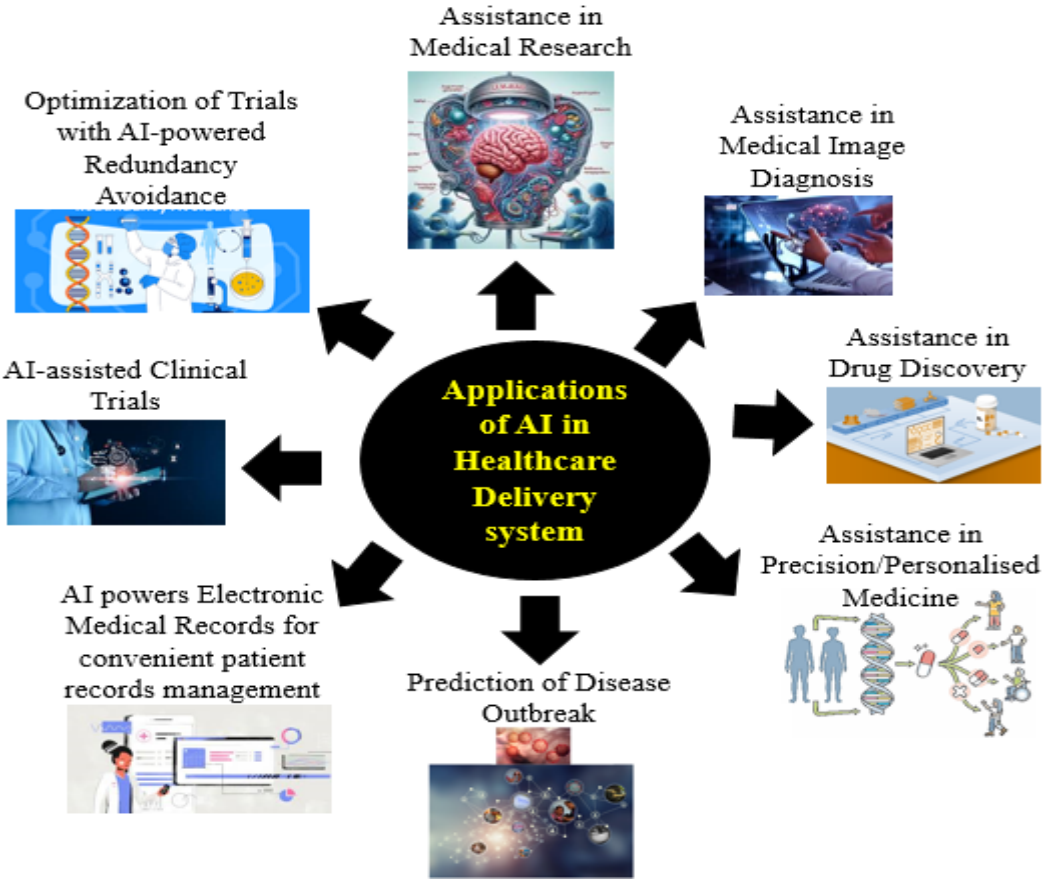

Figure 1:
Applications of Artificial Intelligence in Healthcare Delivery System (Adapted from Pandya *et al.*, 2021)

Diagnosis and Early Detection

Concerning the aspects of diagnosis in healthcare, AI has succeeded tremendously in breaking barriers regarding the screening and treatment strategies of various illnesses and disorders plaguing mankind (Davenport and Kalakota, 2019). Researchers have been able to implement AI models that can improve the prognosis of conditions such as cancer as shown by Mitsala *et al*., (2021). Their research focused specifically on colorectal cancer and how the assistance of AI in the detection of colorectal polyps and optical diagnosis in colonoscopy can help doctors make more accurate diagnoses. Genetic testing also plays a good part alongside AI as shown by Hu *et al*. (2015). They conducted a simulation experiment using gene expression to categorize various colon cancer patients with the I into two groups: relapse and no relapse after surgery (Hu *et al*., 2015). The researchers examined the classification accuracy of three neural networks: S-Kohonen (91%), Back-propagation (66%), and SVM (70%), and proposed that the S-Kohonen neural network is more suited for colon cancer categorization. Some researchers were also able to use gene markers in other to equip predictive models with the ability to measure the rate of disease survival, chemotherapy response, and even a re-occurrence of the cancer (Mitsala *et al*., 2021)

AI technologies are also expected to help healthcare practitioners improve diagnostic accuracy and specificity (Mitsala *et al*., 2021). Medical fields that use pictures for diagnosis, such as radiology, are particularly suited to AI-aided diagnosis. ML is effective at detecting abnormalities in photos. It has been hypothesized that what would take an expert radiologist 30 years to master radiology-pathology correlation might require an AI system hours or days to assess and learn in the future. As AI systems improve and are proven trustworthy in visual diagnosis, clinicians in the diagnostic fields may find it less essential to read photos, possibly only doing so on rare occasions (Stanfill and Marc, 2019).

There are other factors to consider, including whether the reporting of diagnostic specificity utilizing diagnostic test findings should differ based on the AI application (Jiang *et al*., 2017). Providing precision based on AI findings may also rely on whether the AI application uses supervised or unsupervised ML approaches as unsupervised ML is well recognized for feature extraction, but supervised ML, which undergoes a training procedure to select the optimal outputs, is better suited to modelling predictions and is often regarded as providing more therapeutically meaningful outcomes. Hence, the sort of AI and the way the AI application is employed in the clinical workflow may influence subsequent reporting requirements for diagnostic code specificity (Jiang *et al*., 2017).

AI can also help in the early diagnosis of possible or impending illnesses, allowing for more timely action. Machine learning algorithms are proven to be excellent at inferring particular health concerns and forecasting health occurrences. For example, methods based on neural networks are useful in identifying strokes (Jiang *et al*., 2017). The algorithm's input variables include stroke-related signs such as arm or leg paresthesia, acute disorientation, visual changes, mobility issues, and so on. Additional instances include hospital readmissions, infections, and surgical complications (Bertsimas *et al*., 2018; Saqib *et al*., 2018).

Another good example is in the world of electrophysiology in healthcare. Wearable photoplethysmographic sensors have revolutionized the possibilities for arrhythmia screening by allowing long-term, passive measurement of pulse rate and regularity to detect an irregular pulse associated with arrhythmia (Mukhopadhyay *et al*., 2022). Beyond photoplethysmographic pulse detection, the Food and Drug Administration has certified Kardiaband and Apple Watch Series 4-5 to employ wearable ECG recording abilities for on-demand ECG validation of photoplethysmography-based arrhythmia identification (Feeny *et al*., 2020). On Apple Watch Series 4-5, irregular rhythm detection via photoplethysmography encourages the wearer to capture a single-lead ECG using the digital crown's sensor. The Kardiaband algorithm employs pedometer and photoplethysmographic sensors on an Apple Watch Series 2 or 3 to track heart rate and activity levels continually (Feeny *et al*., 2020). Other wearable devices powered by AI are summarized in Table 1.

Healthcare Management and Telemedicine

AI can also assist in the areas of healthcare management and resource optimization. This is significant as its effect could forecast disease outbreaks, cause effective allocation of hospital resources and even affect the management of patient traffic through its various analytics and models (Wang and Preininger, 2019).

Making healthcare records digitally accessible is an effective tool for remotely documenting and sharing healthcare information, and incorporating machine learning-based modelling designed specifically for administrative datasets can aid in the detection of potential complications, as well as improve healthcare resource utilization and outcomes on a personalised level (Lipkova *et al*., 2022).

Table 1:
Summarized table of common commercial smart wearables devices and their various cardiovascular clinical applications. BP, blood pressure; ECG, electrocardiogram; HR, heart rate; PPG, photoplethysmography; SaO2, oxygen saturation (Adapted from Boccuto *et al*., 2023).

Type of Wearable Device	Sensors	Measurements Available	Clinical Application
Earbuds	PPG	HR; BP; SaPO2; cardiac output; stroke volume; rhythm and sleep evaluation	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection Long QT diagnosis; HF management; Hypertension screening and management
Smart ring	PPG	HR; BP; SaPO2; cardiac output; stroke volume; rhythm and sleep evaluation	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management
Patch	ECG	Single-lead and multi-lead ECG; continuous ECG-monitoring; QTc measurement; arrhythmia detection	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management
Chest strap	ECG	Single-lead and multi-lead ECG; continuous ECG-monitoring; QTc measurement; arrhythmia detection	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management
Clothing and shoe sensors	ECG	Single-lead and multi-lead ECG; continuous ECG-monitoring; QTc measurement; arrhythmia detection	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management
Smartwatch	PPG; ECG	HR; BP; SaPO2; cardiac output; stroke volume; rhythm and sleep evaluation. Single-lead and multi-lead ECG; continuous ECG-monitoring; QTc measurement; arrhythmia detection	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management
Smart band	PPG; ECG	HR; BP; SaPO2; cardiac output; stroke volume; rhythm and sleep evaluation. Single-lead and multi-lead ECG; continuous ECG-monitoring; QTc measurement; arrhythmia detection	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management
Smart ring	PPG; ECG	HR; BP; SaPO2; cardiac output; stroke volume; rhythm and sleep evaluation. Single-lead and multi-lead ECG; continuous ECG-monitoring; QTc measurement; arrhythmia detection	Risk assessment and prediction; Cardiac telerehabilitation; Arrhythmia detection; Long QT diagnosis; HF management; Hypertension screening and management

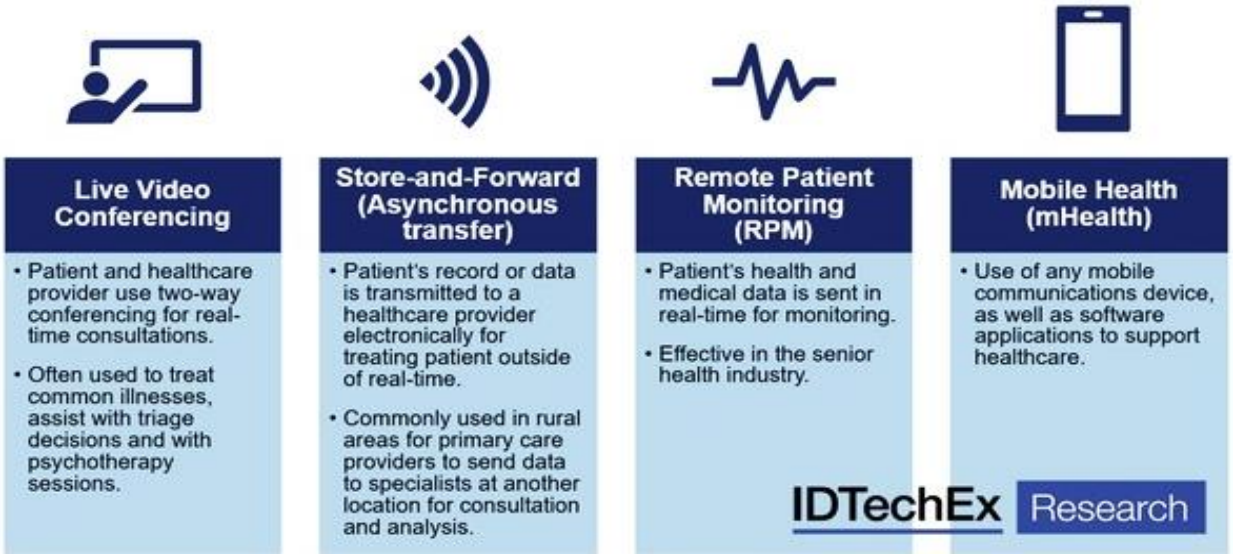

Figure 2:
Types of Telemedicine

One example is the use of machine learning on healthcare data to predict outcomes in sepsis patients. Samad *et al*. (2019) were able to achieve a 96% accuracy in predicting patient survival using echocardiography and digitally supplied data.

Its analytics, as noted, may also be employed in chronic disease management including the prediction of a disease outbreak, and might be characterized by multi-organ involvement, acute variable events, and extended illness progression latencies. One example is the use of predictive analysis in cases of retinopathy (Gulshan *et al*., 2016). Deep learning was used to train two validation datasets to detect and grade diabetic retinopathy and macular edema, and the results were highly specific and sensitive for detecting moderately severe retinopathy and macular edema (Gulshan *et al*., 2016).

Telemedicine is another sector of healthcare that can be positively affected by healthcare. Defined as the use of various communication technologies to form an interaction between and health practitioner and patient to discuss and send information concerning the diagnosis, treatment, and prevention of diseases in patients, it has been a tremendous help in cases of geographical distance between the two parties involved (Shen *et al*., 2021). This includes phone calls, virtual messaging, video calls, and data transfer systems (Figure 2). This has been mostly used in the field of ophthalmology, particularly in the case of diabetic retinopathy (Ting *et al*., 2018). Unfortunately, little research has been done into the potential relationship between AI and telemedicine, and the articles found mostly focused on hypothetical implications. An article focused on the usage of TOSCA, a telemedicine technology that allowed data to be sent between countries such as England and Denmark. The image analysis technique begins with picture polynomial transformation, which allows for blood vessel alignment, followed by preprocessing and retinal lesion extraction. Next, the picture was classified using supervised algorithms. Finally, the platform intends to create a normative reference database to assess algorithms and conduct additional research (Schneider *et al*., 2005).

Saeed *et al*. (2019) study laid out a cloud-based ophthalmic system for the Polish population. The system contained picture preparation, which involved converting the image to grayscale with a green channel, histogram stretching, medial filtering, and gamma correction. The steps involved extracting vascular patterns utilizing vessel segmentation and binarization, as well as removing vascular and optic discs (Saeed *et al*., 2019). Finally, pathogenic alterations were recognized and classed as healthy if no abnormalities were discovered. The published results are based on a 100-image dataset with 98% accuracy, 100% sensitivity, and 96% specificity in recognizing abnormal pictures. The suggested system demonstrated good sensitivity, specificity, as well as accuracy in the validation set (Saeed *et al*., 2019).

Drug Therapy and Discovery

Drug therapy is the treatment with any substance other than food used to avoid, diagnose, treat, or alleviate the symptoms of an illness or abnormal state (National Cancer Institute, 2011) and it is most well-known for aiding in disorders such as cancer, anxiety-related disorders, and depression and it offers patients high benefits of efficacy, minimal adverse reactions, and low drug resistance (Shabani and Hojjat-Farsangi, 2016). AI helps it to be more effective by making the identification of the best match for a given type of therapy less stressful. This is needed to reduce the risks of poor outcomes in terms of prognosis and the high costs of treatment. Despite the limited use of AI due to data unavailability, a study by Johannet *et al*. (2021) was able to show its gradual expansion using CNN models to predict responses to checkpoint immunotherapy in advanced melanoma patients.

To clarify the molecular mechanisms driving this type of therapy, interactome data may be organized and represented as network architectures, with components representing biological entities and edges representing associations/interactions among them (Zhang and Zhang, 2017; Song *et al*., 2022). AI biology analysis algorithms are an effective method for processing biological network data, and they can effectively address the complexity of illnesses

caused by relationships among genes as well as their products in biological network structures, thereby improving our understanding of the root causes (Zhou *et al*., 2020). A very good example is in cancer target identification.

Network-based biology analysis applications begin by reconstructing networks by calculating molecular differential expressions and correlations (Hernández-de-Diego *et al*., 2018). Then, gene set enrichment analysis is used to find network modules that execute diverse biological activities. The found network modules are then utilized to identify important genes that might be targets of therapy (or biomarkers) for cancer (Hernández-de-Diego *et al*., 2018). WGCNA195 is a popular network-based biology analysis program that accepts numerous gene expression matrices as input. It then produces several gene network modules as well as the biological network's fundamental genes (You *et al*., 2022). Though network-based biology analytic approaches are valuable in discovering anticancer targets, they have several drawbacks, such as the inability to efficiently manage multiomics data, which results in high false-positive rates for discovered targets (You *et al*., 2022). AI has also been shown to predict drug efficacy as evident in a study done by Iloro *et al* (2016), which measured the responses of various cancer cell lines and showed that the models were able to almost perfectly predict the effectiveness of a drug. DL has also become a widely popular choice in terms of this subject as a study was able to show how easy the identification and prediction of drug efficacy by the deep neural networks, although there was a notable setback which was the inability of the model to properly interpret the biological mechanism driving such predictions (Chiu *et al*., 2019). This shows more gaps in the knowledge of AI and its relationship to pharmacology and there is a hope that more research will be done on this subject.

In terms of drug discovery, AI provides a quicker process of development and optimization of the design of drugs. Its usefulness in the aspect of pharmaceuticals can span from drug design, polypharmacology, chemical analysis, drug repurposing, and even drug screening (Sellwood *et al*., 2018). It can predict drug-protein interactions, design multitarget drug molecules, and even classify the targeted cells for drug delivery. A good example of this is the quantitative structure-activity relationship model (QSAR) which can predict large numbers of compounds within short periods and can also identify potential drug candidates (Paul *et al*., 2020). DLs also play a role as they can be implemented for evaluations of the safety and effectiveness of drug molecules based on data modelling and analysis (Zhu, 2019). Predictive models have also been shown to be able to predict the needed chemical structure of a specific compound (Pereira *et al*., 2016). All these various AI models can effectively change the way we view the production and discovery of pharmaceutical drugs.

Challenges And Future Directions

Improving the utilization of AI in healthcare in the rapidly evolving world is a huge issue due to increasing socioeconomic and environmental elements, as well as the advent of novel illnesses. However, we must understand the significant problems that might arise while leveraging AI's opportunities.

Monetary funding for AI is an important component in determining its usefulness. The achievement of AI technology development and application in healthcare systems is dependent on the availability of financing and budgetary assistance (Shaw *et al*., 2019). In underdeveloped nations with limited government funds, it is critical to assess the expenses and assets required for AI adoption, as well as the possible cost-to-care effectiveness ratio (Pongtriang *et al*., 2023).

Data mining becomes another critical component in improving this developing system with AI since it increasingly impacts policy choices. For example, patient data analytics are used for monitoring, prediction, and treatment planning to address health concerns in a variety of populations (Janett and Yeracaris, 2020). However, AI integration continues to encounter obstacles in terms of precision, patient confidentiality, and information security, as well as ethical issues. Furthermore, several countries suffer from data-related issues (Rajpurkar *et al*., 2022). These obstacles include a lack of experience in healthcare data mining, limited studies on AI's function in healthcare, and imprecise rules governing the application of machine learning in health and research.

In terms of ethical considerations, patients are more likely to trust a human caregiver than a computer, and healthcare staff are concerned about the susceptibility of AI to errors as a result of data breaches and cyber hacking. There are also crucial rules based on ethical values such as fairness, damage reduction, and justice that are lacking (Abdullah *et al*., 2021). A reassessment of these standards is required to enhance patients' and even medical practitioners' trust in the security and efficacy of AI, and patients should be offered the option of incorporating AI into their treatment care

Furthermore, data gathered by healthcare systems at the basic, secondary, as well as tertiary health service levels are ineffectively integrated due to the multiplicity of platforms utilized for health information collecting. This restricts the application of such databases for efficient care planning and coordination. As a result, government regulations must be modified to tackle the incorporation of AI in healthcare, as well as improve the effective use of medical data for prediction, investigation, as well as care planning, to achieve long-term outcomes (Jassar *et al*., 2022).

CONCLUSION

Incorporation of AI into healthcare constitutes a watershed moment with far-reaching ramifications. It has emerged as a transformational technology, changing how we perceive, analyse, and use various data relating to a patient’s health and well-being. AI uses machine learning algorithms, neural networks, and others to extract important insights from complicated biological systems, allowing for more accurate diagnosis, tailored therapies, and predictive interventions ((Jiang *et al*., 2017; Wang and Preininger, 2019; Johnson *et al*., 2020). Furthermore, AI-powered tools streamline research methods, boosting the rate of discovery and innovation in healthcare. However, ethical factors must be carefully addressed to guarantee that AI is deployed responsibly and equitably. Despite these challenges, AI's position in the health system is expected to grow and usher in a period of extraordinary medical discoveries. The use of AI is transforming healthcare by improving the accuracy

and efficiency of diagnosis, developing new medical technologies, and helping with early treatment (Noorbakhsh-Sabet *et al*., 2019). AI has the potential to massively improve the way we view healthcare if ethical regulations are strictly followed.

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Niger. J. Physiol. Sci. 39 (June 2024): 9 – 13
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

The Types and Utilization of Galactagogues in Nigeria: A Case Study of People Residing in Lagos State.

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Summary: One of the factors characterizing suboptimal breastfeeding is insufficient human milk production. In most African settings, special food items are regularly used to promote human milk production. These food items, called galactagogues, are used in Nigeria, but their use is undocumented and the amount of intake among lactating mothers is unknown. The study aims to document galactagogue consumption habits and describe the food items some lactating mothers use in Nigeria. A cross-sectional study was conducted among 112 mothers who breastfed exclusively in Lagos Nigeria. A pretested, self-reported administered questionnaire was used. All data were coded in a computer program file. Qualitative data were presented as frequencies and percentages while quantitative data were represented as mean ± standard deviation (SD). A $p < 0.05$ was considered statistically significant. The most reported galactagogue was pap (80.8%). Others were tea (46.0%), milk (19.1%), and amala (9.5%). Information concerning galactagogues was mainly from the participants' mothers (35.9%), while media/ internet had little influence (4.2%). Galactagogues are common in Nigeria, and they signify an important part of the nutrition of lactating mothers. These results could contribute to the knowledge of breastfeeding activities in Nigeria and encourage further research to produce robust facts about galactagogues' safety and scientific efficacy to support evidence-based approaches and advance breastfeeding concerns.

Keywords*: galactagogues, human milk, breastfeeding habits, pap, Nigeria*

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Manuscript received- December 2023; Accepted- March 2024

DOI: https://doi.org/10.54548/njps.v39i1.2

INTRODUCTION

The act of exclusively breastfeeding infants for the first 6 months of their lives cannot be overemphasized. This practice is recommended by the World Health Organization (WHO, 2003). An all-inclusive implementation plan on maternal, infant, as well as young child nutrition, was also endorsed by WHO. The plan itemized six global nutrition objectives that should be attained by the year 2025. The Resolution sought among other things to increase rates of exclusive breastfeeding among infants less than 6 months by at least 50% (WHO, 2014).

Exclusive breastfeeding is important to check infant malnutrition and prevent infant mortality in children under five years worldwide (Azuine *et al*., 2015). Reduced danger of infant infections, protection against the development of Type-2 diabetes, obesity, and other non-communicable diseases, and benefits in the educational performance of school children are other advantages of breastfeeding (Krol and Grossmann, 2018). Breastfeeding culture is well-enshrined in various ethnic groups in Nigeria; however, the promotion of optimal exclusive breastfeeding practices has not been attained and should be encouraged (Akadri and Odelola, 2020).

The complex interplay between hormones, emotions, and physical factors is a key component of breastfeeding physiology. Inadequate human milk production is one of the important factors that increase the rate of suboptimal breastfeeding among lactating mothers (Masaba *et al*., 2021, Huang *et al*., 2022). There are reports of perceived human milk insufficiency across the globe (McBride *et al*., 2021) and even in African countries like Nigeria (Akadri and Odelola, 2020). This perceived insufficient human milk production has resulted in different approaches like medication, herbal preparations, and intake of some food substances to solve the problem of inadequacy in human milk production.

Galactagogues (or Lactogogues) are substances used to assist initiation, maintenance, or augmentation of the rate of the mother's milk synthesis (Ali *et al*., 2020). Galactagogues include pharmacological constituents, herbal medicines, and food preparations. There are however concerns about the safety of pharmacological galactagogues (Grzeskowiak *et al*., 2019) while herbal medicines and food substances, however, have been suggested to be effective (Buntuchai *et al*., 2017, Ali *et al*., 2020). Different herbs and food-based substances are either eaten or used for medicinal purposes

around the world and in Africa. There are views that these practices of assisting the mothers' milk to flow exist in the local settings alone. This might not be correct as the migration of people from local settings to cities exists thus the information concerning galactagogue use would be available also in urban settings. The practices of galactagogue consumption in Nigeria are not documented or preserved. This study aims to identify the food items and herbs used by lactating mothers as well as to scientifically document galactagogue consumption practices among lactating mothers in Lagos, a cosmopolitan city that accommodates people from different geopolitical zones in Nigeria. The outcomes from this study could help to understand the level of usage and stimulate further study into the scientific competence of common foods and herbs used as galactagogues.

MATERIALS AND METHODS

Study Design, Setting, Sampling: This study is a cross-sectional study using self-report surveys and researcher-administered surveys that involved the administration of questionnaires to record the specific galactagogues used, usage frequency, and perceived effectiveness. This study was conducted in Lagos State in the South-West geopolitical zone of Nigeria. Different ethnicities reside in Lagos, but the people still have a traditional lifestyle, with traditional foods and traditional pregnancy and postpartum practices even though Lagos is a sophisticated city. The mothers and their infants visited the hospitals for primary nursing care, routine vaccines, etc.

Lactating mothers who are Nigerians living in Lagos were eligible for this study. One hundred and twelve (112) breastfeeding mothers ages 20-50 years and their infants, (0-24 months) took part in the study. The selected sample was based on the following criteria. The mothers who had full-term infants, and who volunteered, participated in the study. The mothers were exclusively breastfeeding at the time of the survey, and some had other babies whom they had breastfed before this survey. No monetary compensation was given to participants.

Data Collection: A self-administered questionnaire was used to collect demographic characteristics, specific galactagogues used, usage frequency, perceived effectiveness, and information on galactagogues use and experiences of galactagogue use among lactating mothers practicing exclusive breastfeeding. The number of galactagogues utilized and the number of people from whom the experiences were learned could both be freely mentioned. The data were collected between August and November 2019. The study took place at a Teaching Hospital as well as two Private Clinics in Lagos, Nigeria. The participants signed an informed consent form before the interview. The section of the questionnaire on food items recorded the frequency and type of galactagogues consumed during the last 2 months. The mothers exclusively breastfed during the last 2 months parallel to the time that galactagogues were consumed. The list of foods was compiled according to texture and type.

Data Analysis: All data were coded in a computer program file (STATA Statistical software, version 15.0). Presentation of the quantitative data was by simple statistics of frequencies, percentages, and mean with standard deviation (SD) used to categorize the variables. Food items were classified by similar nutrients.

Ethical Considerations: Ethical approval CMUL/HREC/11/22/1125 was obtained from the College of Medicine, University of Lagos Ethical Review Committee. Written informed consent was obtained from the participants before the interview.

RESULTS

Maternal Demographic Characteristics: The general characteristics of the mothers are presented in Table 1. The mean age of the mothers was 33.7 ± 6.7 years. The mothers with a total of one child had the highest percentage (34.8%) and whose pregnancies got to term and resulted in breastfeeding. The participants with bachelor's degrees were (50.8%), while those employed were (62.5%).

Table 1
General Characteristics (N=112)

Characteristics		N (%)
Mother's age (years)	< 34	59 (52.6)
	> 34	53 (47.3)
Family status	Live with spouse	108 (96.4)
	Live with unmarried partner	2 (1.7)
	Single mother	2 (1.7)
Education attainment	Less than bachelor's degree	38 (33.9)
	Bachelor's degree	57 (50.8)
	Postgraduate degree	17 (15.1)
Employment status	Employed	70 (62.5)
	Self-employed	23 (20.5)
	Unemployed	19 (16.9)
Number of parities resulting in breastfeeding	1	39 (34.8)
	2	35 (31.2)
	3	21 (18.7)
	4	14 (12.5)
	5	2 (1.7)
	6	1 (0.8)
Region of Origin	North Central	8 (7.2)
	South East	17 (15.4)
	South South	30 (27.2)
	South West	53 (48.1)
	Didn't say	4 (3.57)

Galactagogue Food Items Used Among Lactating Mothers: The food items were classified into four groups: the galactagogues in liquid forms, the galactagogues taken as food, the galactagogues prepared as soups, and another group that included hot foods, fruits, vegetables, and local spices. The drinks-related galactagogues included pap (80.8%), tea (46.0%), water (17.3%), and milk (19.1%). The food-related galactagogues included Amala (9.5%), Pounded yam (6.0%), Yam (5.2%), and Rice (2.6%). The soup-related galactagogues included Pepper soup (8.6%), Bitterleaf soup (5.2%), and Ewedu (Jute) soup (4.3%). The foods listed under 'others' included Fruits at (8.6%), Vegetables at (2.6%), and Hot foods at (1.7%) (Table 2).

Table 2
Classification of the food items used as galactagogues (N=115)

Class of galactagogues		N (%)
Drinks	Pap	93 (80.8)
	Tea	53 (46.0)
	Water	20 (17.3)
	Malt Drink	1 (0.8)
	Fluids	10 (8.6)
	Milk	22 (19.1)
	Yogurt	2 (1.7)
	Soybean products	1 (0.8)
	Palm wine	9 (7.8)
	Kunu	4 (3.4)
Food	*Pounded* yam	7 (6.0)
	Amala	11 (9.5)
	Yam	6 (5.2)
	Ice-cream	1 (0.8)
	Fufu	1 (0.8)
	Semovita	3 (2.6)
	Rice	3 (2.6)
Soups	Pepper	10 (8.6)
	Banga	1 (0.8)
	White	1 (0.8)
	Bitterleaf	6 (5.2)
	Ewedu (Jute)	5 (4.3)
Others	Hot foods	2 (1.7)
	Fruits	10 (8.6)
	Vegetables	3 (2.6)
	Local spices	1 (0.8)
	Tiger nut+coco nut+date	1 (0.8)
	Tigernut only	1 (0.8)
	Moringa	1 (0.8)

Galactagogue Experiences: The galactagogue food items were learned from different experiences of people as follows: Mothers (35.9%), Health workers and Antenatal programs (14.7%), Personal experience (10.5%), Friends (10.5%), Past and present nursing mothers (6.3%), Grandmothers (5.6%), social media/internet (4.2%) (Table 3).

Experience and Reasons for slow milk let down: The responses to the reasons for slow milk let-down are as follows: some mothers had no idea (28.9%), others said it was due to being a first-time mother (5.2%), the poor diet of the lactating mothers (12.2%), illness (10.5%), stress (7.8%). Of some lactating mothers, (61.3%) had personal experience of slow milk let-downs, while others learned about it from experienced family and friends (57.6%) (Table 3).

DISCUSSION

In Nigerian cultures, there are pregnancy and postpartum practices mothers observe strictly (Alabi, 2022). This includes mothers shunning certain foods like okra, and roasted plantain but eating other selected foods like fruits, vegetables, and carbohydrate foods to aid maternal and infant health. This study investigated the foods used to increase lactation and the perceived efficacy of galactagogue practices among Nigerians living in Lagos State who were from various geopolitical zones in Nigeria. The investigators found that there is an awareness of the use of selected foods widely used to aid human milk production in Nigeria and that these foods constitute a vital part of the diet of the mothers. This is comparable to an Australian study (Brodribb, 2018).

Table 3
Factors related to the use of galactagogues to enhance breastmilk production

Variable	N (%)
Where did you learn about the use of special drinks, foods, and other substances for breast milk production? (N= 142)	
Mother	51 (35.9)
Health workers & Antenatal programs	21 (14.7)
Personal experience	15 (10.5)
Friends	15 (10.5)
Colleagues at work	5 (3.5)
Past &Present Nursing mothers	9 (6.3)
Grandmothers	8 (5.6)
Mother-in-law	3 (2.1)
Sister-in-law	2 (1.4)
Popular saying	1 (0.7)
Sisters	2 (1.4)
Social media	6 (4.2)
Poster	1 (0.7)
Clergy	1 (0.7)
Herb sellers	1 (0.7)
Family	1 (0.7)
Experience of slow milk letdown (N=118)	
Personal	65 (61.3)
Family/Friend	53 (57.6)
Reasons for slow milk letdown (N=114)	
No idea	33 (28.9)
First-time mother	6 (5.2)
Poor diet of lactating mother	14 (12.2)
Body make-up	4 (3.5)
Illness	12 (10.5)
Stress	9 (7.8)
Mode of delivery	2 (1.7)
Delay in breastfeeding because of a sick child	1 (0.8)
Others	33 (28.9)

The consumption of pap stood out having been reported by the largest percentage of participants. Pap is a cereal pudding made from corn, millet, or sorghum. The cereal to be used is soaked for a few days to allow it to be softened after which it is washed in cold water and milled to an extremely smooth paste. With the aid of a sieve, the pureed corn is run through with lots of water. After which it is prepared with hot water and served hot. It is taken in liquid form. Corn-based preparations have recently been reported to act as galactagogues which could be used to stimulate milk production in lactating women (Azevedo *et al*., 2022). Amala is a local indigenous food native to the Southwestern part of Nigeria. Amala, which is made from yam, cassava, or plantain flours is cooked and eaten along with soups like Ewedu soup and meat or fish. Saponins, and flavonoids, have been reported to be the major phytochemicals in yam (*Dioscorea alata*), cassava (*Manihot esculenta*) (Olaniyan and Ajayi, 2021), and plantain (*Musa paradisiaca*) (Arun *et al*., 2017), used in making the flour cooked into Amala. The

participants also mentioned pounded yam, yam, and fufu also made from yam and cassava respectively as galactagogue food substances. The reported mechanism of action of these phytochemicals is that they raise milk production by acting as dopamine antagonists (Buntuchai *et al*., 2017).

Phytochemicals, saponins, tannins, polyphenols, isoflavones, and alkaloids impact milk production because they stimulate milk ejection, improve milk protein levels, and improve lactation by increasing prolactin levels. Hypotheses exist that galactagogues might raise milk volume through an estrogenic effect (Buntuchai *et al*., 2017). First, phytoestrogens have been reported to function as dopamine antagonists by preventing the pathway triggered by a dopamine receptor and increasing prolactin secretion to bring about milk production. Second, phytoestrogen from galactagogue origin has been reported to bind with receptors in the mammary glands to induce alveolar cell propagation. The anterior pituitary lactotrophic cells and phytoestrogens then bind with a β-estradiol receptor through an α-isoform of the membrane-associated estrogen and this may induce prolactin gene expression (Buntuchai *et al*., 2017).

A study has shown significant correlations between carbohydrate intake and milk volume (Czosnykowska-Łukacka *et al*., 2018). Pap, amala, pounded yam, fufu, semovita, and rice are energy-giving foods and are taken several times a day and this aligns with a study that suggested that mothers should not consume less than 1600 kcal per day because it predisposes to a reduction in milk production (Marangoni *et al*., 2016).

In line with another study (Ali *et al*., 2020), participants interviewed in this study, indicated that yogurt, milk, and soybean products were galactagogue food items. It is believed that foods high in protein can explain the lactogenic effect since they offer a supply of fatty acids for human milk production or through the action of specific amino acids which are absorbed into the bloodstream and synthesized into milk proteins like casein and whey in the mammary glands (Ali *et al*., 2020). In contrast to a study conducted in the United States that revealed intense awareness of the use of fenugreek (an herb) to enhance human milk production (Bazzano *et al*., 2017), it was observed that participants in this study indicated dietary items with little emphasis on herbs (Moringa). Some participants mentioned palm wine as a form of galactagogues, however given its high alcohol concentration, this should be avoided during breastfeeding.
According to research findings by Buntuchai *et al*., (2017) that support this study, there is a belief in Nigeria that consuming fruits and vegetables helps assist milk letdown. Flavonoids and saponins are found in soups such as white soup, ewedu, bitter leaf, pepper in pepper soup, and various ingredients in Banga soup (Hamzah *et al*., 2013). Barley used in making malt beverages, as well as tea, and kunu are high in phenol, saponins, and flavonoids (Kris-Etherton and Keen, 2002, Raj *et al*., 2023) suggesting they assist in milk letdown.

Certain participants made hints about the need to consume galactagogue foods while they are hot. Food items suggested to be consumed hot include pap, tea, amala, pounded yam, semovita, and soups. The explanation for this was that these foods either quickly increase blood circulation or stimulate circulation to let down milk more quickly (Ali *et al*., 2020).

According to this study, participants primarily learned about certain galactagogue foods from their mothers and other family members, which is in line with findings from another study (Ali *et al*., 2020). Some other studies, however, have indicated that their primary source of information on galactagogue foods was the internet and social media (Bazzano *et al*., 2017, McBride *et al*., 2021) which runs counter to the results of this study. Health professionals served as the primary information source according to other studies (Steyn *et al*., 2017). In this present study, it was observed that most cultures pass on information about postpartum experiences from generation to generation. These postpartum experiences include knowledge of human milk production when it is in low supply to the baby. This generational transfer of information is due, in part, to the fact that family support is evident during lactation, which is in line with other research (Agunbiade and Ogunleye, 2012, Akadri and Odelola, 2020). Additionally, even though the participants gave birth and visited hospitals after giving birth, this study demonstrates that some aspects of the participants' culture remain preserved despite the demands of modern life. When compared to the overall percentage of participants who received knowledge on galactagogue food ingredients from family members, the proportion of participants who received information from health professionals and prenatal programs was lower.
In this study, some participants reported that they experienced slow milk letdown. The galactagogue food items were thus employed as a cure for this set of participants. These foods are however mostly included in the standard postpartum diet. This is consistent with research from other parts of Nigeria where low milk production was reported (Agunbiade and Ogunleye, 2012, Akadri and Odelola, 2020).

A study found that participants with higher levels of education knew more about breastfeeding (Dukuzumuremyi *et al*., 2020). A significant portion of the participants in this study had bachelor's or postgraduate degrees, and a few of them were aware of the causes of the delayed milk letdown. It is common knowledge that a mother's health and ability to produce milk are impacted by her nutritional state. It is also commonly recognized that oxytocin, which aids in the induction of milk ejection, is inhibited by stress, anxiety, and related causes. Consequently, some participants' perceptions were accurate.

This study's findings are necessary for documentation to enlighten nursing mothers. Healthcare providers also require knowledge about galactagogue foods and views of efficacy to assist them in producing content for educational initiatives aimed at lactating mothers which may be utilized during pregnancy or postnatal visits. This is crucial due to the high regard for efficacy in these goods, which lack any sort of well-known scientific evidence. Awareness about palm wine for example as a galactagogue should be discouraged because of its alcoholic content that could harm the infant. The other galactagogue food items acknowledged in this study could be conjectured as safe as these food items are commonplace in Nigeria and appear to be healthy and this could signify some level of safety. There is thus the need for further research to determine their level of efficacy and

safety. To encourage mothers with sluggish milk letdown to seek professional support rather than rely entirely on their beliefs and practices to improve efficient breastfeeding, it is also crucial for healthcare professionals to be aware of these beliefs.

The exclusion of women who were not presently breastfeeding, likely introduced some prejudice in galactagogue habits, because practices of current lactating mothers and non-lactating women, may differ.

In conclusion, the use of galactagogue food items widely used to boost human milk production is commonplace in Nigeria and these food items constitute an important part of the nutrition of lactating mothers. The study into these galactagogue-containing foods could contribute to comprehending breastfeeding practices and inspire advanced research into the efficacy and safety of these food items available in Nigeria.

Acknowledgement

The authors thank all the participants who volunteered to partake in the study. The authors are also grateful to Prof. Victoria A. Alabi, Department of English, University of Ilorin for editorial support.

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Niger. J. Physiol. Sci. 39 (June 2024): 15 – 22
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Prevalence of Acetabular Dysplasia and its Associated Factors: Major Determinants of Hip Function.

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Summary: The hip joint bears a considerable proportion of the human weight, and the ability to efficiently perform its plethora of functions depends on the status of the acetabulum. Morphological alteration of the acetabulum results in acetabular dysplasia which adversely affects the hip joint. The objectives of this study were to determine acetabular morphology, factors that are associated with acetabular dysplasia which compromise hip joint functions and the prevalence of acetabular dysplasia in apparently healthy adults in Calabar. This prospective cross-sectional study was carried out in the Radiology department of the University of Calabar teaching hospital, Calabar, Nigeria over a 5-months period and involved 100 apparently healthy subjects. Pelvic radiographs were conducted for all the subjects and afterwards center edge angle of Wiberg and acetabular angle of Sharp evaluations were done on all the images on a direct digital radiography viewer. Chi square, T-test and Pearson's correlation were used to analyze the data. Mean center edge angle of Wiberg and acetabular angle of Sharp were 27.41 ± 0.730 (SEM) and 39.00±0.860 (SEM) respectively. Center edge angle of Wiberg and acetabular angle of Sharp were significantly associated with age (P=0.000 & P=0.000), marital status (P=0.002 & P=0.000) and employment status (P=0.001 & P=0.002). BMI was only significantly associated with center edge angle of Wiberg (P=0.004). Abnormal center edge angle of Wiberg and acetabular angle of Sharp were mostly seen in individuals below 40 years (64% & 88%, respectively), married (65% & 63%, respectively) and employed (75% & 53%, respectively). Most of the individuals with BMI above 25 had abnormal center edge angle of Wiberg (62%). Mean BMI in males was significantly greater than that of females (P=0.000). Acetabular dysplasia was noted in 4 males and the prevalence of acetabular dysplasia was 4%. It is concluded that the center edge angle of Wiberg and the acetabular angle of Sharp in our population are similar to the values in some European and Asian populations, and the prevalence of acetabular dysplasia is low in this locality. Being a male that is married, employed, overweight and below 40 years are significantly associated with the occurrence of acetabular dysplasia and by implication, are determinants of hip joint function.

Keywords*: Center edge angle, Acetabular angle, Acetabulum, Acetabular dysplasia, Hip joint function, Radiograph*

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Manuscript received- March 2024; Accepted: May, 2024

DOI: https://doi.org/10.54548/njps.v39i1.3

INTRODUCTION

The hip joint is a ball and socket joint that is formed by the acetabulum and the femoral head (Hassa *et al*., 2023). It plays a substantial role in the weight-bearing mechanics of the human body (Vuralli *et al*., 2022). Since the center of gravity of a human being is located around the hip joint, it aids in the maintenance of trunk balance and generally improves postural stability (Jeremic *et al*., 2011). For an efficient performance of the array of hip joint functions, which include flexion, extension, adduction, abduction, circumduction, external rotation and internal rotation (Böker *et al*., 2020; Vuralli *et al*., 2022), the acetabular morphology (depth and width) and its orientation has to be within normal limits (Hassa *et al*., 2023).

Over the past 20 years there has been an evolving body of evidence that lucidly illuminates the sequence of events which results as a consequence of the development of acetabular dysplasia that culminates to an early failure of hip joint function (Welton *et al*., 2023). In the onset of acetabular dysplasia, the roof becomes progressively shallow and vertically oriented. The effect of this mal-orientation is a reduction in the surface area for weight-bearing between the acetabulum and the femoral head making the joint's force per unit area to significantly increase during walking and other activities. The fibrocartilaginous acetabular labrum then undergoes

hypertrophy, to compensate for deficient bony acetabular coverage, and gradually partial or full-thickness tears occur which is accompanied by an accelerated degeneration of the hyaline articular cartilage that ultimately leads to pains, osteoarthritis and impaired hip joint function (Pun, 2016; Khaliq *et al*., 2022; Hong *et al*., 2023). This impairment limits the hip joint's range of motion and causes an obvious marked limp when walking (Pun, 2016).

Acetabular dysplasia exists when one of the two criteria are met; The first criterion is when the center edge angle of Wiberg is equal to or below 250 and the second criterion is when the acetabular angle of Sharp is equal to or above 420 (Umer *et al*., 2009; Jeremic *et al*., 2011; Mannava *et al*., 2017; Hofmann *et al*., 2017; Soydan *et al*., 2021). High values of center edge angle of Wiberg reflects a deep acetabulum while low values are indicative of both a shallow acetabulum and a significant reduction in the anterior, superior and lateral coverage of the femoral head (Tuğrul *et al*., 2020; Hassa *et al*., 2023).

It has been proven over the years that racial and geographical variations have profound influence on the anthropometric parameters of the bones, therefore it is essential to learn and understand the average dimension of the acetabulum as a part of the hip joint in this locality. Furthermore, the findings of this study will potentially provide a scientific basis for a better understanding of the pathogenesis of some sociodemographic and socioeconomic situations that initiate or contribute to the emergence of common diseases of the hip joint. The ortho-pediatricians and prosthetists will benefit immensely from the findings of this study since it will potentially enhance accurate fabrication of prostheses (Jeremic *et al*., 2011; Busato *et al*., 2021; Hassa *et al*., 2023). Moreover, awareness of the average dimensions of the hip joint bones in both sexes will be of prodigious assistance to forensic experts (Jeremic *et al*., 2011).

In most climes, the standard image evaluation of the acetabulum, in clinical practice, is ideally the antero-posterior view of plain pelvic radiographs (Böker *et al*., 2020; Nishimura *et al*., 2023). The parameters employed in the radiographic evaluation for acetabular dysplasia are center edge angle of Wiberg and acetabular angle of Sharp (Cevik and Cicek, 2020). There is no literature in this region of the country on acetabular morphology in adults and the factors that predispose the acetabulum to undergo dysplastic transformation.

We aimed to assess acetabular morphology, determine factors that are associated with acetabular dysplasia which compromise hip joint functions and the prevalence of acetabular dysplasia in apparently healthy adults in Calabar, Nigeria.

MATERIALS AND METHODS

Study design: This prospective cross sectional single center study was conducted in the Radiology department of the University of Calabar Teaching Hospital, Calabar, Nigeria. The duration of the study was from January 2023 to May 2023. The study population was obtained from the patients who were referred to the Radiology department from other departments within the confines of the University of Calabar Teaching Hospital, Calabar and from other health facilities within the city and beyond, to perform pelvic radiographs.

In strict compliance with the Helsinki declaration, approval was obtained from the health research ethics committee of the University of Calabar teaching hospital before the study commenced. The protocol number assigned to this study by the ethical committee was UCTH/HREC/33/557. Convenience sampling method was employed for the study.

Exclusion criteria: Radiographs that were not obtained in the accurate plane, radiographs that demonstrated pathologies, history of pelvic trauma/fracture, pelvic radiographs that are not symmetrical, history of pelvic surgery, family history of developmental dysplasia of the hip, known hip disease, low back pain, groin pain, thigh pain, those wearing pelvic implants or prothesis, pregnant women, < 18 years of age, breast feeding mothers, individuals with sickle cell disease, metabolic disorder such as hyperuricemia.

Sample size: The study involved 100 subjects who met the enrollment criteria and voluntarily filled the consent form and questionnaire out of 338 patients. Most of the cases were excluded on the basis of fracture/history of trauma, avascular necrosis of the femoral head, hip joint asymmetry during measurements, hip joint osteoarthritis, osteomyelitis, congenital anomalies, fibrous dysplasia, osteoporosis and pelvic lesions suspected to be tumors or metastases.
The information obtained from the subjects which included age, gender, center edge angle of Wiberg, acetabular angle of Sharp, socio-demographics were recorded as data for this study.

Operational definition
Acetabular dysplasia: In the context of this study, acetabular dysplasia is said to exist when the acetabulum of an evaluated hip joint has both a center edge angle of Wiberg equal to or less than 250 and acetabular angle of Sharp equal to or above 420. The prevalence of acetabular dysplasia will be determined thus,

$$\textit{Prevalence of Acetabular dysplasia} = \frac{\textit{Number of subjects with both abnormal center edge angle and abnormal acetabular angle}}{\textit{Total number of subjects}} \times 100$$

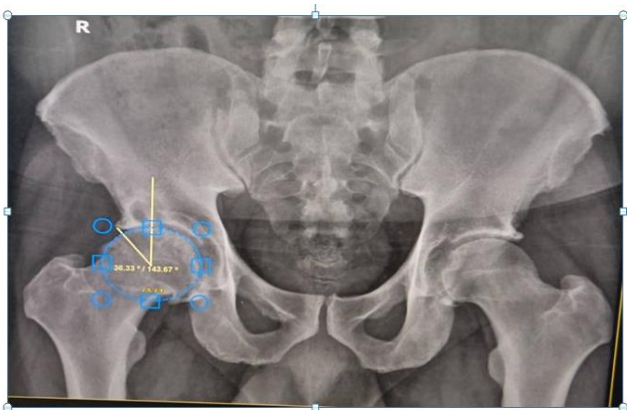

Figure 1
A pelvic radiograph that displays the lines utilized to measure the center edge angle of Wiberg

Center edge angle of Wiberg evaluation: A circle is drawn round the edges of the femoral head. A horizontal line is drawn from the center of the circle to emerge from the

medial outer margin of the circle and another line, which is at right angles to the first line, is drawn vertical upwards from the center of the circle. A third line is drawn from the center of the circle to the lateral margin of the acetabulum. The angle formed at the center of the circle between the second and third lines is regarded as the center edge angle of Wiberg (Figure 1) (Tuğrul *et al*., 2020).

Acetabular angle of Sharp evaluation:
A horizontal line is drawn to traverse the pelvic tear drop on one side and extend to the pelvic tear drop on the other side to ensure symmetry. Then a second line is drawn from the pelvic tear drop on one side to the lateral margin of the acetabulum. The angle between these two lines at the pelvic tear drop is regarded as the acetabular angle of Sharp (Figure 2) (Baharuddin *et al*., 2011).

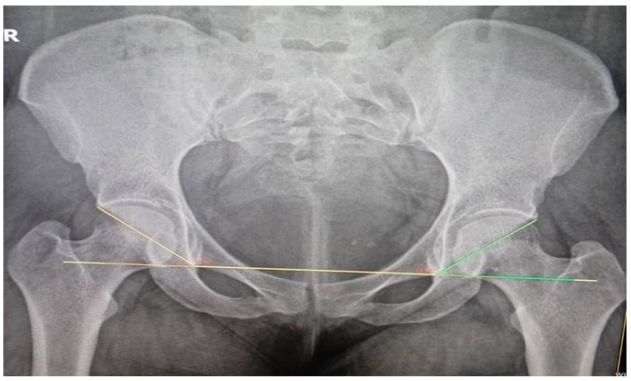

Figure 2:
A pelvic radiograph that displays the lines utilized to measure the acetabular angle of Sharp

Protocol: BRIVO XR575, a digital x-ray machine manufactured in 2021 by General Electric in CHINA and installed in the Radiology department in 2022, was utilized for this study. An antero-posterior pelvic radiograph was obtained with the subjects lying in a supine position. Gonad shields were utilized. The subjects' legs were extended and internally rotated by 200 with a distance of 100 cm between the image detector and the x-ray tube. The vertical x-ray beam was made to get to the image detector at a point that is 5 cm above the pubic symphysis (Baharuddin *et al*., 2011; Jeremic *et al*., 2011). For a pelvic radiograph to be deemed appropriate for the study, there should be symmetry of both obturator foramina and iliac crest, and the femoral necks must appear in true AP views (Ege *et al*., 2016). The center edge angle of Wiberg and the acetabular angle of Sharp were measured on the direct digital radiography viewer.
Two Radiography interns performed all the measurements and a vastly experienced Radiologist, who was blinded to the study, randomly selected 50 images to confirm the reliability of the prior evaluation and rule out inter-observer bias. The center edge angle of Wiberg and the acetabular angle of Sharp were obtained from the right and left hip joints and the mean values for each parameter were recorded.

Statistical analysis: The statistical packages for social sciences version 23 (SPSS Inc., Chicago, Il, USA) was employed to analyze the data obtained. The values were reported as means ± standard error of mean (SEM). Appropriate tables and scatterplot were the means of displaying results where applicable. Chi square analysis and T-test were done to assess the relationship of all the variables with abnormal center edge angle of Wiberg and acetabular angle of Sharp, respectively and to measure the differences in the values of these variables. P value < 0.05 was considered statistically significant.

RESULTS

The study group was made up of 54 males and 46 females. Majority of the male and female subjects had acetabulum with normal center edge angle of Wiberg (n=34 & n=28, respectively) and acetabular angle of Sharp (n=24 & n=34, respectively). Age (P=0.000 & P=0.000), marital status (P=0.002 & P=0.000) and employment status (P=0.001 & P=0.002) were significantly associated with center edge angle of Wiberg and acetabular angle of Sharp, respectively but gender was not (P=0.441 & P=0.879). No subject above 40 years had abnormal center edge angle of Wiberg value and only eight (8) had abnormal acetabular angle of Sharp.

Table 1
Distribution and association of sociodemographic characteristics with center edge angle of Wiberg and acetabular angle of Sharp (n=100)

		CEA (Degrees)		P value	SHARP (Degrees)		P value
		≤ 25 (n/%)	> 25 (n/%)		≥ 42 (n/%)	< 42 (n/%)	
Gender	**Males**	20 (37)	34 (63)	0.441	18 (39)	28 (61)	0.879
	Females	22 (48)	24 (52)		20 (37)	34 (63)	
Age (Years)	**< 40**	42 (64)	24 (36)	0.000*	30 (88)	4 (12)	0.000*
	> 40	0 (0)	34 (100)		8 (12)	58 (88)	
Educational status	**Educated**	18 (39)	28 (61)	0.704	16 (35)	30 (65)	0.665
	Uneducated	24 (44)	30 (56)		22 (41)	32 (59)	
Marital status	**Married**	30 (65)	16 (35)	0.002*	34 (63)	20 (37)	0.000*
	Unmarried	12 (22)	42 (78)		4 (9)	42 (91)	
Employment status	**Employed**	24 (75)	8 (25)	0.001*	36 (53)	32 (47)	0.002*
	Unemployed	18 (27)	50 (73)		2 (6)	30 (94)	
BMI (kg/m^2)	**> 25**	32 (62)	20 (38)	0.004*	22 (42)	30 (58)	0.514
	< 25	10 (21)	38 (79)		16 (33)	32 (67)	

(*) – P value less than 0.05 is significant; BMI – Body mass index; CEA – Center edge angle of Wiberg; SHARP – Acetabular angle of Sharp

Most of the subjects with abnormal center edge angle of Wiberg and abnormal acetabular angle of Sharp were below 40 years (n=42 & n=30), married (n=30 & n=34) and employed (n=24 & n=36), respectively. The subjects' BMI was significantly associated with center edge angle of Wiberg (P=0.004) but was not associated with acetabular angle of Sharp (P=0.514) and most of them with BMI > 25kg/m2 had acetabulum with abnormal center edge angle of Wiberg (n=32). Educational status was also not significantly associated with the center edge angle of Wiberg (P=0.704) and acetabular angle of Sharp (P=0.665), and most of the subjects within this variable had acetabulum with normal center edge angle of Wiberg (n=58) and acetabular angle of Sharp (n=62) (Table 1). Only four male subjects had both abnormal center edge angle of Wiberg and abnormal acetabular angle of Sharp. Therefore,

$$\text{Prevalence of acetabular dysplasia} = \frac{4}{100} \text{ x } 100 = 4\%$$

The mean center edge angle of Wiberg and acetabular angle of Sharp in the study were 27.41±0.730 (SEM) and 39.00 ± 0.860 (SEM) with a range of 20.120 to 35.440 and 30.320 to 51.350, respectively. The age of the subjects was from 18 to 60 years and the mean age was 33.58±1.71 years (SEM). The mean value of the BMI was 29.59±1.41 kg/M2 which shows that the subjects in this study were generally overweight (Table 2).

Table 2:
The mean values of the general descriptive statistics of the subjects

	n	Mean ± SEM	Min	Max
AGE (Years)	100	33.58 ± 1.71	18.00	60.00
BMI (Kg/m^2)	100	29.89 ± 1.41	16.60	38.30
CEA (Degrees)	100	27.41 ± 0.73	20.12	35.44
SHARP (Degrees)	100	39.00±0.86	30.32	51.35

BMI – Body mass index; CEA – Center edge angle of Wiberg; SHARP – Acetabular angle of Sharp

The mean BMI in males, 34.03±1.72 Kg/M2 (SEM), was shown to be significantly more than that in females, 29.15±1.35 Kg/M2 (SEM), (P=0.000), even though the least BMI was recorded in a male, 16.60 Kg/M2. In this study, the mean center edge angle of Wiberg for males was 26.57±0.740 (SEM) with a range of 20.310 to 33.330 and for females was 28.39±1.320 (SEM) with a range of 20.120 to 35.440. The mean values of the acetabular angle of Sharp in males was 39.18±1.230 (SEM) with a range of 30.330 to 51.350 and for females it was 38.80±1.210 (SEM) with a range of 30.320 to 48.330. The differences in the mean values of the center edge angle of Wiberg and the acetabular angle of Sharp for both genders were insignificant (Table 3). The scatter plot showed that the two variables, center edge angle of Wiberg and acetabular angle of Sharp, had a linear negative relationship. The gradient of the scatter plot was 0.462 depicting that both variables had a moderate strength of association. The Pearson's correlation between center edge angle of Wiberg and acetabular angle of Sharp was significant (r=-0.344, P=0.000) (Figure 3).

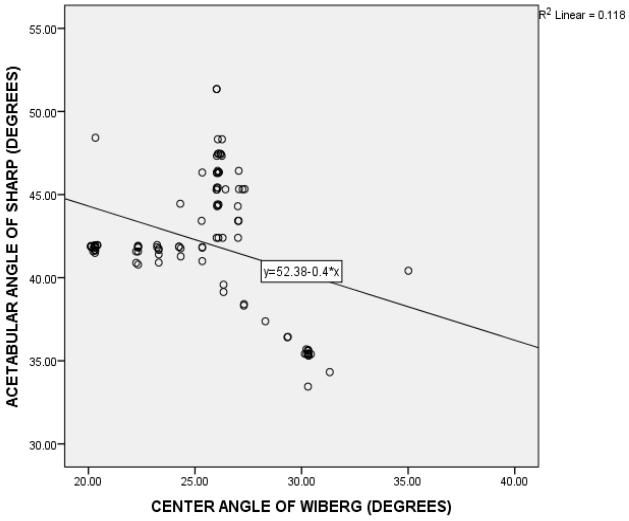

Figure 3
A scatter plot showing the relationship of the center edge of Wiberg and the acetabular angle of Sharp

DISCUSSION

The mean center edge angle of Wiberg in this study was observed to be 27.41±0.730 which was in consonant with previous report of researches conducted by Hassa *et al*., (2023) in Turkey (29.89±2.950) and Nishimura *et al*., (2023) in Japan (27.9±4.800). Incongruent to our findings are an array of reports with higher values in diverse climes and these include the studies of Vuralli *et al*., (2022) in Turkey (32.78±2.0500), Jeremic *et al*., (2011), whose study included Serbian subjects that had been treated for fractures before (33.50±6.500), Umer *et al*., (2009) in Pakistan (35.50±6.600), Khaliq *et al*., (2022) also in Pakistan (33.190), Hofman *et al*., (2017) in Germany (35.81±9.560), Han *et al*., (1998) who focused on Koreans above 20 years (32.50±6.400), Baharudeen *et al*., (2011) in Malaysia (31.69±5.480), Werner *et al*., (2012) in USA (33.600) and Jacobsen and Sonne-Holm (2005) in Denmark (34.600).

Table 3
The differences in the mean values of the general descriptive statistics of the subjects based on gender

	MALES			FEMALES				
	Mean ± SEM	**Min**	**Max**	**Mean ± SEM**	**Min**	**Max**	**T-test**	**P value**
AGE (Years)	34.70±2.34	19.00	60.00	32.26±2.55	18.00	58.00	-0.707	0.483
BMI (Kg/m^2)	34.03±1.72	16.60	38.30	29.15±1.35	20.50	36.70	5.96	0.000*
CEA (Degrees)	26.57±0.74	20.31	33.33	28.39±1.32	20.12	35.44	1.251	0.217
SHARP (Degrees)	39.18±1.23	30.33	51.35	38.80±1.21	30.32	48.33	-0.222	0.825

(*) – P value less than 0.05 is significant; BMI – Body mass index; CEA – Center edge angle of Wiberg; SHARP – Acetabular angle of Sharp

Acetabular angle of Sharp has been widely credited with the capacity to compensate for the replete shortcomings of the center edge angle of Wiberg and as a result its evaluation is considered more reliable for the diagnosis of acetabular dysplasia (Jeremic *et al*., 2011; Baharudeen *et al*., 2011), however, Soydan *et al*., (2021) reiterating the relevance of center edge angle of Wiberg, succinctly stated that it has a significant correlation with the development of osteoarthritis. It was observed in this study that the mean acetabular angle of Sharp was 39.00±0.860. Similar value was noted in the studies done by Hassa *et al*., (2023) in Turkey (37.8±2.330), Jeremic *et al*., (2011) in Serbia (38.00±3.800), Vuralli *et al*., (2022) in Turkey (39.22±2.600), Umer *et al*., (2009) in Pakistan (37.80±4.370) and Han *et al*., (1998) in Koreans (37.00±3.700). In contrast, higher values of the acetabular angle of Sharp have also been reported such as those of Nishimura *et al*., (2023) in Japan (44.40±3.100) and Baharudeen *et al*., (2011) in Malaysians (42.35±3.260). Generally, the outcome of the comparisons of the acetabular parameters obtained in this study lucidly shows that our results displayed substantial similarity with studies done on populations in Turkey, Japan, Serbia, Pakistan and Korea.

Gender wise, we found out that the mean center edge angle of Wiberg was lower in males (26.57± 0.740) than females (28.39± 1.320) but this difference was not significant (P=0.217). In consonance with our results Vuralli *et al*., (2022) in Turkey observed in their study that the mean value of the center edge angle of Wiberg was marginally higher in females than males (33.68±1.860 vs 32.0±1.890) and this difference was significant (P<0.001). Busato *et al*., (2021) also in agreement noted that the mean center edge angle of Wiberg was significantly higher in Brazilian females (35.50±9.500 vs 32.60±7.100; P=0.013). In a dissimilar trend, several literatures reported that males had a higher center edge angle of Wiberg. The compendium included the reports of Jeremic *et al*., (2011) in Serbia (33.60±5.800 vs 33.30±6.900), Hong *et al*., (2023) in Korea (37.41±6.500 vs 31.67±5.400; P <0.001), Nishimura *et al*., (2023) in Japan (29.80±4.600 vs 27.50±5.000; P<0.001), Umer *et al*., (2009) in Pakistan (36.28±6.440 vs 34.57±6.780; P=0.004), Busato *et al*., (2021) in Brazil (35.50±9.500 vs 32.60±7.100; P=0.013) and El-Heis *et al*., (2018) in Jordan (38.00±5.810 vs 38.00±5.790; P=NS).

On the other hand, the acetabular angle of Sharp was observed to be higher in males in our study (39.18±1.230 vs 38.80±1.210) and the difference was also not significant (P=0.825). In accordance with our finding, Vuralli *et al*., (2022) in Turkey, shared similar opinion as they demonstrated that the mean acetabular angle of Sharp was significantly greater in males (39.89±2.350 vs 38.45±2.660; P<0.001), Hutabarat *et al*., (2018) in Indonesia, also observed that the mean acetabular angle of Sharp was higher in males than females (59.76±7.740 vs 58.08±6.350). In a deviation from our finding, females were shown to possess a higher acetabular angle of Sharp compared to males as stated by the reports of Jeremic *et al*., (2011) in Serbia (38.50±3.900 vs 37.50±3.600), Nishimura *et al*., (2023) in Japan (44.30±3.100 vs 43.3±3.100; P<0.001), Lavy *et al*., (2003) in Malawi (38.60±4.900 vs 36.90±4.000; P<0.005) and Ege *et al*., (2016) in Turkey, (38.50±2.100 vs 37.90±2.500; P=0.0001). It can be inferred that the acetabular morphology of the male population in the region of this study was more susceptible to develop acetabular dysplastic changes than the hips of the female population.

The prevalence of acetabular dysplasia was found to be 4% in this study which was in divergence with other literatures. Slightly higher prevalence rates of acetabular dysplasia were observed in the researches conducted by Soydan *et al*., (2021) in Turkey (8.63%) and Busato *et al*., (2021) in Brazil (6%). At the other spectrum of findings, lower prevalence predominated in the reports of Jeremic *et al*., (2011) in Serbia (2.9%) and El-Heis *et al*., (2018) in Jordan (2.2%). It can be inferred that the prevalence of acetabular dysplasia in this study was lower than the prevalence in Turkey and Brazil but higher than what was reported in Serbia and Jordan. In this study the prevalence of acetabular dysplasia was 4% in males and 0% in females. A contradictory postulation with respect to the gender inclination observed in this study was highlighted by Soydan *et al*., (2021) in Turkey and Jeremic *et al*., (2011) in Serbia, who reported a prevalence of 7.26% in males and 10.30% in females, and 2.20% in males and 3.60% in females, respectively. However, Hassa *et al*., (2023) discovered in their research that the prevalence of acetabular dysplasia was similar in both gender (males - 1.9% vs females - 1.4%; P=1.000). Our findings suggests that 4% of the population within the age group of this study are afflicted with acetabular dysplasia and are currently at risk of having functional impairment of the affected hip joint.

This study buttressed the validity of employing either the technique of center edge angle of Wiberg evaluation or acetabular angle of Sharp evaluation in the determination of acetabular dysplasia by demonstrating the existence of a significant relationship between both methods (r=0.722; P=0.000). Umer *et al*., (2009) in Pakistan, aligned with our study as they concisely reported that the center edge angle of Wiberg correlates significantly with the acetabular angle of Sharp (r=0.393, P<0.001). Still in accordance with the findings of this study, Soydan *et al*., (2021) in Turkey noted that there was a moderate correlation between center edge angle of Wiberg and acetabular angle of Sharp (r=0.650). On this premise both methods can be regarded as complementary in the diagnosis of acetabular dysplasia. In variance, Jeremic *et al*., (2011) in Serbia, reported that there was no significant relationship between the acetabular angle of Sharp and the center edge angle of Wiberg (P>0.05).

In this study, it was observed that age was significantly associated with center edge angle of Wiberg and acetabular angle of Sharp (P=0.000, respectively) and furthermore, abnormal values following acetabular morphological evaluation were predominantly exhibited by individuals below 40 years. In the same vein, Tugrul *et al*., (2020) whose study was done on healthy children between 5 and 14 years in Turkey, found out that center edge angle of Wiberg significantly correlated with age (r=0.511, P=0.000). In addition, Hofmann *et al*., (2017) in Germany, lucidly observed that age had a significant correlation with center edge angle of Wiberg (r=0.31; P<0.001) and acetabular angle of Sharp (r=-0.38; P<0.001). Nishimura *et al*., (2023) in Japan, observed that there was a significant correlation between age and the acetabular angle of Sharp (r=-0.006; P=0.040) and the center edge angle of Wiberg (r=0.007; P=0.030). Similarly, Umer *et al*., (2009) in Pakistan, observed that abnormal values of the acetabular angle of Sharp was significantly associated with young age groups

(r=-0.825; P=0.022). Nishimura *et al*., (2023) in Japan, opined that the acetabular angle of Sharp in young age group was demonstrated to have larger angles in their study which was in keeping with the development of acetabular dysplasia. Further corroborating our findings, Adanir and Zorer, (2018) in Turkey, explicitly opined that as the center edge angle of Wiberg reduces to abnormal values, which is in keeping with the onset of acetabular dysplasia, the age at which osteoarthritis occurs proportionately reduces. In complete disagreement, Jeremic *et al*., (2011) in Sebia, found out that there was no significant association between age and the development of acetabular dysplasia.

Amazingly, we observed that marital status was significantly associated with center edge of Wiberg (P=0.002) and acetabular angle of Sharp (P=0.000) and most of the married subjects in this study had abnormal values. As a way of unravelling this relationship, Polenick *et al*., (2015) found out that the severity of osteoarthritis, which might have been a result of acetabular dysplasia, generally increases when there is a corresponding reduction in spouse life satisfaction over a 6 months period (P=0.020). However, when spousal closeness persists, an increase in spouse life satisfaction leads to a reduction in the severity of osteoarthritis (P=0.049). This suggests that family disharmony has a place in the modulation of the severity of osteoarthritis and consequently determines the capacity of the hip joint to function properly. Moreover, Jorgensen *et al*., (2011) realized that being married, marginally heightened the risk of hip osteoarthritis in the Danish population than being unmarried.

In this study it was realized that employment status was significantly associated with center edge angle of Wiberg (P=0.001) and acetabular angle of Sharp (P=0.002). A preponderant number of the subjects who were employed exhibited abnormal values of center edge angle of Wiberg and acetabular angle of Sharp. Illuminating the possible underlying basis for this association, Coggon *et al*., (1998) in England, found out that males whose jobs entailed lifting loads weighing 10 kg or more on a regular basis for a long duration had a higher incidence of hip osteoarthritis, likely as a sequela of acetabular dysplasia, and its severity was directly proportional to the weight of the loads and length of exposure to such jobs. There was however, no such association noted in women. Work posture was also demonstrated to have a significant place in the occurrence of hip osteoarthritis, as affirmed by Pratama *et al*., (2023) in Indonesia, (P=0.002).

Croft *et al*., (1992) in UK, also observed that the odds of acquiring hip osteoarthritis likely through acetabular dysplasia, substantially increased in males whose jobs involved standing for more than 2 hours in a day over a period of about 40 years (OR 2.7; CI 95%: 1.0 – 7.3). Furthermore, Coggon *et al*., (1998) inferred that the risk of acquiring hip osteoarthritis was increased in women whose jobs entailed walking a distance of 3.2 km per working day. In addition, long period exposure to frequent climbing of stairways during working hours was shown to greatly increase the risk of hip osteoarthritis in both males and females. Jorgensen *et al*., (2011) in Denmark and Ali *et al*., (2021) in Pakistan, concisely stated that males and females with low-income jobs were frequently exposed to conditions that lead to the development of osteoarthritis (P=0.001) (Adanir and Zorer, 2018). Teichtal *et al*., (2015) in Melbourne, Australia, observed that exposure to heavy lifting in individuals aged between 18 and 30 years was significantly associated with the risk of bone marrow lesions around the femoro-acetabular region (OR 3.9; CI 95%: 1.6 – 9.8; P < 0.010) and the risk of cartilage defects at the supero-lateral aspects of the femoral head (OR 1.6; CI 95%: 1.0 – 2.5; P = 0.04). These combined outcomes of exposure to heavy lifting induces a remodeling of the acetabular morphology in young age group to promote the onset of hip osteoarthritis. Acetabular dysplasia has been proven without any modicum of doubt to have a significant role in the manifestation of hip osteoarthritis (Soydan *et al*., 2021).

In this study we noticed that body mass index (BMI) was significantly associated with center edge angle of Wiberg (P=0.000) with the BMI of the male subjects significantly greater than their female counterparts (P=0.000). Most of the subjects in this study with abnormal center edge angle of Wiberg were overweight. Aligning with our findings, Adanir and Zorer, (2018) in Turkey attributed the extremely high rate of acetabular dysplasia, to the high BMI of the female participants in their study. In fact, they recorded an immense number of females with a center edge angle of Wiberg below 200 and the female to male ratio observed in this category was 4.1:1. However, in this study the male population rather had a higher BMI. In tangent to this finding, Nishimura *et al*., (2023) in Japan, found out that BMI had no correlation with center edge angle of Wiberg (P=0.22) and acetabular angle of Sharp (P=0.21). Cevik and Cicek (2020) in Turkey, also opined that the BMI of an individual has no bearing on the acetabular morphology.

Evaluation of the acetabular morphology in this study was conducted in one health facility, which was a limitation, and as such may not be regarded as a true representation of the population of this locality. Multi-center study for the evaluation of center edge angle of Wiberg and acetabular angle of Sharp with BMI matched subjects, and with a larger population is highly recommended. This study only included subjects devoid of hip joint abnormality. However, it would have been more enlightening to investigate the center edge angle of Wiberg and acetabular angle of Sharp in subjects with primary osteoarthritis. Even though the 2D image evaluation suffices for the diagnosis of acetabular dysplasia, a 3D image for acetabular volume-surface evaluation will provide more crucial and wholesome information on the status of the acetabulum.

In conclusion, the center edge angle of Wiberg and the acetabular angle of Sharp in our population are similar to the values in some European and Asian populations. Being married, employed, overweight and below 40 years are significantly associated with acetabular dysplasia and by implication are determinants of hip joint function. The prevalence of acetabular dysplasia is low (4%) in this locality and overweight males are highly susceptible to develop acetabular dysplasia.

Long term employment in a low-income job that involves physically carrying heavy items while maintaining abnormal postures, standing for hours, frequent use of stairways and walking long distances to achieve the objectives of one's employers all contribute to alter the morphology of the acetabulum and promote the events that ultimately impair hip functions. In addition, persistent marital disharmony resulting from the spouses living apart or growing apart while living together is a factor, with a

psycho-somatic basis, that regulates the severity of osteoarthritis, which further deteriorates a deformed acetabulum, and also adversely affects the functions of the hip joint as a consequence.

Acknowledgement

We are exceedingly grateful for the commitment and dedication of the staff of the Radiology Department, University of Calabar Teaching Hospital towards the success of this research..

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Niger. J. Physiol. Sci. 39 (June 2024): 23 – 29
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Relationship between Serum Resistin and Insulin Resistance among Obese Non-Diabetic Patients in a Nigerian Tertiary Hospital

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Summary: The prevalence of obesity is soaring globally, particularly in Nigeria, where sedentary lifestyles and dietary changes contribute significantly to its rise. It is linked to various metabolic disorders, including insulin resistance (IR) which is also linked to diabetes mellitus, but the role of resistin, an adipokine, in this context remains contentious. This study aimed to investigate the relationship between serum resistin levels and insulin resistance among non-diabetic obese individuals in Nigeria. This was a hospital-based cross-sectional study involving 200 participants aged 18 to 65 years. Anthropometric measurements, fasting blood glucose, insulin levels, lipid profiles, and serum resistin were assessed. Insulin resistance was calculated using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). Statistical analysis was carried out with Mann-Whitney U-test and Spearman's rank correlation coefficient. The obese group exhibited significantly higher BMI, abdominal circumference, waist circumference, hip circumference, waist-hip ratio, fasting blood glucose, insulin levels, and HOMA-IR compared to controls. Lipid profiles and cardiovascular parameters were also significantly elevated in the obese group. However, serum resistin levels did not differ significantly between groups and showed no correlation with BMI or HOMA-IR. In conclusion, circulatory resistin levels were unaffected in non-diabetic obese individuals, suggesting a limited role in insulin resistance among this population. Other adipokines and inflammatory biomarkers may drive insulin resistance in obesity without diabetes. Resistin may not serve as a reliable predictive marker for insulin insensitivity or glucose homeostasis in non-diabetic obesity. Further research is warranted to elucidate the complex interplay of adipokines in metabolic dysfunction among obese individuals.

Keywords*: obesity, resistin, insulin resistance, adiposity, Nigerian, Type 2 diabetes mellitus*

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Manuscript received- February, 2024; Accepted: May, 2024

DOI: https://doi.org/10.54548/njps.v39i1.4

INTRODUCTION

Globally, the prevalence of obesity has taken an upward trend across all age groups and socioeconomic strata. This trend is not different in Nigeria with the reported rise in the prevalence of obesity, particularly among urban populations and women, a situation attributed partly to sedentary lifestyles and a surge in processed food consumption (Adeloye *et al*., 2021). For instance, the prevalence of overweight and obesity was put at 20.3% and 11.6%, respectively, with around 12 million persons in Nigeria estimated to be obese in 2020. This is indeed worrisome given the socioeconomic burden, psychological impacts, and the heightened morbidity and mortality associated with obesity (Okunogbe *et al*., 2021; Wanjau *et al*., 2023).

Obesity, characterized by excessive accumulation of body fat, places a heavy burden on the metabolic machinery of the body system, thus serving as a potential risk factor for a broad spectrum of debilitating pathophysiological conditions, including insulin resistance (IR), type II diabetes mellitus (T2DM), cardiovascular diseases, and various malignant diseases (Volpe & Gallo, 2023; Yashi & Daley, 2024). The determination and classification of obesity often rely on Body Mass Index (BMI), with the World Health Organization (WHO) defining obesity as a BMI ≥30 kg/m² and overweight as BMI ≥25kg/m² (Woolcott & Seuring, 2022). While BMI serves as a valuable population-scale measure of obesity due to its simplicity and correlation with health risks, other indices such as waist circumference, waist-hip ratio, and body adiposity index provide additional insights into adiposity distribution and associated health risks (Dalton *et al*., 2003).

It is well established that obesity and augmented adiposity contribute to insulin resistance, which in turn cascades into a vicious cycle of glucose intolerance and accumulation of abnormal lipids in the circulation. These interrelated metabolic disturbances significantly increase the risk of developing type 2 diabetes mellitus and

cardiovascular diseases (Kosmas *et al*., 2023; Wondmkun, 2020). The progressive accumulation of fat in adipose cells, independently boosts the synthesis and the release of resistin, one of the range of peptides produced by adipose tissues. This category of molecules, which includes leptin, adiponectin, and other chemokines, have been identified to play pivotal roles in the regulation of appetite, energy expenditure, inflammation, and insulin sensitivity(Clemente-Suárez *et al*., 2023; Yamauchi & Kadowaki, 2013). To this end, a rise of circulatory resistin has been linked with heightened risk of glucose intolerance, dyslipidemia, inflammatory responses, and a variety of unpleasant cardiovascular outcomes (Lehr *et al*., 2012; Park *et al*., 2017).

Specifically, resistin acts by interacting with various proteins involved in inflammatory and metabolic pathways, and interferes with insulin signaling pathways, leading to decreased phosphorylation of insulin receptor substrate 1 (IRS-1) and the inhibition of insulin action in target tissues, consequently, contributing to insulin resistance in various tissues. As such, suppressed adiponectin levels and reciprocal elevation of circulatory resistin levels have been proposed as predictive markers for future diabetes risk in obese individuals (Su *et al*., 2019).

Furthermore, insulin resistance, characterized by decreased responsiveness of target tissues to insulin, is a central feature of obesity-related metabolic disturbances. The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), a measure derived from fasting glucose and insulin levels, serves as a marker for evaluating insulin resistance and impaired insulin sensitivity in excess adiposity (Matthews *et al*., 1985). Therefore, in obese individuals, the dysregulation of glucose and lipid metabolism leads to elevated HOMA-IR scores, which could be interpreted as impaired insulin signaling pathways and glucose homeostasis. Furthermore, studies suggest that resistin levels are positively correlated with HOMA-IR values, indicating a potential role in exacerbating insulin resistance (Steppan *et al*., 2001).

As it stands, the relationship between resistin, IR, and lipid profile in humans is enveloped in equivocation, with some studies suggesting positive correlations between resistin with IR and lipid profile in obese individuals (Pandey, 2018), while other investigations suggest otherwise (Amirhakimi *et al*., 2011). Also, there is paucity of information on the possible role of resistin in the obese non-diabetic in our environment. Although the prevalence of obesity is on the upward trend in Nigeria accompanied by a range of metabolic disorders like insulin resistance and type 2 diabetes mellitus (T2DM), the specific factors contributing to insulin resistance in obese individuals are not well understood in the study population. Investigating the relationship between resistin and insulin resistance in obese Nigerian patients is crucial to the understanding of the underlying mechanisms of metabolic dysfunction in this population. Therefore, this current study attempts to investigate the relationship between serum resistin and insulin resistance among non-diabetic obese patients.

MATERIALS AND METHODS

Ethical Consideration: Approval was obtained from the Health Research and Ethical Committee of the Lagos University Teaching Hospital with protocol number ADM/DCST/HREC/APP/2775 on 3rd April 2019, and was conducted in compliance with the Declaration of Helsinki (Ehni & Wiesing, 2024). Prior to inclusion in the study and data collection, informed and written consent was obtained from all participants. Each recruited participant was assigned numerical codes, and their clinical data and test results were securely stored to maintain confidentiality during the study.

Study Design and Setting: This was a cross-sectional study conducted at Lagos University Teaching Hospital (LUTH), Lagos State, Nigeria, a tertiary hospital which serves as a referral center with approximately 800-bed spaces. The study was conducted between May 2019 and May 2020 (a period of 12 months).

Participants in this study included male and female subjects aged 18 to 65 years attending the LUTH General Outpatient Clinic and the Obesity and Metabolic Clinic of the Department of Clinical Pathology who were recruited using a simple random sampling method based on the order of registration. The minimum sample size was determined to be 97 using the formular ($n = [Z2.P.(1 – p)] \div E2$) (Daniel & Cross, 2019) where N is the sample size, Z is the standard deviation (1.96), p is the prevalence of obesity in Nigeria (6.8%)(Chigbu, 2018), and d is the level of significance (0.05). A sample size of 200 (100 each for the obese and control groups) was used.

The study included apparently healthy men and women aged 18 to 65 years with a BMI equal to or greater than 30 kg/m², classified as obese participants, while control participants comprised apparently healthy individuals with a BMI ranging from 18.5 kg/m² to 24.9 kg/m². Exclusion criteria included diabetic individuals, cancer patients, smokers, and pregnant women due to the potential confounding factors impacting resistin levels and BMI calculations.

Data Collection: Patient demographic and clinical data was obtained by the use of a structured interviewer-administered questionnaires. Anthropometric measurements like weight was measured using a weighing scale, height using a stadiometer, and BMI was calculated from the weight and height measurements.

Waist circumference was measured using a stretch-resistant tape, wrapped around the subjects at a level parallel to the floor, midpoint between the top of the iliac crest and the lower margin of the last palpable rib in the mid auxiliary line while the hip circumference was at a level parallel to the floor, at the largest circumference of the greater trochanter. Both measurements were taken three times for each participant. Abdominal obesity was defined using the recommended criteria of waist circumference >88 cm in women and greater than 102cm in men while Waist-hip ratio was computed by dividing the waist circumference by the hip circumference, both in centimeter.

Measurement of cardiovascular parameters: Following a 5 minute of rest in sitting position, systolic and diastolic blood pressure (SBP and DBP) were recorded three times within 1-2 minutes interval with automated sphygmomanometer (Omron), which has been standardized with mercury sphygmomanometer prior to the study. The

mean of the two closest readings for SBP, DBP and pulse rates are then recorded for the participants. The mean arterial pressure (MAP) was subsequently calculated as follows: DBP + 1/3(SBP - DBP), while myocardial oxygen demand (MOD) was derived as the product of pulse rate and SBP.

Sample Collection and Assays: Ten milliliters of venous blood were collected from each participant and stored at -20°C for up to three months before analysis. Glucose levels were measured using a quantitative-enzymatic-spectrophotometric method involving the glucose oxidase (GOD) method. Insulin levels were determined using a Calibiotech ELISA kit, which involves a solid-phase enzyme immunoassay with monoclonal antibodies against insulin, measured at 450 nm. Insulin resistance was calculated using the HOMA-IR formula: (glucose × insulin)/22.5.

Serum lipids, including total cholesterol, triglycerides, and HDL-cholesterol, were also measured using quantitative-enzymatic-spectrophotometric methods, with absorbance measured at 500 nm. Remnant cholesterol was calculated from a standard lipid profile as total cholesterol minus the LDL cholesterol and HDL cholesterol (Nordestgaard & Varbo, 2014). Serum resistin levels were analyzed using a Human RETN ELISA kit, employing a sandwich-ELISA method with pre-coated microplate wells containing antibodies specific to human resistin, and absorbance was measured at 450 nm.

Statistical Analysis: Data were presented as medians and interquartile ranges (IQR) to describe the distribution of variables in the two groups of participants. The Mann-Whitney U test, also known as the Mann-Whitney-Wilcoxon test, was used to compare differences between the medians of the two groups with non-parametric data distribution and it was conducted with a confidence interval of 95%. Statistical analysis was performed using SPSS GraphPad 5 software package (USA). Additionally, correlations between serum resistin levels and various clinical parameters were analyzed using Spearman's rank correlation coefficient. All statistical tests were two-tailed, and p-values less than 0.05 were considered statistically significant.

RESULTS

There were no significant differences in mean age, gender distribution, education level, or religion between the obese and control groups. However, a marginally significant difference was observed in marital status, with a higher percentage of the obese group being married compared to the control group (n=81 versus n=69; $\chi^2 = 3.840$, p = 0.050) (Table 1). Significant differences were found in several anthropometric and glycemic parameters between the obese and control groups.

Table 1:
Sociodemographic characteristics of the study population (n= 200)

Variable		Obese (N=100)	Control (N=100)	X^2	p-value
Age *(year; mean* ±SD*)*		4 1.5± 11.7	40.5 ± 12.8	-0.600	0.549
Gender	Male	39 (39.0%)	45 (45.0%)	0.739	0.390
	Female	61 (61.0%)	55 (55.0%)		
Marital status	Single	19 (19.0%)	31 (31.0%)	3.840	0.050
	Married	81 (81.0%)	69 (69.0%)		
Education	None	1 (1.0%)	3 (3.0%)	4.532	0.209
	Primary	4 (4.0%)	6 (6.0%)		
	Secondary	29 (29.0%)	39 (39.0%)		
	Tertiary	66 (66.0%)	52 (52.0%)		
Religion	Christian	85 (85.0%)	87 (87.0%)	0.166	0.684
	Muslim	15 (15.0%)	13 (13.0%)		

independent t-test statistic,Chi-square statistic,*
[a]Fisher's exact test, adjusting for cells containing values less than 5

The obese group had significantly higher median BMI (33.4 kg/m² versus 25.3 kg/m², p < 0.0001), abdominal circumference (104.0 cm versus 86.0 cm, p < 0.0001), hip circumference (112.0 cm versus 98.0 cm, p < 0.0001), waist circumference (100.0 cm versus 84.0 cm, p < 0.0001), and waist-hip ratio (0.88 versus 0.84, p = 0.0002). Additionally, median fasting blood glucose (5.20 mmol/L versus 4.60 mmol/L, p = 0.0001), insulin levels (13.0 µU/mL versus 4.81 µU/mL, p < 0.0001), and HOMA-IR (3.02 versus 0.93, p < 0.0001) were significantly higher in the obese group. Notably, there was no significant difference in serum resistin levels in both groups of participants (Table 2).

In terms of lipid profiles and cardiovascular parameters, the obese group had significantly higher median triglyceride levels (1.00 mmol/L versus. 0.88 mmol/L, p = 0.0004), total cholesterol (4.34 mmol/L versus. 4.21 mmol/L, p = 0.017), LDL cholesterol (2.73 mmol/L versus. 2.54 mmol/L, p = 0.009), and remnant cholesterol (0.45 mmol/L versus. 0.40 mmol/L, p = 0.002).

Table 2:
Arthrometric and Glycemic parameter in non-obese and obese Nigerians

Parameters	Non-obese median	IQR	Obese median	IQR	P value
BMI (kg/m2)	25.3	23.3-27.1	33.4	33.34 -36.31	<0.0001
Abdominal circumference	86.0	81.0-94.0	104.0	96-112.5	<0.0001
Hip circumference	98.0	93.0-104.0	112.0	108-119.0	<0.0001
Waist circumference	84.0	79.0-91.0	100.0	93.0-107.0	<0.0001
Waist-hip ratio	0.84	0.81-0.90	0.88	0.84-0. 0.94	0.0002
FBG (mg/dl)	4.60	4.2-5.2	5.20	4.55-5.90	0.0001
Insulin (mIU/ml)	4.81	3.30-6.77	13.0	9.44-19.31	<0.0001
HOMA-IR	0.93	0.67-1.41	3.02	2.16-4.71	<0.0001
Resistin	1.63.	1.45-1.74	1.63	1.52-1.70	0.586

*Data presented as median, and the distribution between the non-obese and obese compared with Mann -Whitney test; *p<0.05 vs non-obese is significantly higher. MOD: myocardial oxygen demand of oxygen*

Conversely, HDL cholesterol was significantly lower in the obese group (1.07 mmol/L versus. 1.23 mmol/L, p = 0.0003). The obese group also had significantly higher median systolic blood pressure (132.0 mmHg versus. 125.0 mmHg, p = 0.033), diastolic blood pressure (86.0 mmHg versus. 81.0 mmHg, p = 0.033), mean arterial pressure (96.0 mmHg versus. 88.0 mmHg, p = 0.033), and myocardial oxygen demand (10578.0 versus. 9690.0, p = 0.015) (Table 3).

Regarding serum resistin levels, there were no statistically significant differences across BMI categories or HOMA-IR levels. There was no significant correlation between serum resistin levels and BMI (r = 1.780, p = 0.152). as well as serum resistin level and HOMA-IR (r = -0.613, p = 0.540) (Table 4).

Table 3:
Relationship between serum resistin, body mass index and HOMA-IR among the study participants

Variable	n	Serum resistin (Mean±SD)	Spearman's correlation	p value
BMI				
Normal	100	1560.53±285.04	1.780	0.152
Obese class I	66	1465.66±436.04		
Obese class II	23	1591.81±200.17		
Obese class III	11	1641.67±55.72		
HOMA-IR				
<2	110	1524.25±345.92	-0.613	0.540
≥2	90	1553.22±314.64		

Table 4:
Lipid Profiles and Cardiovascular parameter in non-obese and obese participants

Parameters	Non-obese(n=100)	IQR	Obese (n=100)	IQR	p value
Triglycerides (mmol/L)	0.88	0.68-1.12	1.00*	0.85-1.42	0.0004
Total Cholesterol(mmol/L)	4.21	3.62-4.73	4.34*	3.79-5.28	0.017
HDL Cholesterol(mmol/L)	1.23	1.03-1.42	1.07*	0.89-1.24	0.0003
LDLCholesterol(mmol/L)	2.54	1.97-2.95	2.73*	2.24-3.57	0.009
Remnant Cholesterol	0.40	0.31-0.51	0.45*	0.85-1.42	0.002
SBP (mmHg)	125.0	118.0-142.0	132.0*	121.5-144.5	0.033
DBP (mmHg)	81.0	75.0-91.0	86.0*	80.0-92.0	0.033
MAP (mmHg)	81.0	75-91	86.0*	80.0-92.0	0.033
MOD	9690.0	8160-11543	10578*	9348.0-12077	0.015

Data presented as median, and the distribution between the no-obese and obese compare with Mann -Whitney test ; *p<0.05 versus non-obese is significantly higher. MOD: myocardial oxygen demand of oxygen

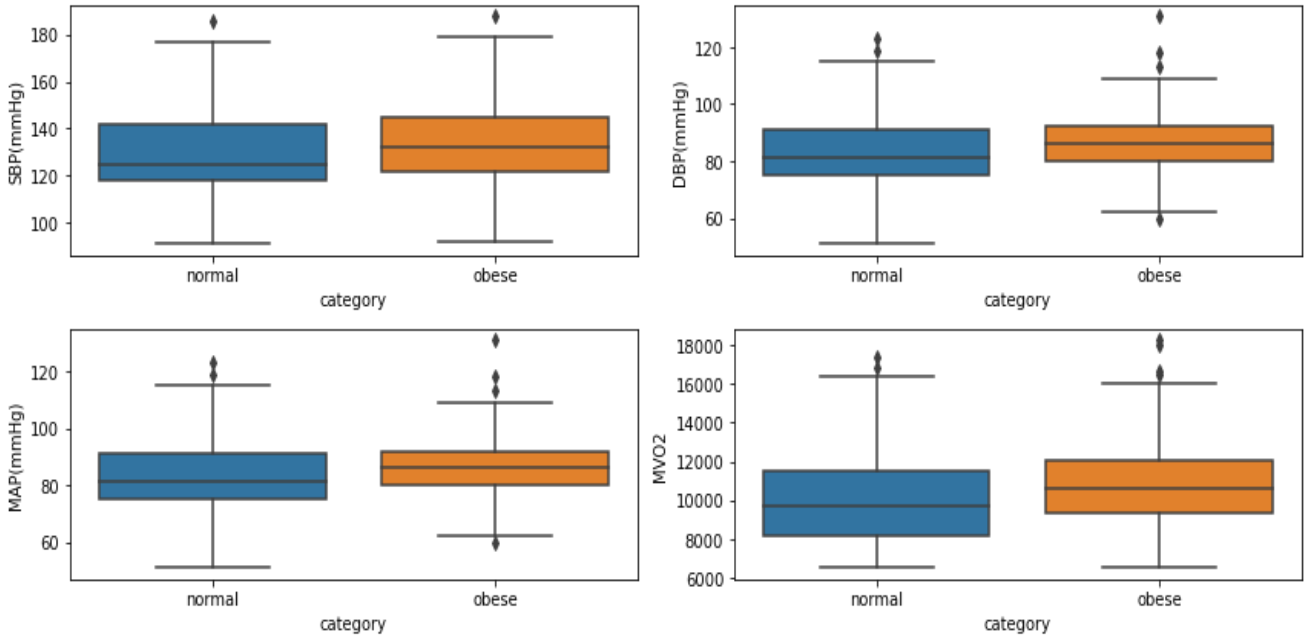

Figure 1:
Distribution of FBG, serum insulin ,HOMA-IR I and Resistin in obese and non-obese participants, with the obese showing significantly higher median value with the exception of resistin ,*p<0.05, Manny-Whitney test

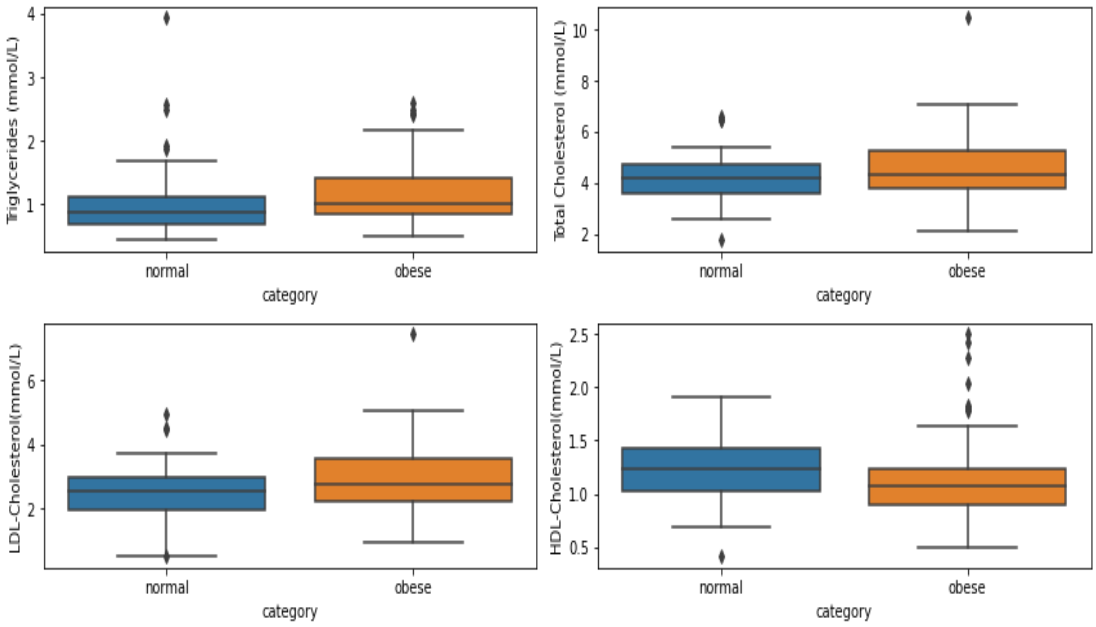

Figure 2:
Distribution of SBP, DBP, MAP and MOD in obese and non-obese participants, with the obese showing significantly higher median value,*p<0.05, Manny-Whitney test.

DISCUSSION

The obese group in our current study demonstrates clear evidence supporting the presence of insulin resistance otherwise referred to as insulin insensitivity, as reflected by higher HOMA-IR and corresponding elevated fasting serum insulin level in them compared to the records in the non-obese group. The higher HOMA-IR indicates that insulin resistance was significantly higher in the obese condition compared to the non-obese condition, an observation that is in line with previous study reporting an unprecedented high prevalence of insulin resistance in young non-diabetic obese persons (Elrayess *et al*., 2020). This is indeed instructive, as our finding invariably suggests that the obese participants in our study are currently prediabetic and could be on their ways to full-blown diabetic condition.

Enhanced tissue adiposity, characterized by excessive fat in visceral tissue, is a fundamental hallmark in obesity with the potential to drive insulin resistance (Hocking *et al*., 2013). In particular, hyperinsulinemia typically results from attenuated peripheral responses of adipose tissue and muscle cells to insulin signal (Saltiel & Kahn, 2001). Without equivocation, the participants with obesity clearly presented with evidence of augmented adiposity as supported by their higher abdominal circumference, waist circumference, hip circumference, waist-hip-ratio, and BMI characteristic of increased adiposity (Després & Lemieux, 2006; Jeong *et al*., 2023; Ross *et al*., 2020).

Aside from serving as a potential breeding ground for insulin resistance, exaggerated tissue adiposity especially in visceral cells is also a trigger for dyslipidemia (Fabbrini *et al*., 2009; Vega *et al*., 2006), a metabolic condition characterized by accelerated release of fatty acids from adipose tissue, suppressed clearance of blood cholesterol resulting from reduced HDL and reciprocal elevated LDL. The picture above of lipid dysregulation with overall accentuation of total blood cholesterol, LDL cholesterol, remnant cholesterol and triglyceride in the obese group in our study aligns with previous study (Finn *et al*., 2010; Vekic *et al*., 2019). It is also imperative to state that insulin resistance influences the accumulation of bad fat in blood as observed in this current study, and the plausible mechanisms advanced for this is the disruption of lipid metabolism via the potentiation of hepatic fat release, blunted lipoprotein lipase activity, and increased LDL cholesterol at the expense of HDL cholesterol (Chung *et al*., 2022; Liu & Li, 2015). Overall, the dyslipidemia engendered by insulin resistance and augmented adiposity is an important cardiovascular risk factor implicated in arterial pathological condition like coronary heart disease. In particular, high concentrations of remnant cholesterol as seen in the obese group in our study, has been identified as a potential residual risk of cardiovascular disease even when LDL cholesterol is lowered (Langsted *et al*., 2020).

It is therefore not surprising to observed a higher level of systolic, diastolic and mean arterial blood pressure with corresponding rise in myocardial oxygen demand in the obese group in our study. Most importantly, the combined elevation of remnant cholesterol and myocardial oxygen demand in the obese group further accentuates the risk of coronary heart disease among this group. The central role played by insulin resistance in driving blood pressure involved interconnected mechanisms which increases sympathetic nervous system activity (Russo *et al*., 2021), endothelial dysfunction (Muniyappa *et al*., 2020), renal sodium retention (Brosolo *et al*., 2022), renin-angiotensin-aldosterone system activation, proinflammatory oxidative stress (Sarafidis & Ruilope, 2006), disruptions in vascular growth and remodeling (Muniyappa *et al*., 2007). Furthermore, while obesity results in adiposity dysfunction, insulin resistance is also known to promote adipose tissue dysfunctions. Therefore, it is held that insulin resistance and adipose tissue dysfunction works in vicious cycle, with each condition exacerbating the other to worsen the progression of metabolic syndrome in obese patients.

Furthermore, adipose tissue performs pertinent metabolic regulatory functions through the actions of different molecules referred to as adipokines that could be potentially exaggerated in obesity, insulin resistance and other metabolic conditions. These molecular peptides

include resistin, adiponectin, leptin, chemerin, visfatin, and vaspin, adipokines alongside other inflammatory cytokines and chemokines. Surprisingly, resistin was unaltered in the obese group when compared with the non-obese group, a finding that is at variance with a previous study that reported significantly higher level of circulatory resistin among obese non-diabetic Nigerians (Onyemelukwe *et al*., 2022), same with the result obtained in a study conducted among Saudi Arabian women (Al-Harithy & Al-Ghamdi, 2005).

Most importantly, some studies have indicated that resistin levels are often elevated in conditions related to insulin resistance, including condition like obesity and type 2 diabetes (Reilly *et al*., 2005; Yang *et al*., 2005). Therefore, contrary to our expectation resistin level was apparently unaffected in our non-diabetes obese group. Although the reason for this is somewhat elusive, some works have nonetheless provided empirical evidence that exaggerated insulin level possesses the potential to modulate and suppress resistin level (Lee *et al*., 2003). Perhaps, insulin resistance self-regulated the level of resistin it produces in some subsets of obese individuals who have not developed full-fledged type 2 diabetes mellitus. It is therefore safe to infer that the insulin resistance in our obese groups could have likely attenuated the anticipated exaggerated resistin response to insulin resistance among the investigated obese participants.

In conclusion, obesity did not significantly alter circulatory resistin among a cross-section of non-diabetic obese individuals in this study, rather the level was comparable to the non-obese group. Also, no association was found between circulatory resistin and insulin resistance. Other adiposity indices like BMI, waist-hip ratio and abdominal circumference were not also significantly correlated with circulatory resistin in the obese non-diabetic state. This invariably supports the preponderant of interplay of other adipokines and inflammatory biomarkers as the likely engine behind the observed insulin resistance in obesity with no associated diabetes mellitus. The apparent silence of circulatory resistin in obesity may explain the moderation in the non-diabetic fasting glucose level amidst this condition although noticeably high. Most likely resistin may not be the primary driver of insulin resistance until a threshold cut off is attained and exceeded. As such, circulatory resistin may not offer a high predictive value for insulin insensitivity and glucose homeostasis in the obese non-diabetic individual.

List of Abbreviations

BMI: Body Mass Index; IR: Insulin Resistance; T2DM: Type 2 Diabetes Mellitus; WHO: World Health Organization; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; LUTH: Lagos University Teaching Hospital; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; MAP: Mean Arterial Pressure; MOD: Myocardial Oxygen Demand; LDL: Low-Density Lipoprotein; HDL: High-Density Lipoprotein; IQR: Interquartile Range

Acknowledgements

We acknowledge the management of Lagos University Teaching Hospital, and College of Medicine University of Lagos, Nigeria and the Department of Clinical Pathology LUTH for granting access to patients attending the Obesity and metabolic clinic.

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Niger. J. Physiol. Sci. 39 (June 2024): 31 – 38
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Age-Related Effects of Carbohydrate-, Protein- And Fat-Rich Diets on Healing of Acetic Acid-Induced Gastric Ulcers in Rats.

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Summary: In this study, we compared the ulcer healing effect of carbohydrate-, protein- and fat-rich diets on acetic acid-induced ulcers in young and aged rats. Male Wistar rats (40 each at 3-, 6-, 12-, and 18 months old) were grouped into four to receive basal diet (control), carbohydrate-, protein-, or fat-rich diets for 21 days before acetic acid-induced gastric injury. After this, the various feedings continued for 3- and 7 days. Planimetry was used for the ulcer healing study. We estimated the redox status, pepsin, mucin, and nitric oxide activities by UV/Vis-spectrophotometer while the Epidermal Growth Factor-Receptor (EGF-R) was by immunohistochemistry. Data was analyzed (two-way ANOVA) and was considered significant at $p \leq 0.05$. Percentage ulcer healing by day 7 relative to day 3 decreased with advancing age in other diets but increased in the aged rats fed a protein-rich diet. Gastric carbonyl, malondialdehyde, and pepsin activities increased significantly with age, while superoxide dismutase, catalase, mucin, Nitric-oxide and EGF-R expression significantly decreased with age. Protein-rich diets modulated the age-related alterations. These findings suggest that a protein-rich diet facilitates the healing of acetic acid-induced gastric injury by enhancing gastroprotective activity to favour EGF-R expression in the ulcerated stomach.

Keywords*: Ageing, ulcer healing, protein, carbohydrate, fat, rats*

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Manuscript received- January 2024; Accepted: May 2024

DOI: https://doi.org/10.54548/njps.v39i1.5

INTRODUCTION

The percentage of the population over 65 years old is rising and is expected to increase to 21% by the year 2040 in the United States, with an increase in age-related diseases (Alan, 2017). During ageing there are structural and functional changes in multiple organs with associated increased inflammation (Guarner and Rubio-Ruiz, 2015) in the skin, lungs, digestive system and kidneys (Goldacre, 2009; Alan, 2017). Among these systems, the digestive system is the primary system that has the largest contact area with ingested pathogens and environmental factors (Soenen *et al*., 2016), which incessantly exposes gastric mucosa to the action of various irritants capable of injuring its structural and functional integrity to cause ulceration.

Gastric ulceration is a disease of chronic development characterized by necrotic abrasion, laceration, or injury in the mucosal and muscularis mucosae layer of the digestive tract. Considering its morbidity and mortality rates, digestive tract ulcer is among the most prevalent diseases affecting people worldwide (Xie *et al*., 2022). The leading cause is not well-known, but an imbalance between aggressive and cytoprotective factors in the mucosa is broadly recognized (Zhang *et al*., 2020). The multifactor-associated etiology includes Helicobacter pylori infection, genetic, free radicals formation, prolonged and excessive use of non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, cigarette smoking, and stress (Akpamu *et al*., 2016). Others may include nutritional deficiencies and poor dietary habits (Yegen, 2018). These conventional risk factors cannot explain several digestive tract ulcers, thereby supporting the possibility of other factors.

Reports show that digestive tract ulceration and its associated disorders and dysfunctions increase with ageing (Polo *et al*., 2012) due to changes in the defense and repair processes (Campos *et al*., 2000). The healing of ulcerated gastric mucosa, assessed by area and depth in rats, has been reported to be facilitated in young rats compared to aged rats (Ajayi and Olaleye, 2015). The ulcerated digestive tract healing is a dynamic process involving alleviating aggressive factors, deposition of epithelial and connective tissue cells, and mucosal reconstruction (Tarnawsaki *et al*., 2001). These processes are controlled by interacting with factors like diet, feeding habits, and pattern (Ogias *et al*., 2010) and growth factors (Osaki *et al*., 2011). Previous studies have reported epidermal growth factor receptor (EGF-R) as one of the ideal growth factors for wound healing, which can mitigate ulceration through its mechanism in the gastrointestinal tract (Khanbanha *et al*., 2014).

There are existing facts that ageing correlates positively with deceased mucosa protective factors (Polo *et al*., 2012) and ulcer healing (Ajayi and Olaleye, 2015). Therefore, there is concern about this population's digestive tract health status. Considering that reports on the investigation of the interaction between substances ingested, such as diet, their influence on gastric ulcer healing and gastric protective factors is unavailable in different ages. The main objective of the present study is to compare the effect of the carbohydrate-, protein- and fat-rich diet on the healing process of acetic acid-induced ulcers in young and aged rats.

MATERIALS AND METHODS

Animals and ethical approval of the study: Forty male Wistar rats were assigned and used per phase in this study at 3-, 6-, 12-, and 18 months old. To ascertain the ages of the rats, they were bred from birth in the Postgraduate Animal House, Department of Physiology, University of Ibadan, Ibadan, Nigeria, from parents obtained at the Central Animal House, College of Medicine, of the same institution. The housing of the rats was clean wire meshed plastic cages in a day-light and night-dark cycle. The rats were fed with designated feeds and allowed drinking water ad libitum.

Ethical approval was sought and obtained for the study from the Animal Care and Use Research Ethics Committee of the University of Ibadan with assigned number UI-ACUREC/18/0100. The investigation was conducted strictly following the approved guidelines and regulations of UI-ACUREC and in compliance with the International Guidelines for Laboratory Animal Care and Use Act of the National Institute of Health (NIH, 1985).

Experimental design: Rats were randomly grouped into four diet schemes for four age groups (Figure 1) as follows;

- Group 1: Control (10 rats each at 3-, 6-, 12- and 18-months); rats fed formulated standard diet (basal diet).
- Group 2: Carbohydrate group (10 rats each at 3-, 6-, 12- and 18-months); rats fed on a carbohydrate-rich diet.
- Group 3: Protein group (10 rats each at 3-, 6-, 12- and 18 months); rats fed on a protein-rich diet.
- Group 4: Fat group (10 rats each at 3-, 6-, 12- and 18 months); rats fed a fat-rich diet.

The diet formulation were based on different macro-nutrients, as described in a previous study by Adedeji and Olapade-Olaopa (2018). Rats were fed on these diets for 21 days before ulcer induction and continued for 3 and 7 days, during which we harvested five rats' stomachs for ulcer healing study.

Induction of acetic-acid ulcer model: The acetic acid ulcer model was used in this study because the model easily and reliably produces round, deep ulcers and high resemble human ulcers in both pathological features and healing processes (Okabe and Amagase, 2005). Acetic acid ulcer was induced as described by Ajayi and Olaleye (2015) with some modifications. Laparotomy was performed under a cocktail of anaesthesia (Ketamine, 0.0015ml/100g bwt and Xylazine, 0.0005ml/100g bwt). The stomach was exposed and gastric ulcer was induced by clamping the anterior and posterior glandular stomach walls with 9mm eye forceps, and 0.2 mL of 30% acetic acid solution was slowly injected into the secured area for 60 seconds and withdrawn. After rinsing the abdomen with normal saline, the abdominal openings were sutured back. Appropriate topical antibiotics were applied for 3 days on the surgical site to prevent infection. The rats were weighed every other day to determine their body weight, and the ulcerated stomachs were harvested 3- and 7 days post-ulceration, weighed, and harvested for macroscopic scoring and biochemical assays.

Asseement of the gross gastric ulcer scores: The stomachs were excised and cleansed using a phosphate buffer and placed it on filter paper to dry. The vertical and horizontal diameters of the formed gastric ulcer were measured using a plastic ruler under a magnifying lens. Ulcer Indexes (UI), Ulcer Area (UA), and Ulcer Healing Rate (UHR) were determined as described by Xiaoyum *et al*. (2007) with some modifications. The sum of the longest vertical and horizontal diameters across the formed ulcer was calculated as UI.

UA (mm^2) = π x (sum of vertical diameters / 2) x (sum of transverse diameters / 2).

Percentage ulcer healing (%H) was calculated by day 7 relative to day 3 as:

%H = ((UI at day 3 - UI at day 7) / (UI at day 3)) x 100%.

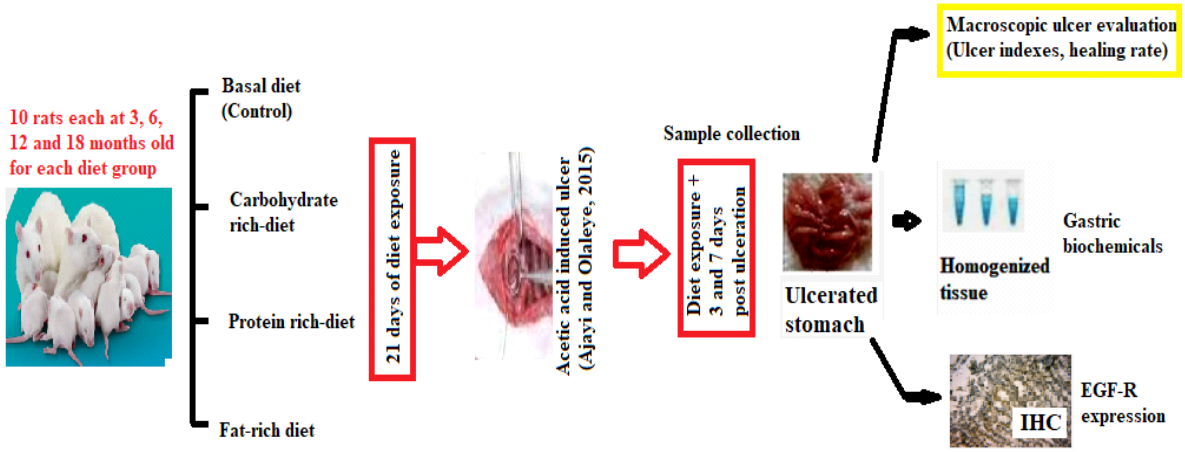


Figure 1:
Experimental design

Gastric biochemicals analysis: The cleansed harvested ulcerated stomachs were minced in ice-cold 0.1M phosphate buffer solution (10% of volume) and centrifuged (via a cold centrifuge) at 4°C (5,000 rpm for 15 minutes) as previously described (Salami *et al*., 2021). The supernatants were used for the estimations of gastric biochemical assays. Gastric tissue protein was estimated using the Biuret method. Malondialdehyde (MDA) levels were assessed by evaluating thiobarbituric acid reactive substances (TBARS) and mucin was estimated based on hexose component determination (Salami *et al*., 2021). The gastric carbonyl, catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), nitric oxide (NO) and pepsin activities were according to standardized method in Salami *et al*. (2021).

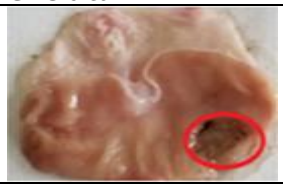
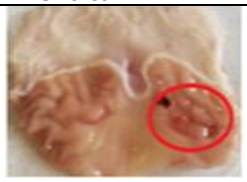
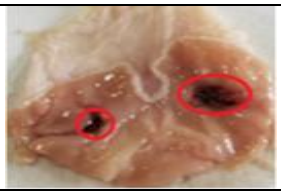
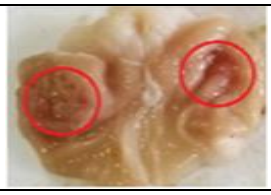
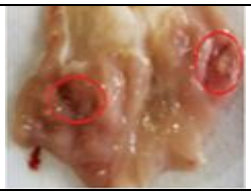
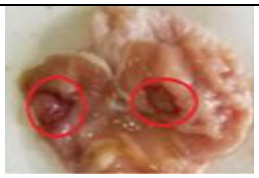
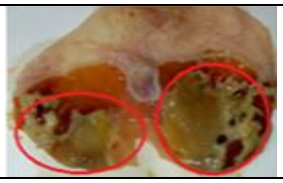
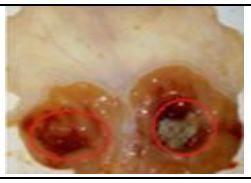
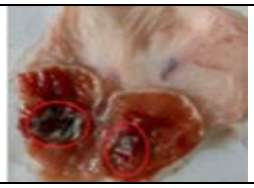
Immunohistochemistry study: Immunohistochemical staining was performed on the ulcerated stomach (5µm thickness of fixed stomach tissues embedded in paraffin) using EGF-R (Catalog No.: E-AB-31285) monoclonal antibodies in immunoperoxidase techniques as modified by Oyagbemi *et al*., (2018). The prepared slides were viewed and photomicrographs were taken at x40, x100 and x400 magnification with a digital microscope (VJ-2005 DN MODEL BIO-MICROSCOPR(R) and the immunoreactivity expression was quantified using TS View XC Image (R) Software, File version 6.2.5.3 and ImageJ (version 1.51).

Statistical analysis: Data was analyzed and presented as mean ± SEM of 5 rats in a group. Using GraphPad Prism (Version 5.0), two-way variant analysis and the Bonferroni post hoc test were considered significant at $p \leq 0.05$.

RESULTS

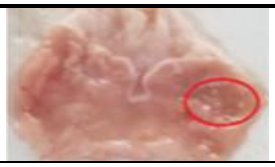
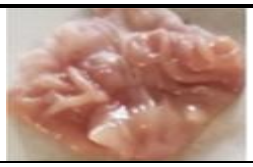
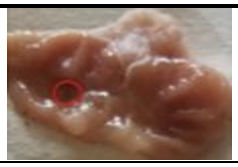
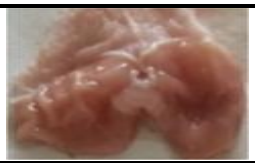
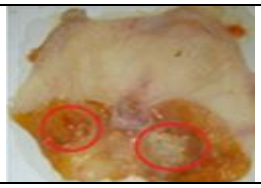
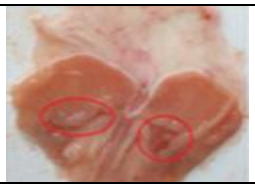
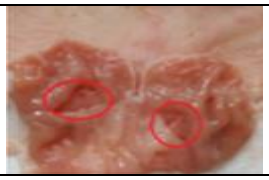
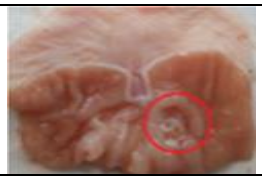
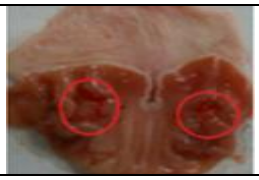
Effect of carbohydrate-, protein- and fat-rich diets on relative stomach weight, gross ulcer area, and percentage ulcer healing in rats of different ages with acetic acid-induced gastric ulcer: The relative stomach weight decreases with ageing; however, there is no significant difference in the relative weight of the stomach between or within groups (Tables 1 and 2). The mean gross ulcer area of the control, carbohydrate- and fat-rich diet groups increased significantly with age. In contrast, that of the protein-rich diet decreased with age and was significantly lower than the control. On the other hand, the percentage of ulcer healing by day 7 relative to day 3 was increased considerably in the aged rats fed a protein-rich diet compared to the control.

Table 1:
Effect of carbohydrate-, protein- and fat-rich diets on relative stomach weight, gross ulcer area and percentage healing by day 3 of acetic acid ulcer healing in rats of different ages

Age	Standard diet	CHO diet	PRO- diet	FAT diet
3 months				
RSW	1.09±0.07	0.87±0.00	1.12±0.09	0.91±0.08
GUA	12.83±0.66	5.76±2.92*	5.24±4.12*	7.86±2.98
6 months				
RSW	0.93±0.11	0.78±0.02	1.09±0.09	0.87±0.08
GUA	24.88±0.94[a]	2.10±0.52*	3.67±1.71*	4.98±0.69*
12 months				
RSW	1.13±0.04	0.89±0.04	1.10±0.04	1.04±0.16
GUA	43.74±1.12[ab]	26.98±2.23[ab*]	32.48±0.79[ab*]	25.67±0.43[ab*]
18 months				
RSW	1.11±0.11[a]	0.93±0.08	0.92±0.10	1.06±0.13
GUA	46.10±1.32[ab]	42.43±1.08[abc]	28.29±1.69[ab*]	36.67±1.22[ab*]

The values are shown as mean ± SEM; α= 0.05; n = 5.
** versus age-matched basal diet, a versus 3 months, b versus 6 months, c versus 12 months. Basal = Standard diet, CHO = Carbohydrate rich-diet, PRO- = Protein rich-diet, FAT = Fat rich-diet, RSW = Relative Stomach Weight, GUA = Gross Ulcer Area.*

Table 2:
Effect of carbohydrate-, protein- and fat-rich diets on relative stomach weight, gross ulcer area and percentage healing by day 7 of acetic acid ulcer healing in rats of different ages

Age	Standard diet	CHO diet	PRO- diet	FAT diet
3 months				
RSW	1.21±0.05	0.80±0.03	1.08±0.11	1.16±0.07
GUA	6.29±6.29	0.00±0.00	8.38±4.19	0.00±0.00
%H	88.89±1.11	100.00±0.00	69.94±3.79*	100.00±0.00
6 months				
RSW	1.04±0.09	0.71±0.05	1.08±0.05	1.09±0.08
GUA	13.62±1.05	9.43±3.14	13.62±2.10	20.95±2.02[a]
%H	85.11±3.03	68.33±4.4[a*]	77.78±11.11	56.94±1.95[a*]
12 months				
RSW	0.96±0.09	0.72±0.04	0.97±0.03	0.91±0.10
GUA	53.43±3.65[ab]	49.24±3.49[ab]	4.19±2.10*	60.10±1.56[ab]
%H	63.03±2.13[ab]	55.99±2.18[a]	96.68±1.68*	54.23±1.65[a]
18 months				
RSW	0.80±0.04	0.75±0.04	0.93±0.05	0.83±0.04
GUA	74.38±2.09[abc]	68.10±4.88[abc]	0.00±0.00[a*]	62.86±1.43[ab*]
%H	57.82±5.27[ab]	54.86±5.62[a]	100.00±0.00[a*]	61.16±0.67[a]

Values are expressed as mean ± SEM; α= 0.05; n = 5. versus age-matched Standard diet, a versus 3 months, b versus 6 months, c versus 12 months*
Keys: *Basal = Standard diet, CHO = Carbohydrate rich-diet, PRO- = Protein rich-diet, FAT = Fat rich-diet, RSW = Relative Stomach Weight, GUA = Gross Ulcer Area, %H = percentage Ulcer Healing.*

Effect of carbohydrate-, protein- and fat-rich diets on gastric redox level in rats of different ages with acetic acid-induced gastric ulcer: Gastric carbonyl and MDA levels increased significantly with age in all the treated groups. However, the gastric MDA level increased considerably in the aged rats fed carbohydrate- and fat-rich diets compared with the control. Gastric SOD and CAT decreased substantially with age, while gastric GSH was insignificant with age. The different fed diets significantly increased gastric SOD levels in the 18 months old compared to the age-match control by day 7 of ulcer healing. In contrast, CAT levels increased significantly in the 18-month-old fed fat-rich diet and the 3-month-old of the different diets compared with the control (Tables 3 and 4).

Effect of carbohydrate-, protein- and fat-rich diets on gastric pepsin, mucin, and nitric oxide levels in rats of different ages with acetic acid-induced gastric ulcer: Gastric pepsin level increased significantly with age by days 3 and 7 of ulcer healing but was insignificant between the other diets compared with the control by day 7; however, it increased significantly in the 6- and 18-months fed protein- and fat-rich diet and in the 6-months old carbohydrate-rich diet compared with the control by day 3, (Tables 5 and 6). On the other hand, gastric mucin and nitric oxide levels decreased significantly with age by days 3 and 7 of ulcer healing. Gastric mucin levels increased significantly in the 12-nth-old fed a protein-rich diet, while nitric oxide was lower in the 18-month-old compared with the control by day 7 of ulcer healing. Also, carbohydrate- and fat-rich diets significantly increased nitric oxide levels compared with the control by day 7 of ulcer healing.

Effect of carbohydrate-, protein- and fat-rich diets on gastric expression of EGF-R in rats of different ages with acetic acid-induced gastric ulcer: Gastric expression of EGF-R decreased with ageing in all the groups and was significantly lower by day 3 of ulcer healing in the 12-month-old protein- and fat-rich and 18-month-old of fat-rich diets compared with the control. By day 7 of ulcer healing, gastric expression of EGF-R was 83.76±1.73%, 74.30±2.31%, 72.39±1.20%, and 71.99±0.58% for 3-, 6-,

12-, and 18-months old respectively for the control (Figure 2). Gastric expressions of EGF-R for the protein-rich diet-fed rats were 87.42±1.27%, 87.34±4.06%, 83.26±2.33%, and 74.01±2.31% for the respective ages, while it decreased for carbohydrate- (75.49±3.96%, 69.14±1.63%, 68.21±1.92%, and 63.49±1.75%) and fat-rich diets (77.64±2.88%, 76.71±3.54%, 74.55±2.28%, and 63.62±1.84%) compared with the control (Figure 2).

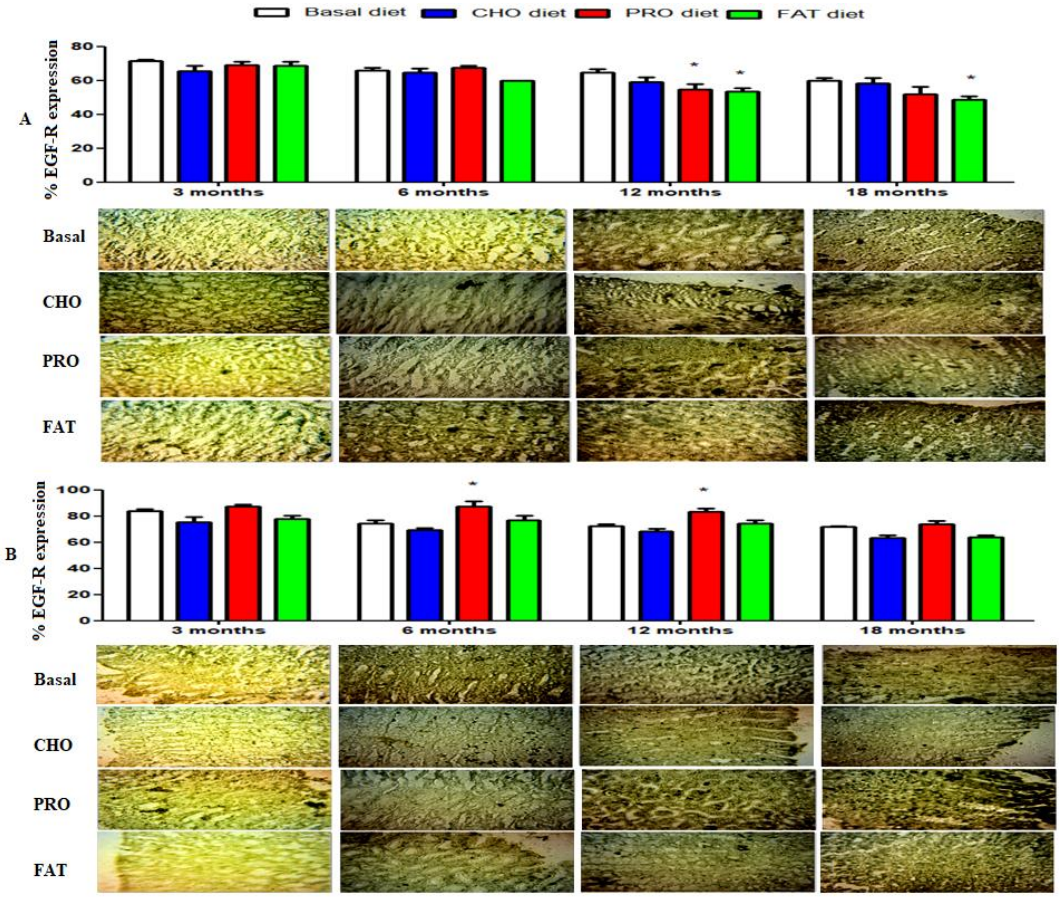


Figure 2:
Effect of carbohydrate-, protein- and fat-rich diets on gastric expression of EGF-R by day 3 (a) and 7 (b) in rat of different ages
Values are expressed as Mean ± SEM; n = 5. significant versus age-matched control diet, Basal = Control diet, CHO = Carbohydrate rich-diet, PRO⁻ = Protein rich-diet, FAT = Fat rich-diet.*

Table 3:
Effect of carbohydrate-, protein- and fat-rich diets on gastric redox level by day 3 of acetic acid ulcer healing in rats of different ages

	Age	Basal	CHO	PRO-	FAT
Carbonyl	**3 months**	1.54±0.03	1.28±0.05	1.53±0.02	1.56±0.02
(x 10^{-7} ng mL^{-1})	**6months**	1.61±0.11	1.41±0.01	1.51±0.02	1.75±0.09
	12 months	1.73±0.06	1.64±0.02	1.58±0.02	1.85±0.06
	18 months	1.85±0.05	1.97±0.03^{a}	1.77±0.06	2.05±0.02^{a}
MDA	**3 months**	3.50±0.24	3.20±0.29	3.68±0.71	3.37±0.48
(x 10^{-6} ng mL^{-1} Protein)	**6months**	4.11±0.46	3.62±0.71	4.48±0.56	3.60±0.17
	12 months	5.41±0.44	5.41±0.79	5.36±0.72	7.06±0.47^{ab}
	18 months	5.94±0.53^{a}	5.99±0.84	6.43±0.45	6.98±0.16^{ab}
SOD	**3 months**	11.34±1.26	11.15±0.33	11.55±0.81	10.94±0.16
(ng mL^{-1} protein)	**6months**	10.50±0.62	9.68±0.44	10.76±0.17	9.41±0.27
	12 months	10.47±0.60	9.41±0.89	9.74±0.76	7.83±0.25^{a*}
	18 months	9.49±0.75	8.23±0.91	9.52±0.71	8.56±0.84^{a}
CAT	**3 months**	6.98±0.20	7.60±0.09	7.98±0.26*	7.75±0.20
(x 10^{2} ng mL^{-1} Protein)	**6months**	6.60±0.39	6.65±0.48^{a}	6.11±0.22^{a}	6.33±0.28^{a}
	12 months	4.86±0.27^{ab}	5.45±0.16^{ab}	4.33±0.22^{ab}	4.25±0.14^{ab}
	18 months	4.26±0.36^{ab}	3.66±0.01^{abc}	3.13±0.15^{abc}	2.69±0.30^{abc*}
GSH	**3 months**	3.79±0.05	3.66±0.03	3.68±0.04	3.72±0.05
(mg dL^{-1})	**6months**	3.65±0.05	3.82±0.01	^{a}3.88±0.02	3.98±0.12
	12 months	3.72±0.07	4.01±0.14	3.83±0.06	3.82±0.05
	18 months	3.77±0.06	3.81±0.16	^{a}3.94±0.01	3.79±0.13

Values are expressed as Mean ± SEM; n = 5. significant versus age-matched control diet, a versus 3 months, b versus 6 months, c versus 12 months. Basal = Control diet, CHO = Carbohydrate rich-diet, PRO⁻ = Protein rich-diet, FAT = Fat rich-diet*

Table 4:
Effect of carbohydrate-, protein- and fat-rich diets on gastric redox level by day 7 of acetic acid ulcer healing in rats of different ages

	Age	Basal	CHO	PRO-	FAT
Carbonyl	**3 months**	1.48±0.07	1.36±0.01	1.42±0.09	1.42±0.01
(x 10^{-7} ng mL^{-1})	**6months**	1.58±0.05	1.63±0.08	1.52±0.01^{a}	1.60±0.01
	12 months	1.79±0.02	1.72±0.07	1.93±0.01	2.15±0.09^{a}
	18 months	2.19±0.08^{ab}	2.44±0.01^{abc}	2.17±0.05^{ab}	2.51±0.02^{ab}
MDA	**3 months**	3.08±0.45	4.27±0.43	4.05±0.17	4.21±0.21
(x 10^{-6} ng mL^{-1} Protein)	**6months**	3.96±0.80	4.43±0.44	4.33±0.42	5.52±0.40
	12 months	4.19±0.23	5.92±0.15*	5.50±0.31	7.72±0.60^{a*}
	18 months	4.58±0.36	6.54±0.31^{ab*}	5.73±0.55	8.16±0.88^{a*}
SOD	**3 months**	10.14±0.53	10.61±0.86	11.51±0.21	12.94±1.03*
(ng mL^{-1} protein)	**6months**	9.01±0.50	10.74±0.50	10.93±0.02*	10.39±0.27
	12 months	8.77±0.04	8.86±0.49	9.78±0.55	10.32±0.25
	18 months	7.08±0.45^{a}	8.98±0.48*	9.46±0.42^{a*}	9.71±0.10^{a*}
CAT	**3 months**	5.34±0.47	6.32±0.22*	6.34±0.16*	6.65±0.14*
(x 10^{2} ng mL^{-1} Protein)	**6months**	5.00±0.23	4.55±0.27^{a}	4.73±0.39^{a}	5.38±0.21^{a}
	12 months	4.45±0.13	4.30±0.17^{a}	4.50±0.19^{a}	4.38±0.25^{ab}
	18 months	2.06±0.21^{a}	1.88±0.26^{abc}	2.12±0.42^{abc}	3.11±0.07^{abc*}
GSH	**3 months**	4.05±0.43	3.78±0.04	3.73±0.05	3.85±0.09
(mg dL^{-1})	**6months**	4.04±0.01	3.84±0.05	3.78±0.10	3.75±0.05
	12 months	3.80±0.07	4.06±0.11	3.78±0.02	3.79±0.07
	18 months	3.72±0.04	3.76±0.06	3.30±0.09^{a}	3.67±0.06

Values are expressed as Mean ± SEM; n = 5. significant versus age-matched control diet, a versus 3 months, b versus 6 months, c versus 12 months. Basal = Control diet, CHO = Carbohydrate rich-diet, PRO^{-} = Protein rich-diet, FAT = Fat rich-diet*

Table 5:
Effect of carbohydrate-, protein- and fat-rich diets on gastric pepsin, mucin and nitric oxide levels by day 3 of acetic acid ulcer healing in rats of different ages

Gastric parameter	Age	Basal	CHO	PRO-	FAT
Pepsin	**3 months**	11.62±0.21	12.49±0.32	12.19±0.69	10.39±0.28
(x 10^{2}µg mL^{-1})	**6months**	11.89±0.15	13.81±0.36*	13.20±0.80*	13.88±0.65^{a*}
	12 months	12.85±0.41	14.20±0.09	13.90±0.27	13.74±0.35^{a}
	18 months	13.71±0.24	13.49±0.57	15.35±0.45^{a*}	15.41±0.23^{a*}
Mucin	**3 months**	56.64±6.71	48.81±4.08	39.34±1.10*	45.89±0.92
(mg dL^{-1})	**6months**	45.70±0.88	40.24±2.03	43.03±4.10	47.51±3.42
	12 months	51.42±5.52	37.04±2.84*	46.43±1.37	31.35±4.80*
	18 months	35.08±4.40	31.94±4.09^{a}	27.48±2.82^{abc}	32.53±3.13
Nitric oxide	**3 months**	40.43±1.15	43.31±1.34	43.93±1.30	50.96±1.41*
(mL organ	**6months**	43.85±4.29	43.97±2.78	42.24±2.33	47.39±1.02
weight^{-1})	**12 months**	38.06±4.55	42.02±1.95	40.72±3.62	41.13±3.41
	18 months	29.11±4.40	32.89±2.17^{a}	35.86±1.86	27.62±1.40^{abc}

Values are expressed as Mean ± SEM; n = 5.* significant versus age-matched control diet, a versus 3 months, b versus 6 months, c versus 12 months. Basal = Control diet, CHO = Carbohydrate rich-diet, PRO^{-} = Protein rich-diet, FAT = Fat rich-diet

Table 6:
Effect of carbohydrate-, protein- and fat-rich diets on gastric pepsin, mucin and nitric oxide levels by day 7 of acetic acid ulcer healing in rats of different ages

Gastric parameter	Age	Basal	CHO	PRO-	FAT
Pepsin	**3 months**	11.31±0.66	12.54±0.30	11.24±0.30	12.17±0.30
(x 10^{2} µg mL^{-1})	**6months**	12.31±0.22	13.07±0.19	11.81±0.43	13.31±0.55
	12 months	13.10±0.17^{a}	14.02±0.23^{a}	12.18±0.60	13.50±0.49
	18 months	14.05±0.10^{a}	14.24±0.14^{ab}	12.97±0.34	13.80±0.16
Mucin	**3 months**	52.51±1.82	57.35±3.53	61.72±1.86	52.21±0.86
(mg dL^{-1})	**6months**	49.10±4.80	45.52±4.89	54.33±2.56	46.39±2.36
	12 months	32.69±1.35^{ab}	37.63±2.62^{a}	43.40±3.31^{a*}	38.24±0.59^{ab}
	18 months	28.67±2.71^{ab}	28.22±2.57^{a}	35.83±1.56^{ab}	27.62±0.85^{abc}
Nitric oxide	**3 months**	57.53±7.24	69.19±2.75*	59.50±3.44	58.42±1.08
(mL organ	**6months**	49.86±1.54	58.94±2.02*	46.91±3.28	52.68±1.56*
weight^{-1})	**12 months**	45.24±3.19	49.98±1.02^{ab}	34.78±2.29^{a}	35.10±1.18^{ab}
	18 months	43.17±2.78	31.07±0.21^{abc*}	27.17±4.78^{ab*}	30.66±0.65^{ab*}

Values are expressed as Mean ± SEM; n = 5.* significant versus age-matched control diet, a versus 3 months, b versus 6 months, c versus 12 months. Basal = Control diet, CHO = Carbohydrate rich-diet, PRO^{-} = Protein rich-diet, FAT = Fat rich-diet.

DISCUSSION

Gastrointestinal ulcer is already known to have a multifactor-associated etiology and in the present study, we investigated the fate of diets rich in carbohydrate, protein and fat on the ulcer healing in young and aged rats. The findings agreed with the previous fact that ulcer healing is delayed in the old rats compared to the young rats if one considers the healing rate in the control, carbohydrate- and fat-rich diets but not so for the protein-rich diet. A protein-rich diet enhances healing in the aged rats and thus suggests that a protein-rich diet may favour ulcer healing for older adults. Based on results in the production and prevention of experimental gastric ulcer in animals, Windwer and Matzner (1938) has previously reported and suggested a high-protein diet to relieve ulcer symptoms in 90% of studied patients. The report concurs with the present findings.

Considering the multifactor aetiology of digestive tract ulceration, including free radicals and oxidative formation, studying the gastric redox system is necessary. In the present study, ageing favours excess ROS over endogenous antioxidants, which may have delayed ulcer healing in the aged rats. However, a protein-rich diet inhibits the gastric concentration of protein carbonyl and MDA as well as stimulates SOD to favour ulcer healing as compared to carbohydrate-and fat-rich diets that promote protein carbonyl and MDA and inhibit the endogenous antioxidants. In line with this finding, high carbohydrate and fat diets generate increased levels of lipid peroxidation and protein carbonylation products and inhibit endogenous antioxidants (Apel and Hirt, 2004).

Pepsin has a mucolytic activity and digests the adherent luminal mucus layer. This may explain the carbohydrate- and fat-rich diets-induced inhibition of mucin levels since they stimulate pepsin activity in the ulcerated stomach during gastric ulcer healing. However, ageing is associated with a reduced capacity of many mucus-secreting cells (Newton et al., 2003). Peptic ulcer diet therapy aims to prevent the hypersecretion of peptic chloride and promote healing based on a complex sequence of events going from the initial trauma to the repair of the damaged tissue. In this study, carbohydrate- and fat-rich diets stimulate gastric pepsin and nitric oxide levels during the healing phase of acetic acid-induced gastric ulcers, while protein-rich diets boost mucin levels. This mucin effect of a protein-rich diet may have protected the gastric mucosa from acetic acid-induced damage or enhanced repair of the ulcerated mucosa.

The findings of the present study suggest that a protein-rich diet up-regulated gastric EGF-R in ulcerated rats. EGF binds to its receptor, EGF-R, to halt acid secretion, exerts a trophic effect on gastroduodenal mucosa, protects gastric mucosa against injury, and accelerates gastroduodenal ulcer healing by stimulating cell migration and proliferation (Tarnawski and Jones, 1998). The down-regulation of EGF-R with ageing by carbohydrate- and fat-rich diets suggests little EGF to perform its protective and healing function. In contrast, the up-regulating role of a protein-rich diet on EGF-R may have increased EGF concentration to stimulate ulcer healing in the present study. In support of this assertion, Reis (2003) suggested that clinicians should adjust the distribution of calories in patients with peptic ulcers.

In conclusion, gastro-aggressive factors increase while gastro-protective factors decrease with age. Furthermore, carbohydrate and fat-rich diets favour the gastro-aggressive factors during ageing. The protein-rich diet facilitates the healing of acetic acid-induced gastric ulcers through enhanced gastric SOD, mucin and EGF-R expression in the ulcerated stomach. Also, protein-rich diets provide improved gastroprotective activity through weakening oxidative and pepsin concentrations and thus may possess anti-inflammatory and anti-secretory properties.

Acknowledgements

The authors of this work are grateful to Dr. A.A. Oyagbemi, of the Cardiorenal Laboratory, Department of Veterinary Physiology and Biochemistry, Dr O.O. Aina, of the Department of Veterinary Anatomy and Mr. Ambrose, O. Nwagbara, of Histopathology Unit, Department of Veterinary Pathology; all in the Faculty of veterinary medicine, University of Ibadan, Ibadan, Nigeria for his assistance towards the immunohistological processing, capturing and analysis.

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Niger. J. Physiol. Sci. 39 (June 2024): 45 – 56
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

D-Ribose-L-Cysteine Protects Against Sodium Arsenite-induced Hepato-Nephrotoxicity in Rats

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Summary: D-Ribose-L-Cysteine (Riboceine)- an antioxidant supplement that may help to raise the glutathione levels by acting as a precursor for glutathione biosynthesis in biological systems. Effect of riboceine (Rb) on sodium arsenite (SA) induced hepatorenal toxicity was investigated in rats. Four groups (A-D) (six per group) were treated thus: Group A (water and normal diet only); while Group B (SA at 5 mg/kg body weight); Group C (riboceine at 10 mg/kg body weight) and Group D (riboceine and SA). The exposure to test substances lasted for a total of 14 days in each case in which pre-treatment was done with riboceine. Exposure to SA triggered a significant reduction in the entire weight and relative organ weight, increase in ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase) activities, decrease in liver total protein and increase in serum levels of urea and creatinine. Furthermore, SA caused a significant reduction in GSH (glutathione) level and CAT (Catalase) activity, while the LPO (lipid peroxidation) and NO (nitric oxide) levels were significantly increased. Pre-treatment with riboceine, restored the levels of the aforementioned parameters. Riboceine also promote restoration of hepatocytes and renal cells integrity. Findings from this study reaffirm the hepatorenal toxicities of sodium arsenite and show the protective role of riboceine against SA-induced toxicities. Protective effects of riboceine may be via the enhancement of the level of glutathione, a natural scavenger of free radicals.

Keywords*: D-Ribose-L-Cysteine, antioxidant, sodium arsenite, hepatotoxicity, nephrotoxicity, lipid peroxidation*

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Manuscript received- August 2023; Accepted- February 2024

DOI: https://doi.org/10.54548/njps.v39i1.7

INTRODUCTION

Arsenicals are considered potent human carcinogens, causing cancer of the skin, lung, bladder, liver and kidney (IARC, 1987; Tchounwou *et al*., 2004). A large population of humans is chronically exposed to arsenic throughout the world, via ingestion, inhalation, dermal contact and the parenteral route (Tchounwou *et al*., 2004). The carcinogenic effect of arsenicals has been linked to the of free radicals' generation and alteration of cellular redox states which cumulates into oxidative stress due to an imbalance between the cellular antioxidant defense and ROS (reactive oxygen species) generation (Xu *et al*., 2017). Exposure to arsenicals can also result to the following health defects: keratosis, hyperpigmentation, hypopigmentation, cardiovascular disease, diabetes mellitus, central nervous disorders etc. (Janga *et al*., 2016). It was earlier proposed that arsenic induction of oxidative stress is by cycling between its metallic oxidation states or by antioxidants imbalance and high inflammation rate, thereby causing cellular accumulation of free radicals (Halliwell *et al*., 2004).

Riboceine is a synthetic compound designed to replenish glutathione levels thereby complementing the anti-oxidant defense system of the body (Chandra *et al*., 2015; Falana *et al*., 2017). D-Ribose-L-Cysteine which is abbreviated as riboceine provides a means of delivering cysteine to the cell. Cysteine, which cannot be delivered directly to the cell due to its fragility, has been proposed as the key player amino acid in the antioxidant effect of riboceine (Falana *et al*., 2017; Adelakun *et al*., 2018). Whole glutathione consumption also cannot be effective because it would be destroyed in the digestion process before reaching the target cell (Falana *et al*., 2017). Riboceine avail the body cells of cysteine for the synthesis of glutathione, while ribose is channeled into producing cellular energy. The glutathione within a cell protects it against destruction from free-radical damage (Flora, 2011).

Variations in the systemic antioxidant have been reported in many pathological conditions involving oxidative stress induced by metals (Beyersmann and Hartwig, 2008). Enzymatic antioxidant response has been shown to play key roles in response to arsenic-induced oxidative stress (De Vizcaya-Ruiz *et al*., 2009). Furthermore, a time-dependent relationship in the enzymatic antioxidant response was observed in the levels of CAT and SOD (superoxide dismustase) as there was an initial increase but latter decrease on further exposure to arsenic (De Vizcaya-Ruiz *et al*., 2009; Nandi *et al*., 2005). Non-enzymatic antioxidants have been shown to be highly efficient in combating metal-induced oxidative stress and

preventing cellular oxidative injury. Non-enzymatic antioxidants are currently in use as therapeutic and preventive agents to combat damages induced by oxidative stress which occur during arsenic exposure (De Vizcaya-Ruiz *et al*., 2009). Such antioxidants include vitamin C, vitamin E, flavonoids, carotenoids, amino acids, for example taurine, methionine, cysteine, and other thiol-containing compounds which include: glutathione, alpha-lipoic acid, thioredoxin and N-acetyl Cysteine (De Vizcaya-Ruiz *et al*., 2009).

Riboceine, as a non-enzymatic antioxidant, helps in combating oxidative stress. There are limited reports in literature regarding the use of riboceine against metal-induced oxidative stress. We designed this study to assess the effect of riboceine on sodium arsenite-induced oxidative stress and toxicity in rats.

MATERIALS AND METHODS

Chemicals and reagents: Sodium arsenite was procured from Sigma Aldrich Co. St. Loius United States and riboceine was obtained from a pharmacy store in Ibadan, Nigeria. Serum alkaline phosphatase (ALP) activity, aspartate aminotransferase (AST), with alanine aminotransferase (ALT) activities were evaluated alongside the urea and creatinine levels using reagent kits obtained from RANDOX Laboratories Ltd., Ardmore, United Kingdom. The kits were used according to the respective manufacturers' protocol for the measurement of enzymes activities.

Experimental Animals: Twenty-four mature male Wistar rats each weighing 160 ± 20g, obtained from the preclinical facilities of Faculty of Basic Medical Sciences, were used for this study. Prior to the commencement of treatment, the rats were made to acclimatize for a period of one week and kept in well ventilated cages stationed in the animal house of our department. All animals had unrestricted access to fresh rat chows and water. Experimental rats were exposed to photoperiod of 12 hrs light/dark cycles in synchrony with natural light cycle, and cared for adequately and were handled in adherence to the guide for the care and use of experimental animals, as specified by the National Institute of Health (NIH publications number 85–93 revised in 1985).

Experimental design:
Group A: This is the control group that received distilled water only.
Group B: Administered sodium arsenite (SA) at 5 mg/kg for a total of 14 days at a day interval.
Group C: Administered riboceine (10 mg/kg) for 14 days
Group D: Pretreated with riboceine (10 mg/kg) daily for 14 days and then administered SA (5 mg/kg) for a total of 14 days at a day interval and this dose was chosen be with reference to previous studies (Falana *et al*., 2017; Adelakun *et al*., 2018) at 30mg/kg and since pre-treatment was utilized a lower dose of 10mg/kg was used for this study.

All treatments were done by oral gavage. Each group has 6 animals per group. Sodium arsenite was administered according to the published report (Gbadegesin *et al*., 2009).

Tissue preparation: Rats were sacrificed by cervical decapitation 24hrs after the last dose of the treatments. Blood samples were collected. Harvested liver and kidney organs were rinsed in ice cold 1.15% KCl solution, blotted with filter paper and weighed to determine their initial weights. Thereafter, the liver samples were sectioned for histological examination and submerged in 10% Formalin. The remaining portions of the harvested liver were homogenized with 0.1M Phosphate buffer (pH 7.4) using a Teflon homogenizer. The homogenates gotten were then centrifuged at 10,000 revolution per minute for 15 minutes in a cold centrifuge (4 degrees) to obtain the post mitochondrial fraction. After centrifugation, the supernatants were collected stored at -20 degrees prior to the biochemical analyses.

Preparation of Serum: Blood samples collected via the venous plexus into plain tubes and left to clot at room temperature. Preparation of serum was done by centrifugation of the clotted blood at 3,000 g for 10 min. The supernatant (serum) was removed and stored at -20 degrees prior to the analyses

Blood analysis: The blood samples were taken via the periorbital sinus into lithium heparinized bottles. The PCV (packed cell volume) was evaluated using the microhematocrit method and the Hb (hemoglobin) concentration was also assessed by the cyanmethemoglobin. The new improved Neubauer hemocytometer was used in the estimation of red blood cells (RBC) and white blood cell (WBC) counts. Differential leukocyte counts were evaluated using standard method Jain (Jain, 1986).

Biochemical assays:

Total protein level assay: Total Protein level was determined following the protocol outlined in Randox Laboratories Limited kits.

Kidney function assay: Serum urea and creatinine levels were estimated by the method of Fawett and Scott (Fawett and Scott, 1960).

Liver function assay: Activities of ALT and AST were evaluated by the method of Reitman & Frankel (Reitman and Frankel, 1957). Estimation of serum ALP activity was evaluated by the method described by (Rec, 1972).

Assays for antioxidant markers: The CAT activity was assessed by the method earlier described by Claiborne, 1985. SOD activity was assessed based on the method described by Misra and Fridovich, 1972. GST (glutathione-S-transferase) activity was estimated by following the method described by Habig,1974. The GPx (glutathione peroxidase) activity was estimated using the method of Rotruck *et al*., 1973. The GSH (glutathione) activity was determined following the method of Beutler *et al*., 1963. Also, LPO activity was evaluated by the method earlier described by Rice-Evans which involves the reaction between 2-thiobarbituric acid (TBA) and malonaldehyde (MDA). MDA level was calculated following the method of (Adam-Vizi and Seregi, 1982).

Assay for markers of inflammation: The MPO (myeloperoxidase) activity was assessed by slight modification of the method described by Trush *et al*., (1994) Nitric oxide (NO) activity was evaluated by the method of Green *et al*., (1982).

Histological evaluations: Sections of tissues were obtained from the liver and kidney and fixed in 10% neutral buffered formalin. The tissues were thereafter processed for histological examinations with the use of a routine paraffin-wax embedded method. The tissue section of 5 micrometer thickness was stained with hematoxylin and eosin and afterwards processed for light microscopy at a magnification of X400.

Statistical analysis: The data obtained were analyzed by the one-way analysis of variance (ANOVA). This was followed by a post hoc test (Bonferroni) to verify the level of significance between groups using GraphPad prism-6 software (version 6; GraphPad Software, La Jolla). Levels of significant difference between mean values were set at $p < 0.05$.

RESULTS

Sodium arsenite caused a reduction in the body and relative liver weight: A significant $p < 0.05$ decrease was noticed in the body weight of the animals in Group B, treated with sodium arsenite only in comparison to the control (Figure 1). Also, there was a significant decrease $p < 0.05$ in the relative liver weight of the Group B and of Group D pretreated with riboceine before sodium arsenite when juxtaposed with the control group. No significant difference in the relative kidney weights across the groups (Figure 1).

Riboceine offered protection against sodium arsenite-induced hepatotoxicity in the treated rats: There was an increase in the mean serum ALT, AST and ALP activities of the rats in the group administered only sodium arsenite (Group B) when compared with the control Group A (Figure 2). Pretreatment with riboceine before sodium arsenite (Group D) however, led to significant reduction in the serum ALT, AST and ALP activities relative to the group treated with sodium arsenite only (Group B).

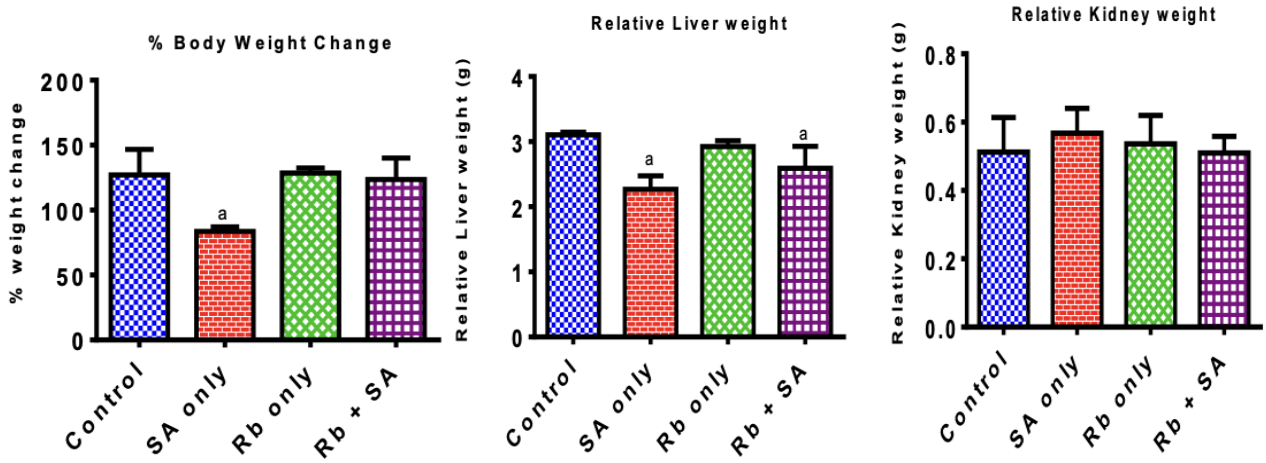

Figure 1:
Effect of Sodium arsenite and riboceine on percentage body weight, and relative liver and kidney weights respectively. Values are expressed as mean ± SD (n=6). a= significant difference ($p < 0.05$) when compared with the negative control.

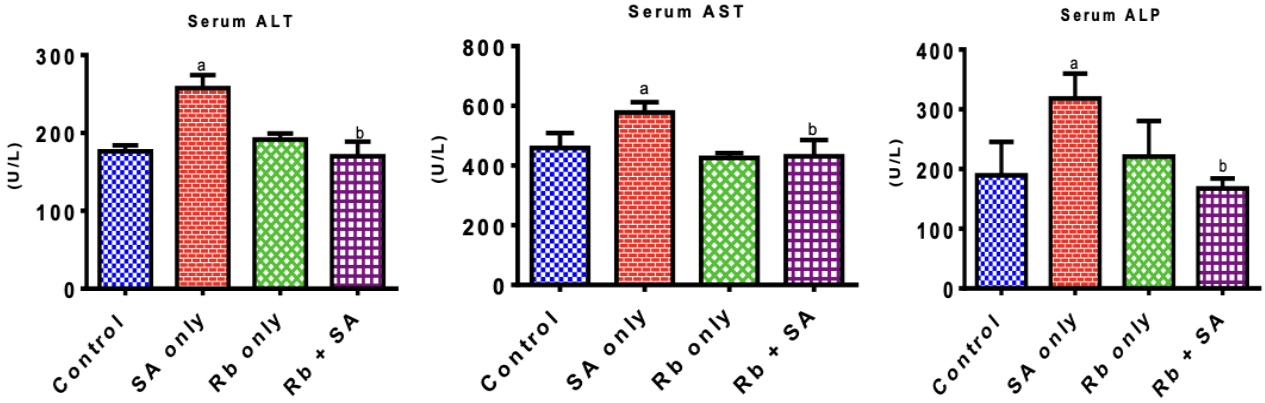

Figure 2:
Riboceine (Rb) protects against sodium arsenite-induced hepatotoxicity in the serum of treated rats. Values are presented as mean ± SD (n=6). a= significant difference ($p < 0.05$) relative to the negative control while b = significant difference ($p < 0.05$) in comparison to the group treated with sodium arsenite (SA) alone.

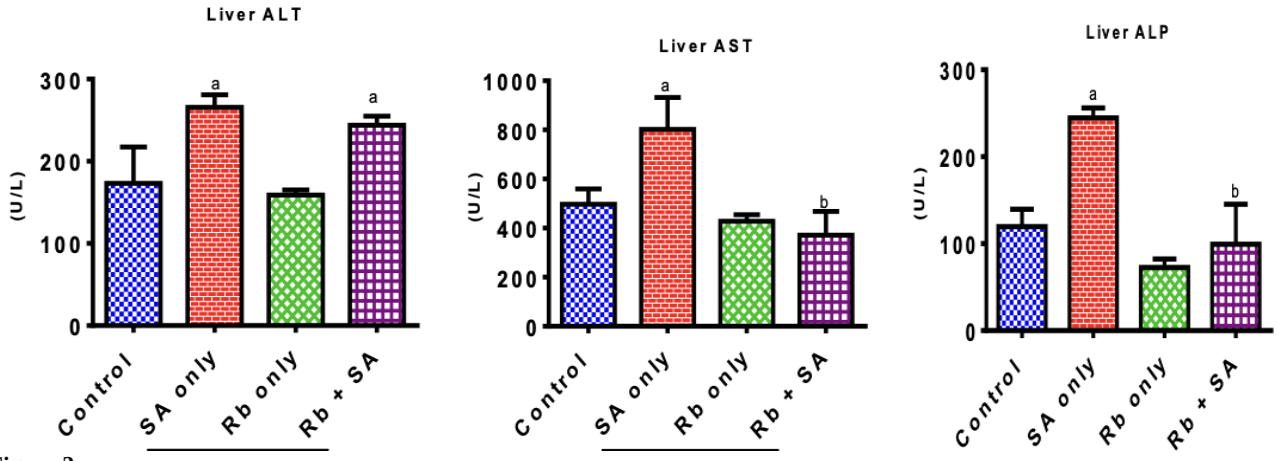

Figure 3:
Riboceine (Rb) protects against sodium arsenite-induced hepatotoxicity in the liver of treated rats. Values are presented as mean ± SD (n=6). a= significant difference ($p < 0.05$) relative to the negative control while b = significant difference ($p < 0.05$) in comparison to the group treated with sodium arsenite (SA) alone.

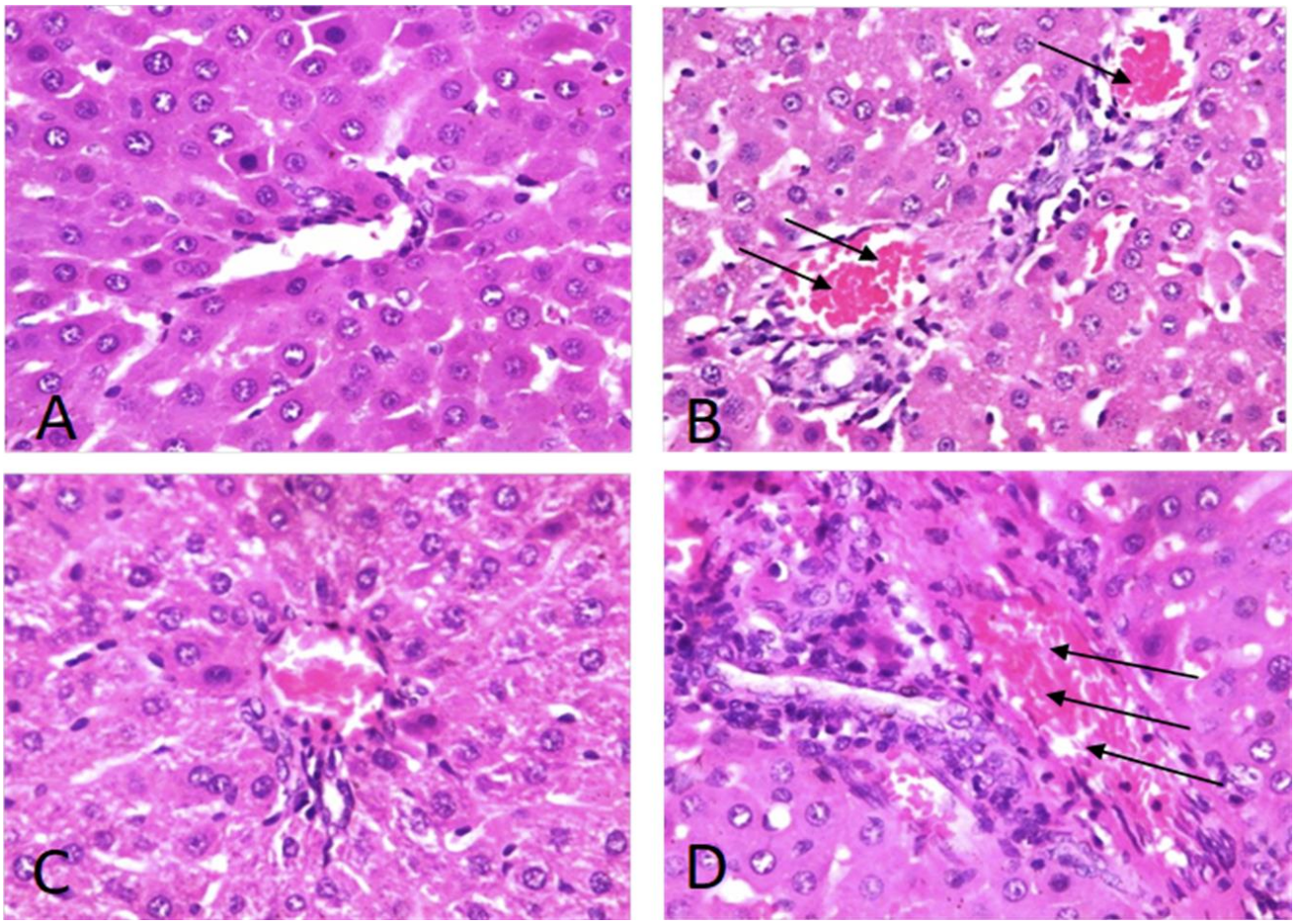

Plate 1:
Photomicrograph of liver sections of rats treated with riboceine and sodium arsenite for 14 days (Mag X400). (A) Control showing normal hepatocytes. (B) SA only (5 mg/kg body weight) showing mild vascular congestion and mild inflammation. (C) Riboceine (10 mg/kg body weight) showing no visible lesion. (D) Rb + SA (10 mg/kg and 5 mg/kg body weight) showing mild inflammation and hepatocytes regeneration

Similar observations were seen in the liver activities of ALT, AST, and ALP (Figure 3). There were increases in the activities of ALT, AST, and ALP in the rats treated with sodium arsenite only (Group B) when compared with the negative control, and pretreatment with ribociene (Group D) resulted in a significant reduction of AST and ALP activities, with a slight decrease in ALT activities (Figure 3)

Histology: Histology results also confirmed the toxicity of sodium arsenite on the liver and prevention of such effects in the liver cells of rats pretreated with riboceine in Group D (Plate 1).

Protective effects of riboceine on sodium arsenite-induced renal toxicity: Administration of sodium arsenite (Group B) caused a significant increase ($p < 0.05$) in serum urea levels when examined along with the negative control (Group A) given distilled water only (Figure 4). Pretreatment of rats with riboceine before the administration of sodium arsenite (Group D), led to significant decrease ($p < 0.05$) in the serum urea in comparison to Group B treated with sodium arsenite only. In addition, treatment with sodium arsenite resulted in significant ($p < 0.05$) increase in creatinine levels in the serum in Group B which received sodium arsenite when examined with the negative control group (Figure 4). Pre-treatment with riboceine before administration of sodium arsenite resulted in significant ($p < 0.05$) reduction in serum creatinine levels in comparison to Group B. Histological analysis showed mild lesions observed in renal tissue in the group exposed to sodium arsenite (Group B). However, pre-treatment with riboceine (Group D) prevented such degeneration in the renal cells (Figure 5).

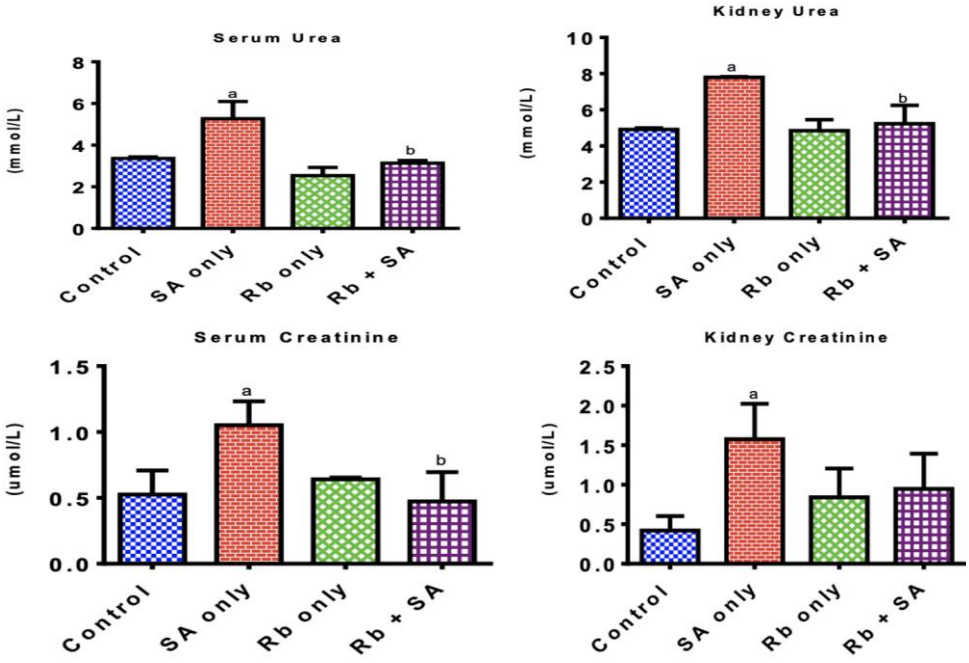

Figure 4:
Protective effects of riboceine (Rb) on sodium arsenite-induced renal toxicity. Each bar represents mean ± SD (n=6). *a* = significant difference ($p < 0.05$) in comparison to the negative control; b = significant difference ($p < 0.05$) relative to the group treated with sodium arsenite (SA) alone.

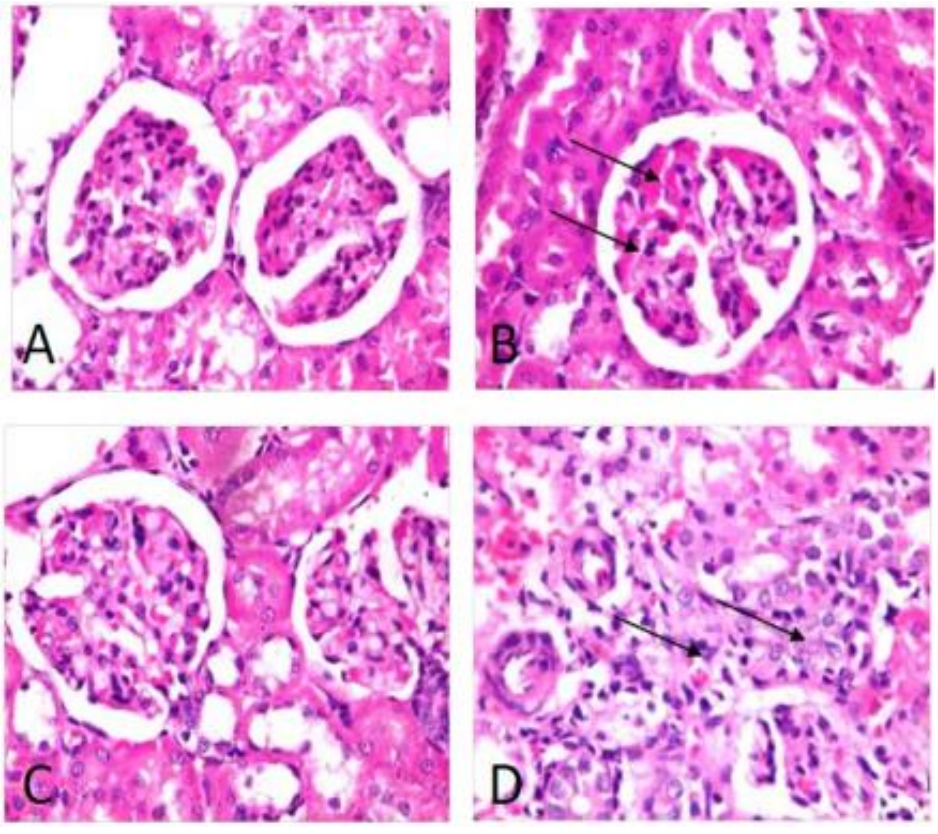

Plate 2:
Photomicrograph of kidney sections of rats treated with riboceine and sodium arsenite for 14 days (Mag X400). (A) Control showing normal renal cells. (B) SA only (5 mg/kg body weight) showing mild vascular congestion and mild peritubular inflammation. (C) Riboceine (10 mg/kg body weight) showing no visible lesion. (D) Rb + SA (10 mg/kg and 5 mg/kg body weight) showing mild peritubular inflammation and renal cell regeneration

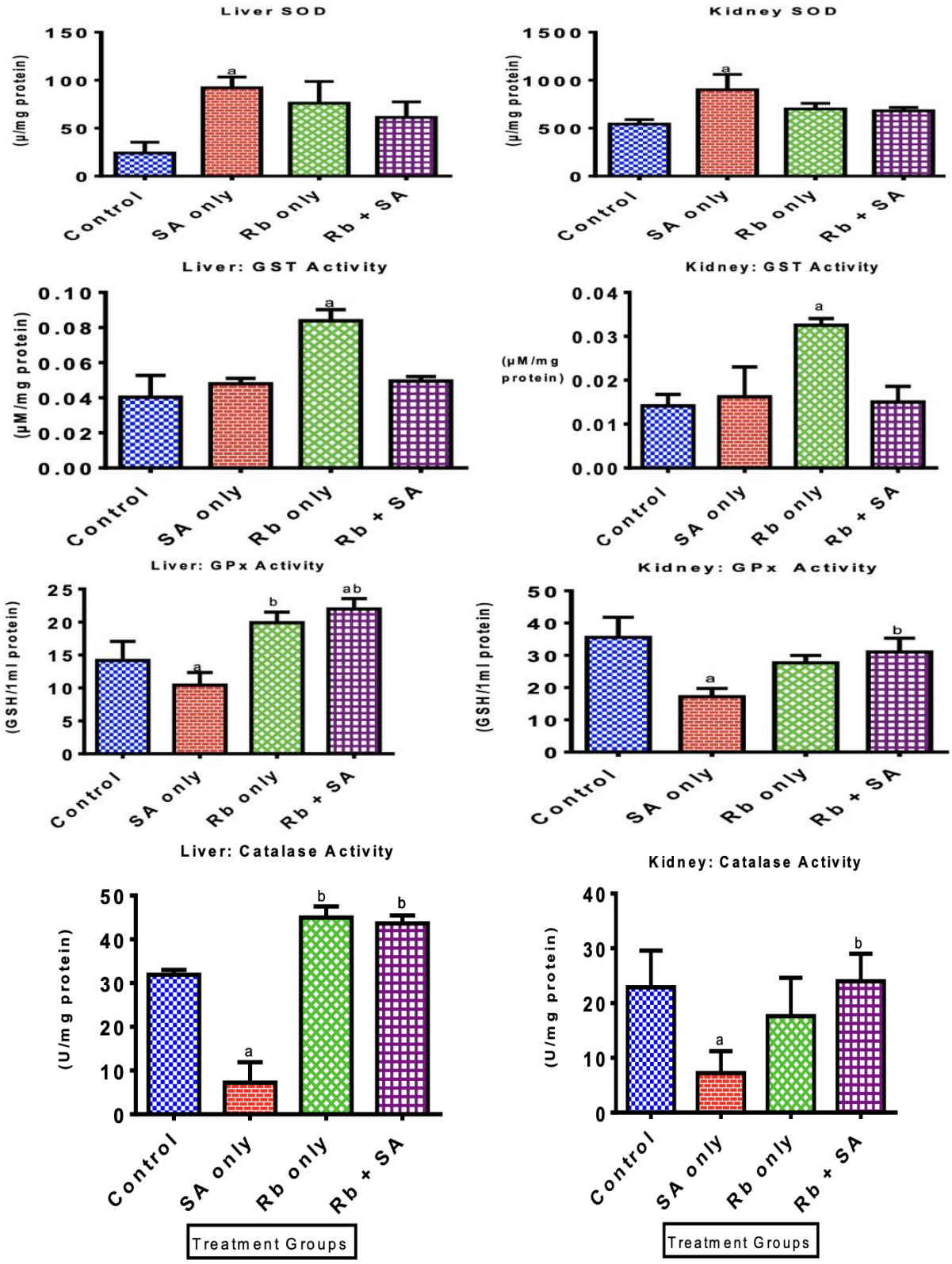

Figure 5:
Riboceine (Rb) ameliorated sodium arsenite induction of distortion of enzymatic antioxidant in liver and kidney of treated rats. Each bar represents mean ± SD (n=6). *a* = significant difference ($p < 0.05$) relative to the negative control; b = significant difference ($p < 0.05$) in comparison to the group treated with sodium arsenite (SA) alone.

Riboceine ameliorated sodium arsenite-induced distortion of antioxidant biomarkers in the treated rats: The activity of SOD in the liver significantly increased $p < 0.05$ in the group treated with only sodium arsenite (SA) in comparison to the control (Figure 5). Pretreatment with riboceine before sodium arsenite (Group D) however led to decrease in the mean SOD activity compared with Group B.

Observations made in the kidney regarding SOD were similar to the findings with the liver described above (Figure 6). In addition, hepatic GSH level decreased significantly in the group given SA only (Group B) when compared with the control (Group A). However, riboceine brought about a significant increase in GSH level ($p<0.05$) in Group D given SA and riboceine compared with Group B. Renal GSH level also decreased significantly in the group given SA only (Group B) when compared with the control (Group A). However, there was no significant change in the group given both SA and riboceine (Group D) when compared with Group B, given SA only. On the other hand, hepatorenal Glutathione-S-Transferase activity was found to increase but not significantly in the group given SA only. Group C (treated with riboceine only) however, showed a significant increase ($p < 0.05$) in the mean GST activity of the liver and kidney when compared with the control and the SA treated groups.

Hepatic GPx activity was found to be higher significantly ($p < 0.05$) in Group B (administered SA only) than the control (Group A). Pretreatment with riboceine before SA (Group D), effectively reversed the effect of SA on the GPx activity seen in Group B (Figure 5). On the other hand, treatment with SA had a contrary effect in the kidney cells with respect to GPx activity (Figure 5). Both hepatic and renal CAT activities decreased significantly ($p < 0.05$) in the group given SA when compared with the control. Pre-treatment with riboceine before SA (Group D) restored with the above parameters to values close to control. Similarly, both liver and kidney malonaldehyde levels were found to increase significantly ($p < 0.05$) in the group treated with SA only (Group B) when compared with the control (Group A), but values were reduced significantly ($p < 0.05$) in the rats pretreated with riboceine before SA exposure (Group D) as compared with Group A (Fig. 6).

Riboceine ameliorates the effects of sodium arsenite on inflammatory biomarkers in rats:

The NO level in both liver and kidney increased significantly $p < 0.05$ in the group given sodium arsenite alone compared with the negative control group (Figure 7). On the other hand, the effect of SA on the NO concentration was effectively reversed in Group D pretreated with riboceine prior to SA exposure when Group B was compared with Group D. Patterns similar to the observation made with NO concentrations were observed with effect of riboceine on SA treatment on liver MPO (Figure 7).

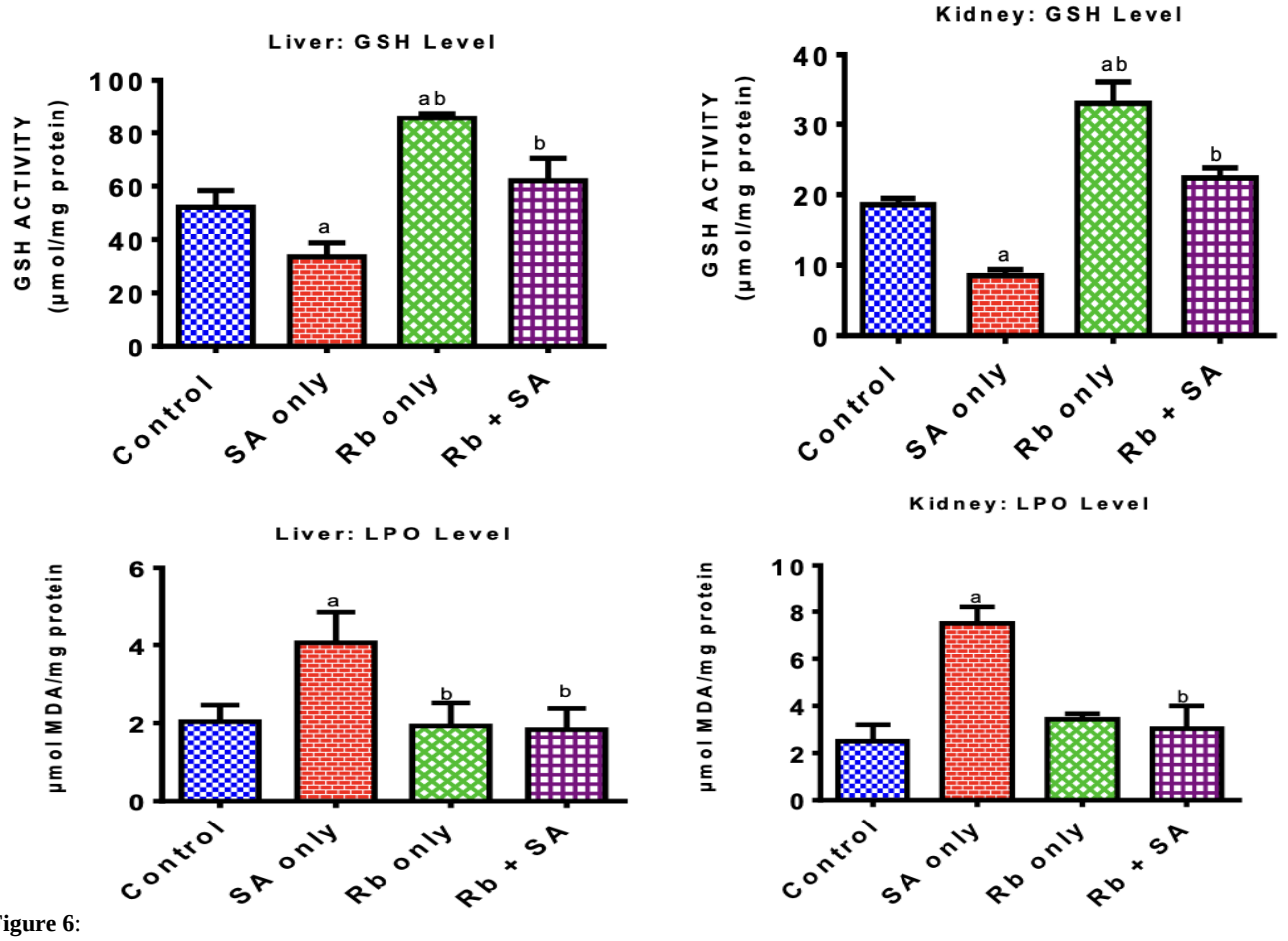

Figure 6:
Riboceine (Rb) ameliorated sodium arsenite induction of distortion of non-enzymatic antioxidant in liver and kidney of treated rats. Each bar represents mean ± SD (n=6). *a* = significant difference ($p < 0.05$) relative to the negative control; b = significant difference ($p < 0.05$) in comparison with group treated with sodium arsenite (SA) alone

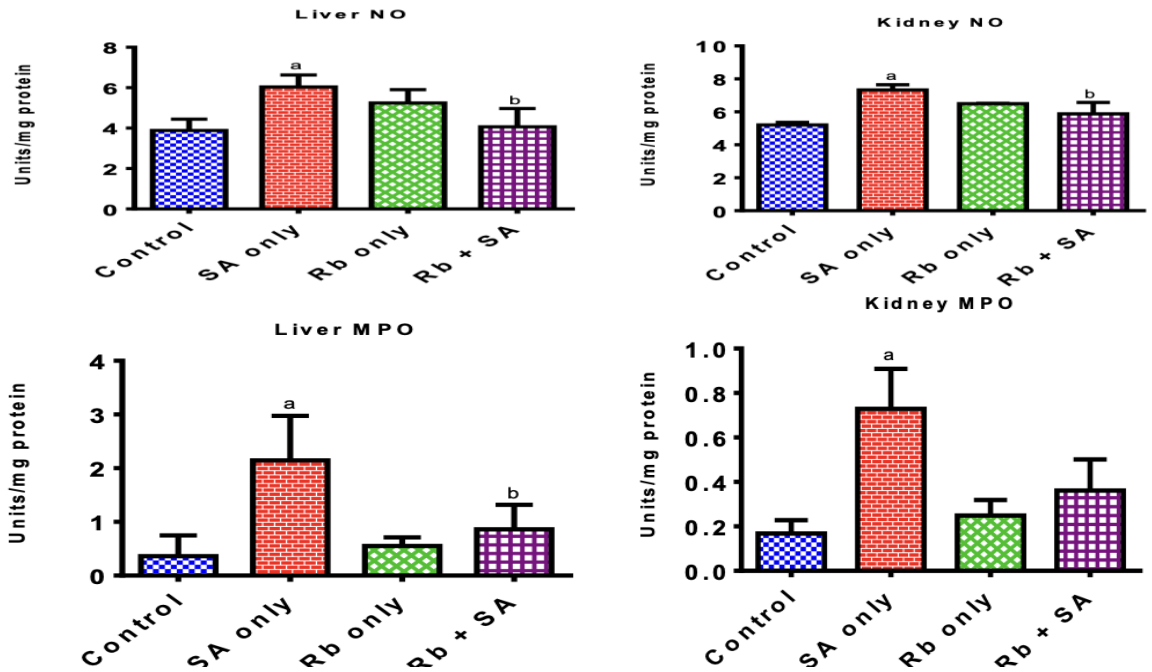

Figure 7:
Effects of sodium arsenite (SA) and/or riboceine (Rb) on biomarkers of inflammation in the liver and kidney of rats. Each bar represents mean ± SD (n=6). *a* = significant difference ($p < 0.05$) relative to the negative control; b = significant difference ($p < 0.05$) in comparison to the group treated with sodium arsenite (SA) alone.

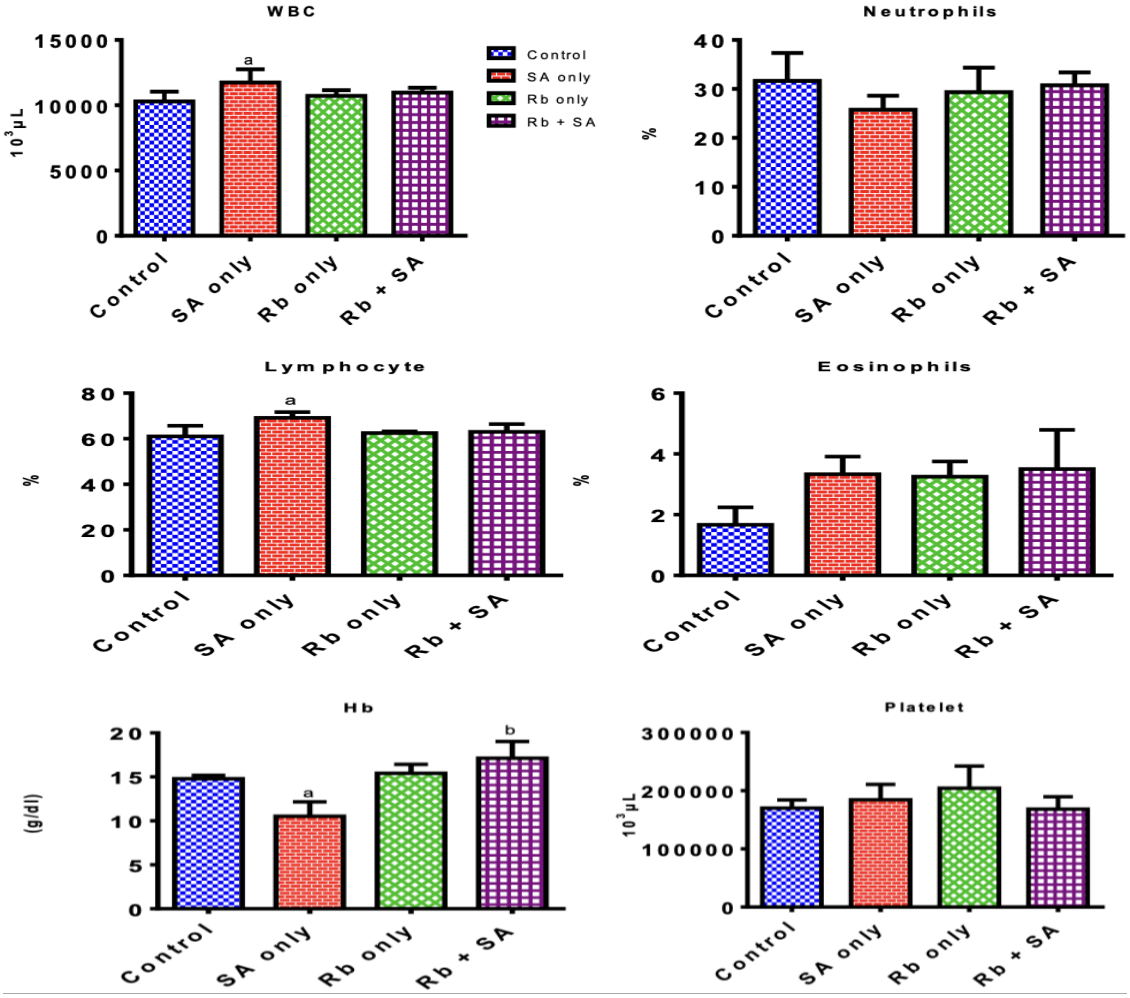

Figure 8:
Effects of sodium arsenite (SA) and/or riboceine (Rb) on hematological parameters in the treated rats. Each bar represents mean ± SD (n=6). *a* = significant difference ($p < 0.05$) relative to the negative control; b = significant difference ($p < 0.05$) in comparison to the group treated with SA alone.

Riboceine restores the normal levels of PCV, Hb and RBCs in the treated rats: The number of neutrophils, eosinophils and platelets were not significantly different across the groups (Figure 8). There was a significant increase $p < 0.05$ in the number of WBC and lymphocytes in the group treated with SA only. In addition, the PCV and RBCs diminished significantly in the SA group when compared with the control. However, both PCV and RBCs increased significantly ($p< 0.05$) in Group D pretreated with riboceine before SA when compared with the group given SA alone.

DISCUSSION

The carcinogenic effect of arsenic has been linked with ROS generation which cumulates into an alteration in cellular redox states and oxidative stress, an imbalance between the cellular antioxidant defense and ROS generation (Xu *et al*., 2017). This decline in the antioxidant capability of the body system affected, has led to the proposition of the use of antioxidants as therapeutic and preventive agents in circumstances of metal-induced oxidative damage and it has been effective in the protection of cells from these detrimental effects (Flora 2011; Nandi *et al*., 2005). The present study examined the effect of D-Ribose-L-Cysteine, a potent antioxidant, on sodium arsenite-induced hepatorenal toxicities in rats. An exposure of rats to sodium arsenite (SA) at 5 mg/kg body weight via oral gavage resulted in notable decrease in the percentage body weight gain of rats in the group treated with SA only when compared with the control. The inhibitory effect of SA on weight gain is consistent with reports of previous studies (Adil *et al*., 2015; Jana *et al*., 2006). More so, SA toxicity has been reported to destroy liver integrity in mouse, rat and goat (Sharma *et al*., 2009; Roy *et al*., 2009). Furthermore, the increased activities of AST, ALP and ALT seen in the serum of rats treated with SA alone when compared with the control, along with the significant decrease in the relative liver weight in the former group point in the direction of SA effect on systemic metabolism related to liver. Moreover, histological analysis of the liver samples confirms the toxic effects of SA which was prevented by pretreatment with riboceine.

The glutathione-stimulating effect of riboceine was also observed in groups treated with riboceine suggesting its cellular restorative capability. This is in consonance with the findings that the glutathione-stimulating effect of riboceine is key in combating oxidative stress in the body as it helps raise the level of this vital anti-oxidant in the cells (Falana *et al*., 2017).

Arsenite toxicity induces several metabolic disorders including urea and creatinine elevation following proximal tubule damage and glomerular injury (Anwar *et al*.,1999; Nandi *et al*., 2005). In the present study, sodium arsenite treatment had deleterious effects on kidney functions as seen by elevation of urea and creatinine levels in the serum in the rats treated with SA alone. Urea, a major waste product of protein catabolism, can rise especially when the kidney is defective (Higgins, 2016). Pretreatment with riboceine was observed to have a significant reversal effect on these parameters. Glutathione (GSH) is a tri-peptide non-enzymatic anti-oxidant that has a beneficiary role in the protection of the cell against metal-induced oxidative stress (Masella *et al*., 2005). It functions by binding metals at sulfhydryl groups, thereby preventing them from creating ROS (Andrews, 2000; Pinto *et al*., 2003). The present study revealed a significant decrease in hepatorenal GSH level on exposure to sodium arsenite. Riboceine treatment on the other hand, produced an increase in GSH levels supporting its capacity in restoring depleted GSH levels and therefore ability as a potent antioxidant agent.

A decrease in CAT activities in the liver and kidney in the group of rats treated with SA alone in this study signifies a decrease in free radical scavenging activities. These findings were in agreement with previous studies, which showed that intracellular ROS levels were increased and GSH contents were decreased and accompanied by reduction of SOD activity in cells after exposure to SA (Shi *et al*., 2003). On the other hand, we observed increased activities of hepatorenal GPx and SOD in the group exposed to SA. The observed difference in the behavior of SOD may relate to the dosage and length of time of exposure adopted in the study as explained in another study (Flora, 2011).
One major effect of ROS is lipid peroxidation that generate many relatively stable decomposition end-products, primarily unsaturated reactive aldehydes, like MDA, HNE and 2-propenal (acrolein) and isoprostanes. As indirect indicators of oxidative stress, these can be measured in plasma and urine (Dalle-Donne *et al*., 2008). In the present study, the lipid peroxidation product levels increased in both liver and kidney of rats treated with SA. This elevated level of MDA was brought to about normal by pretreatment with riboceine thereby showcasing its protective ability against SA-induced peroxidation of lipid and fatty lining membrane of functional organs such as the liver and kidney.

Related to the toxicity of SA is cellular inflammation. During inflammation, NO (nitric oxide) production, by the vasculature is considerably increased and, along with other ROS, add to oxidative stress (Lubos *et al*., 2008). In the present study, NO levels increased significantly in SA treated rats. However, this effect was reduced significantly in the group of rats pretreated with riboceine thereby showcasing the protective effects of riboceine, this is consistent with earlier report (Adil *et al*., 2014). Trends similar to the observation with NO were seen in the MPO levels. There was a significant reduction in MPO levels in the group of rats pretreated with riboceine before SA compare with the group administered SA only.

Considering the hematological parameters in the treated animals, PCV and RBCs were observed to diminish significantly in group treated with SA alone when compared with the control. This reduction in PCV, Hb, and RBC counts might be a result of inhibition of porphyrin or heme synthesis. Arsenic is reported to cause inhibition of aminolevulinic acid dehydratase activity, thereby modifying the heme synthesis pathway (Gupta *et al*., 2005). However, both PCV and RBCs increased significantly when rats were pretreated with riboceine before SA exposure. This is in line with the report by (Ola-Davies and Akinrinde, 2016). The hematinic property of riboceine was observed in reversing anemia induced by sodium arsenite. No significant change in WBC and platelets levels across the treatment groups,

except for Group B animals, treated with SA alone, where a significant increase in the WBC level was observed which could be indicating that sodium arsenite can induce immune response indicated by increase in lymphocytes counts.

In conclusion, this study demonstrated a protective role of riboceine against toxicities induced with sodium arsenite in male Wistar rats. D-Ribose-L-Cysteine was found to be effective in preventing oxidative stress and damage to liver and kidney cells in the animals exposed to sodium arsenite. D-Ribose-L-Cysteine therefore showed strong antioxidant, cytoprotective and anti-inflammatory effects against oxidative stress and hepatorenal toxicity induced with sodium arsenite in rats (Figure 10). This is the first report showing the protective effect of D-Ribose-L-Cysteine on hepato-nephrotoxicity induced by sodium arsenite in rat model.

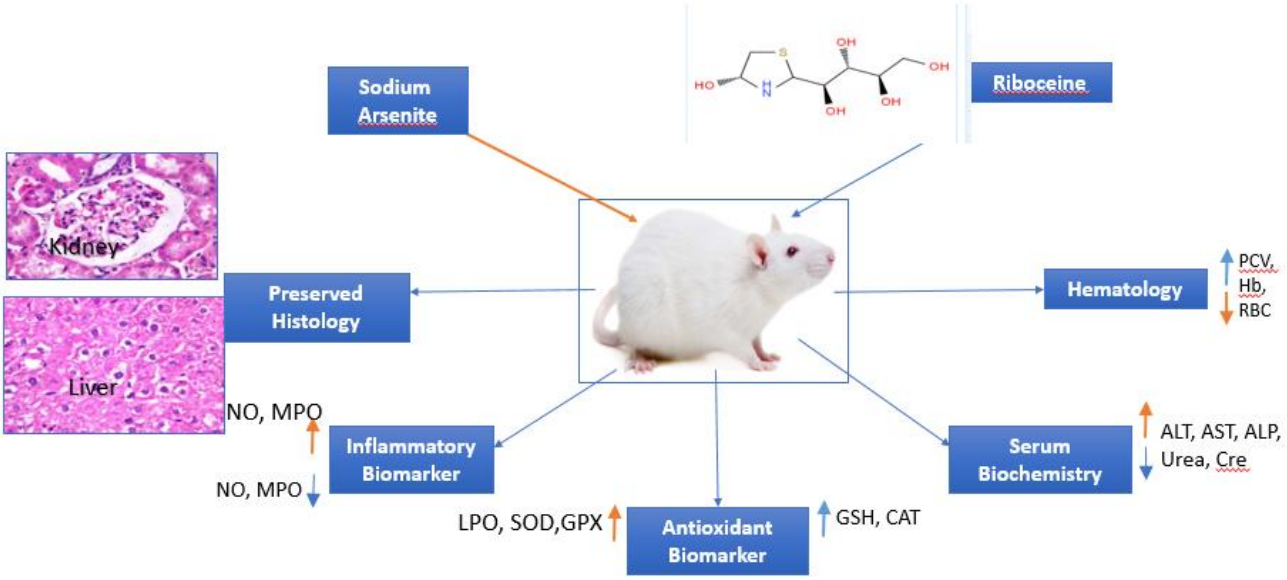

Figure 9:
Graphical summary of the protective role of riboceine against sodium arsenite-induced hepatotoxicity and nephrotoxicity in rats

Acknowledgements
We appreciate the Cancer Research and Molecular Biology Laboratories Team of the Department of Biochemistry, University of Ibadan for their support during the execution of this research

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Niger. J. Physiol. Sci. 39 (June 2024): 57 – 63
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Gastroprotective Biochemicals in Wistar Rats Orally Exposed to Bisphenol A and Co-treated with either *Garcinia kola* (Heckel) Seeds or its Biflavonoid, Kolaviron

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Summary: This study evaluated gastroprotective biochemicals in Wistar rats exposed to both Bisphenol A and either *Garcinia kola* or its biflavonoid, kolaviron. Fifty-six rats (140-160g) divided into 7 groups (n=8), and treated orally for 28 days as follows; Group I was the control (distilled water, 1.5mL/kg) while group II (vehicle control) received corn oil (1.5mL/kg), groups III-V were exposed to BPA (50mg/kg) only and treated with distilled water (1.5mL/kg), *Garcinia kola* (200mg/kg) and kolaviron (200mg/kg), respectively. Animals in groups VI and VII received *Garcinia kola* (200mg/kg) and kolaviron (200mg/kg) only, respectively. Thereafter and under anaesthesia, the stomach was dissected out, estimated for mucin (n=3), homogenized (n=5), centrifuged, and the clear supernatant obtained was analyzed for malondialdehyde, superoxide dismutase, catalase, reduced glutathione, glutathione S-transferase, nitrites, myeloperoxidase, interleukin-6 and tumor necrosis factor-α, respectively. Gastroprotective biochemicals were significantly ($p<0.05$) reduced in animals exposed to BPA while values in animals exposed both BPA and either *Garcinia kola* or kolaviron were elevated. Exposure to *Garcinia kola* and kolaviron alone also showed a potentiation of gastric antioxidant and anti-inflammatory activities. This study shows that *Garcinia kola* and especially its biflavonoid, kolaviron, protects the gastric mucosa against Bisphenol A induced impairment by potentiating gastroprotective biochemicals in male Wistar rats.

Keywords*: Bisphenol A, stomach, Garcinia kola, kolaviron, antioxidant, antiinflammation*

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Manuscript received- November 2023; Accepted- February 2024

DOI: https://doi.org/10.54548/njps.v39i1.8

INTRODUCTION

Bisphenol A (BPA) is one of the most produced industrial chemicals globally. It is extensively used in the production of epoxy resins and polycarbonate plastics that are used in food and drink packaging industry (Manzoor *et al*., 2022). Bisphenol A also belongs to a group of compounds that are categorized as endocrine disruptive chemicals (EDCs) that are capable of mimicking or antagonizing the mechanism of action, synthesis, transportation, receptor interaction, storage and disposal of hormones in the body resulting in multi organ toxicities in both animals and human beings (Yoon *et al*., 2014). Studies have shown that in plastic products, not all monomers and additives utilized during production are sufficiently polymerized and covalently bonded. Hence, some of the chemicals used can potentially leach and diffuse through the polymeric matrix of the product and into the foods stored in these plastics (Muzeza *et al*., 2023). Bisphenol A has been reported to leach out into food when plastics there are contained in are exposed to high temperature and abrasive chemicals (Obuzor and Onyedikachi, 2023).

Bisphenol A has been reported to cause multiple organ toxicities after entering the body through the respiratory, dermal, and / or gastrointestinal tract. It has been reported that the main exposure route to BPA in humans is orally and the stomach as well as the intestine have been observed to be especially vulnerable to its (i.e., BPA) adverse effects (Vandenberg *et al*., 2007). Bisphenol A has been reported to promote inflammatory processes in the stomach and intestine, damage gastrointestinal barrier function, and cause changes in the neurochemical characterization of nervous structures supplying the gastrointestinal tract (Szymańska *et al*., 2020). The ulcerative effect of BPA has also been attributed to its ability to induce gastric oxidative stress (Abo-Elsoud *et al*., 2022). The mechanisms adduced to BPA induced gastric impairment suggests that antioxidant substances, especially natural products such as *Garcinia kola* and kolaviron, may exert beneficial effects.

Garcinia kola, which belongs to the genus "Garcinia", family "Clusiaceae" and order "Malpighiales", is found in tropical African countries and all over Asia. In Nigeria, the seed (commonly referred to as bitter kola) is widely consumed for traditional hospitality as well as at

cultural and social ceremonies (Ogwu *et al*., 2024). In folkloric medicine, it is used to manage and treat hypertension, cancer, malaria, diabetes, and numerous other ailments (Emmanuel *et al*., 2021). Investigations for medicinal properties have also revealed that it exerts antioxidant, antidiabetic, antihypertension, anti-analgesic, anti-inflammatory effects and possesses neuro-, cardio- and gastro- protective properties (Dogara *et al*., 2022). Kolaviron, an active complex of at least 3 compounds in *Garcinia kola* seed, has been reported to possess a wide array of pharmacological properties some of which include antidiabetic, cardioprotective, neuroprotective, haematoprotective, nephroprotective, gastroprotective, and hepatoprotective activities (Oyagbemi *et al*., 2016). Despite the presence of various active principles in *Garcinia kola*, studies have reported most of its pharmacological activity could be attributed to that of its principal biflavonoid, kolaviron (Tauchen *et al*., 2023).

Garcinia kola and kolaviron have both been reported to mitigate, ameliorate, or reverse several toxicities in diverse experimental studies (Olatoye and Akindele, 2021). The likely therapeutic effects of either *Garcinia kola* or kolaviron against BPA induced toxicities are sparse with limited information on gastroprotective potentials. One of such is our previous investigation on the neuroprotection offered by *Garcinia kola* and kolaviron against BPA induced behavioural impairment and hippocampal inflammation (Kayinu *et al*., 2024). In the gastrointestinal tract, gastroprotection is said to be proffered through two main mechanisms which include mechanisms that decrease or counter acid/pepsin secretion and those that afford cytoprotection by virtue of their effects on mucosal defensive factors which include mucin secretion, cellular mucus production, gastric antioxidants, bicarbonate secretion, mucosal blood flow and cell turnover (Goyal and Sairam, 2003, Ige *et al*., 2016).

This study was therefore designed to evaluate gastric cytoprotective biochemicals in male Wistar animals exposed to both Bisphenol A and either *Garcinia kola* or kolaviron.

MATERIALS AND METHODS

Drugs, plant preparation and extraction of kolaviron: Bisphenol A, *Garcinia kola* and kolaviron were obtained as previously described (Kayinu *et al.,* 2024). Briefly, BPA (Sigma-Aldrich, St. Louis, MO, USA) was suspended in corn oil prior to administration. *Garcinia kola* seeds, purchased from a local vendor in Kaduna state, Nigeria, was authenticated at the University of Ibadan Herbarium where a voucher specimen already existed. These seeds were peeled, sliced, air-dried, pulverized and stored in a sterile container until needed. Daily, fresh solutions of *Garcinia kola* suspended in distilled water were prepared and administered at a dose of 200 mg/kg. Kolaviron was extracted from *Garcinia kola* using the method described by Olaleye *et al.,* (2000). Briefly, dried pulverized *Garcinia kola* was defatted with n-hexane (in order to extract nonpolar inactive compounds) using the cold maceration method. The dried marc obtained was subsequently re-defatted twice using n-hexane. Thereafter the defatted filtrate obtained was dried and repeatedly extracted with acetone (for optimal extraction of flavonoids, saponins, phenolic compounds and other extractable solids) thrice to get the kolaviron-rich crude extract. This crude acetone extract was concentrated to about 100mL with a rotary evaporator at 40℃, diluted to twice its volume with distilled water and then partitioned with ethyl acetate (to extract nonpolar flavonoids). The ethyl acetate fraction obtained was concentrated to get a yellow-brown powder known as kolaviron which was administered at a treatment dose of 200 mg/kg (Kayinu *et al.,* 2024).

Animals, groupings and experimental protocol: Fifty-Six (56) rats of Wistar strain (140-160 g), obtained from Central Animal House, College of Medicine, University of Ibadan, were used for this study. The animals were house and acclimatized for 2 weeks prior to experimental procedures in solid bottom plastic cages, under standard environmental conditions at room temperature (approximately 26-30 C), and natural alternating day and nighttime cycles respectively. Animals were maintained under humane conditions in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996). The animals were allowed access to standard rat chow and clean drinking water *ad libitum* throughout the experiment. Thereafter, Animals were divided into seven (7) equal groups and treated as follows. Group I, the control group, received distilled water (1.5 mL/kg), while group II served as vehicle control and was treated with corn oil (1.5 mg/kg). Animals in groups III – V were co-treated with BPA (50mg/kg) (Zhang *et al*., 2013) and either distilled water (1.5 mL/kg), *Garcinia kola* (200 mg/kg), or kolaviron (200 mg/kg), respectively. All treatments were administered via the oral route using an oral cannula, daily for 28 days.

Sample collection, preparation and biochemical assays: At the end of the treatment duration, animals in each group were anaesthetized using an intraperitoneal administration of ketamine (87 mg/kg) and xylazine (13 mg/kg) thereafter a midline incision was carried out and the stomach of each animal (n=5/group) was excised, perfused with cold phosphate buffered saline (PBS) (pH 7.4) to wash away any debris contained therein, weighed, homogenized in PBS (w/v 1:4) and centrifuged at 10,000 rpm for 10min at 4 ^{0}C. The clear supernatant obtained was aliquoted into labeled sample bottles and stored, until use, at -4 ^{0}C. The supernatant obtained was analysed for malondialdehyde (Papastergiadis *et al.,* 2012), superoxide dismutase (Misra and Fridovich, 1972), catalase (Sinha, 1972), reduced glutathione (Jollow *et al*., 1974), glutathione S-transferase (Habig *et al.,* (1974), nitrites (Olaleye *et al*., 2007), myeloperoxidase (Xia and Zweier 1997), interleukin-6 and tumor necrosis factor-α (ELISA**),** respectively. Stomach samples (n=3/group) were also estimated for gastric barrier mucous (as mucin) using the method described by Corne *et al.,* (1974).

Statistical analysis: Data obtained was analyzed using One-way ANOVA, followed by Turkey multiple comparisons with statistical significance between groups at $p < 0.05$.

RESULTS

Gastric biomarkers of oxidative status in control and experimental groups: Gastric malondialdehyde (MDA), a marker of lipid peroxidation, was increased ($p<0.05$) in

group III (96.6%) when compared with control. Values in groups IV and V decreased ($p<0.05$) by 26.3% and 45.7% when compared with group III, while values in groups VI and VII were comparable with controls (Group I). Superoxide dismutase activity (U/mg protein) in group III (0.94±0.04) was significantly reduced ($p<0.05$) compared with groups I (1.21±0.16), IV (1.23±0.03) and V (2.60±0.14) respectively. Compared to controls, SOD values in groups V (2.60±0.14) and VII (1.91±0.11) were increased, while values in group VI (1.50±0.12) were comparable. Catalase, reduced glutathione, glutathione-S-transferase and nitrites were reduced in group III (the BPA only group) compared to controls, while values in groups IV and V increased when compared with the same group (group III), respectively (Table 1).

Gastric mucin and inflammation biomarkers in control and experimental groups: Mucin levels (mg/g protein) reduced in groups III (15.69±5.07), IV (21.81±4.52) and V (27.19±5.91) when compared with controls (55.92±3.48) while values in groups VI and and VII were significantly increased when compared with control and all other treatment groups (Figure 1). Myeloperoxidase (MPO) (a marker of oxidative stress and inflammation), interleukin-6 (Il-6), and tumour necrosis factor – alpha (TNF-α) was significantly increased ($p<0.05$) in group III compared with control and all other experimental groups (Figure 2-4). The values for Il-6 (ρg/mg protein) and TNF-α (ρg/mg protein) were however increased in groups IV (57.36±3.27; 77.59±2.84) and VII (54.55±4.07; 69.15±0.96) compared with control (30.59±1.31; 40.13±3.12). Values for TNF were also elevated in group V (78.66±3.72) when compared with control (40.13±3.12) (Figure 4).

Table 1.
Gastric oxidative status in control and experimental groups

Groups	Antioxidant: Oxidant biomarkers					
	MDA (ηmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GSH (μM/mg protein)	GST (U/mg protein)	Nitrites (μmoles/mg protein)
I	0.89 ± 0.03	1.21 ± 0.16	16.18 ± 1.13	8.24 ± 0.27	0.112 ± 0.010	3.02 ± 0.06
II	1.24 ± 0.04	1.32 ± 0.08	12.33 ± 0.64	6.76 ± 0.49	0.077 ± 0.004	3.11 ± 0.23
III	1.75 ± 0.03*	0.94 ± 0.04*	9.75 ± 0.30*	5.83 ± 0.37*	0.048 ± 0.005*	2.24 ± 0.09*
IV	1.29 ± 0.05*#	1.23 ± 0.03#	13.45 ± 0.27#	10.70 ± 0.50#	0.111 ± 0.008#	4.18 ± 0.29#
V	0.95 ± 0.08#	2.60 ±0.14*#	17.18 ± 0.58#	14.86 ± 0.85#	0.147 ± 0.015#	3.25 ± 0.21#
VI	0.86 ± 0.04	1.50 ± 0.12	12.32 ± 0.50	7.82 ± 0.24	0.122 ± 0.008	2.36 ± 0.16
VII	0.81 ± 0.05	1.91 ± 0.11*	18.11 ± 0.46	9.97 ± 0.32	0.142 ± 0.012	2.57 ± 0.11

*Values are mean±SEM, * indicates values that are significantly different from controls, while # indicates values that are significantly different from group III, the BPA only treatment group.*
I – Control, II – Vehicle group, III – BPA only, IV – BPA+G. kola, V – BPA+kolaviron, VI – G. kola only, VII – kolaviron only. MDA = malondialdehyde, SOD = superoxide dismutase, CAT = catalase, GSH = reduced glutathione, GST = glutathione-S-transferase.

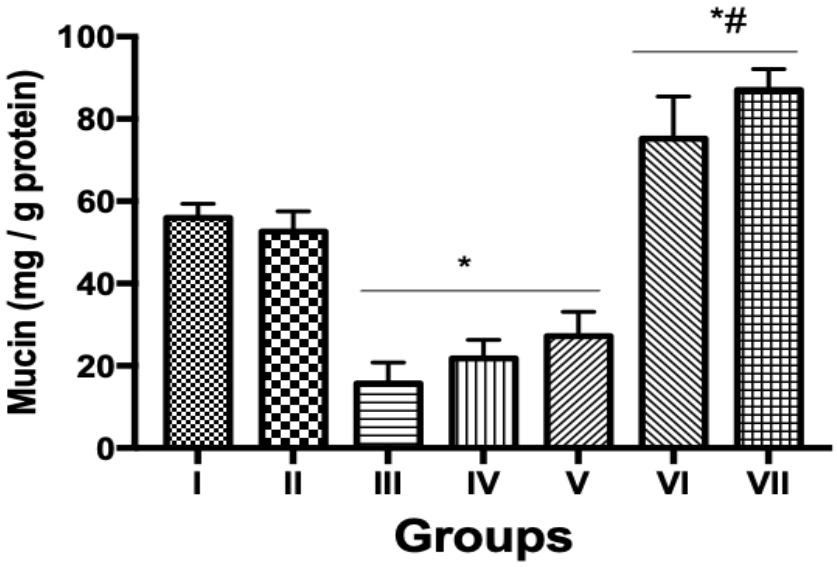

Figure 1.
Gastric mucin levels in control and experimental groups exposed to Bisphenol A and either *Garcinia kola* or kolaviron.
Values are mean±SEM, * indicates values that are significantly different from controls, while # indicates values that are significantly different from group III, the BPA only treatment group. I – Control, II – Vehicle group, III – BPA only, IV – BPA+*G. kola*, V – BPA+kolaviron, VI – *G. kola only*, VII – kolaviron only. MDA = malondialdehyde, SOD = superoxide dismutase, CAT = catalase, GSH = reduced glutathione, GST = glutathione-S-transferase.

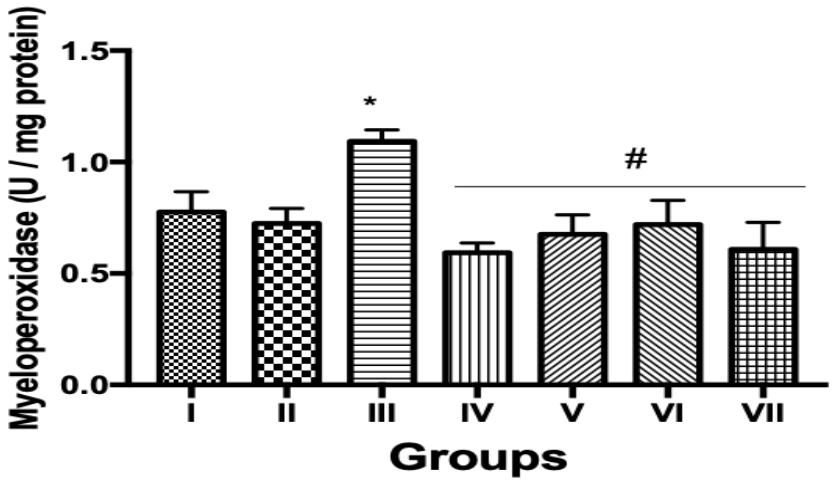

Figure 2.
Myeloperoxidase activity in the gastric tissue of control and experimental groups.
Values are mean±SEM, * indicates values that are significantly different from controls, while # indicates values that are significantly different from group III, the BPA only treatment group. I – Control, II – Vehicle group, III – BPA only, IV – BPA+*G. kola*, V – BPA+kolaviron, VI – *G. kola only*, VII – kolaviron only. MDA = malondialdehyde, SOD = superoxide dismutase, CAT = catalase, GSH = reduced glutathione, GST = glutathione-S-transferase.

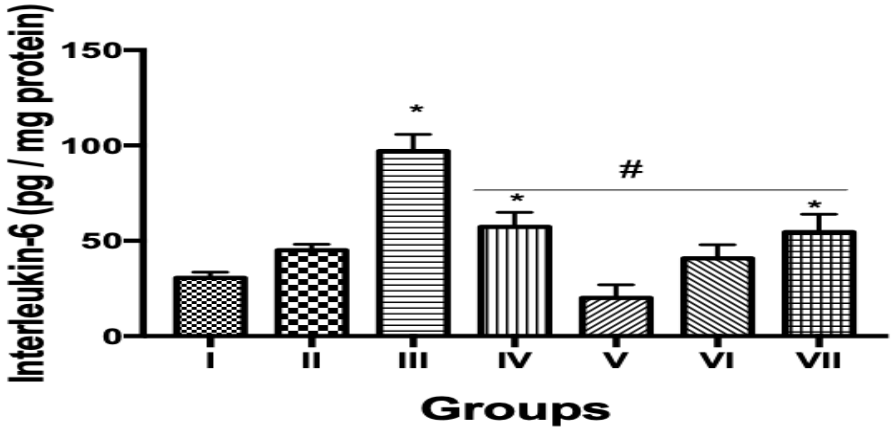

Figure 3.
Gastric interleukin-6 activity in control and experimental groups.
Values are mean±SEM, * indicates values that are significantly different from controls, while # indicates values that are significantly different from group III, the BPA only treatment group. I – Control, II – Vehicle group, III – BPA only, IV – BPA+*G. kola*, V – BPA+kolaviron, VI – *G. kola only*, VII – kolaviron only. MDA = malondialdehyde, SOD = superoxide dismutase, CAT = catalase, GSH = reduced glutathione, GST = glutathione-S-transferase.

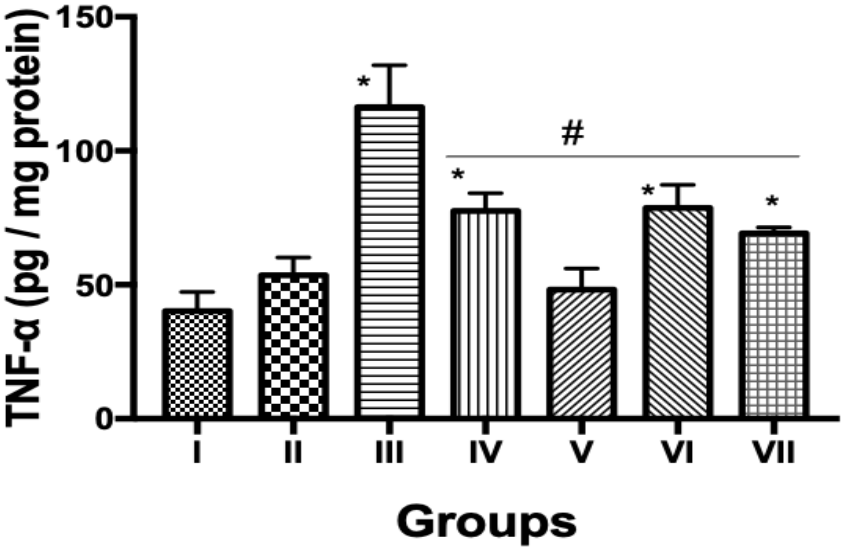

Figure 4.
Tumour necrosis factor – alpha activity in the gastric tissue of control and experimental groups
Values are mean±SEM, * indicates values that are significantly different from controls, while # indicates values that are significantly different from group III, the BPA only treatment group. I – Control, II – Vehicle group, III – BPA only, IV – BPA+*G. kola*, V – BPA+kolaviron, VI – *G. kola only*, VII – kolaviron only. MDA = malondialdehyde, SOD = superoxide dismutase, CAT = catalase, GSH = reduced glutathione, GST = glutathione-S-transferase.

DISCUSSION

Naturally, there is a balance between aggressive forces and protective mechanisms in the stomach, thereby maintaining the integrity of the mucosa (Goel and Sairam, 2003). In cases of an increased aggressor challenge such as exposure to BPA, the defense mechanisms need to be upregulated to avoid pending injury or invasion of the mucosa. Bisphenol A, an "endocrine disrupting" substance found in plastics, has been reported to be absorbed by the gastrointestinal (GI) tract especially the stomach where it has been shown to induce high levels of oxidative stress resulting in a compromise of the gastric structural and functional integrity (Chen *et al.,* 2022). Two important gastroprotective mechanisms involved in maintaining gastric integrity is the protective mucous layer and intrinsic antioxidant defense

system domiciled within the gastrointestinal tract (Goel and Sairam, 2003).

In this study, oral exposure to BPA led to a reduction in the gastric tissue level of mucin and GSH as well as reduced activities of SOD, CAT, and GST suggesting a compromise of gastric integrity and endogenous gastric antioxidant protective mechanisms. Furthermore, and to lend credence to this is the fact that gastric malondialdehyde levels, a marker lipid peroxidation induced by oxidative stress, was elevated in the BPA only treatment group. Myeloperoxidase (MPO), a marker of leucocytes infiltration, which is considered an index of inflammatory and leukocyte-mediated mucosal tissue damage as well as breakdown of the mucosal barrier, was found to be elevated in the gastric tissues of BPA only group, which shows that the integrity of the gastric mucosa in this group may be compromised by mechanisms that can be associated with increased oxidative stress and inflammation. These observations are consistent with finding from other researchers who have reported a gastrotoxic effect of BPA on the gastric mucosa (Ige *et al.,* 2022, Abo-Elsoud *et al.,* 2022).

One of the primary manifestations of increased oxidative stress is inflammation (Sánchez *et al.,* 2015). In this study, the BPA only treated group also exhibited signs of increased gastric inflammation as gastric IL-6 and TNF-α activity were found to be elevated in this treatment group. Bisphenol A has been reported to mimic estrogenic activity resulting in immune dysregulation and thus affect the immune cell signaling pathways and responses. Mucosal injury following exposure to BPA has also been suggested to occur as a result of increased stimulation and production of macrophage TNF-α (Sugimoto *et al.,* 2007). Furthermore, increased TNF-α activates caspase-3 that leads to gastric cell apoptosis (Park *et al.,* 2000). Exposure to BPA has also been linked to an increased expression of gastric IL-6 through mechanisms that are associated with an inflammation mediated amplification of NF-κB production (Zhang *et al*., 2020).

In this study, concomitant treatment of BPA orally exposed animals with *Garcinia kola* or its biflavonoid extract, kolaviron, upregulated the production of gastroprotective biochemicals (SOD, CAT, GSH and GST) when compared with BPA only treated animals suggesting a mitigation of BPA-induced gastric oxidative stress. Furthermore, animals in the BPA+*Garcinia kola*, BPA+kolaviron, *Garcinia kola* only, and kolaviron only treatment groups showed gastric lipid peroxidation values that suggests a potentiation of and increased level of free radical scavenging activity in these treatment groups. The activities of MPO, Il-6 and TNF-α seen in these groups also further suggests a mitigation of BPA induced gastric inflammation. However, mucins which are composed of highly glycosylated proteins that play a tripartite (lubrication, cell signaling, protecting the epithelium) role in the gut, was reduced in all animals exposed to BPA (untreated and treated). This suggest that co-exposure of BPA to either *Garcinia kola* or kolaviron did not attenuate reductions in mucin secretion which have been reported following exposure of the gut to BPA (Gonkowski, 2020). Exposure to either *Garcinia kola* or kolaviron alone, however resulted in increased mucin secretion suggesting a potentiation of gastroprotection in these treatment group.

Isolates of *Garcinia kola* that have been implicated as being responsible for its plethora of its medicinal potentials includes biflavonoids, benzophenones, benzofurans, benzopyran, vitamin E derivatives, xanthones, and phytosterols (Emmanuel *et al.,* 2021). *Garcinia kola* and its major biflavonoid extract, kolaviron, have also been widely touted in various reports for their strong antioxidant and anti-inflammatory potentials (Olatoye and Akindele, 2021). Furthermore, though *Garcinia kola* contains a plethora of medicinal phytochemicals, most of its medical and pharmacological activity could be ascribed to its major biflavonoid, kolaviron (Farombi *et al.,* 2022), which is an active complex of at least 3 compounds consisting of Garcinia Biflavanoid-1 (GB-1), kolaflavanone, and Garcinia Biflavanoid-2 (GB-2). This kolaviron is well known for its potent antioxidant and anti-inflammatory properties that have been explored for therapeutic potentials in several disease models ranging from reproductive toxicity, cardiotoxicity, diabetes mellitus, gastrotoxicity and hepatotoxicity (Erukainure *et al.,* 2021). The potent antioxidant potentials of kolaviron may therefore be responsible for the increased activity of gastric antioxidant biochemicals in the groups co-exposed to BPA and kolaviron when compared with animals co-treated with BPA and *Garcinia kola.*

Taken together, it is likely that the gastroprotection and mitigation against BPA induced gastrotoxicity proffered by both *Garcinia kola* and kolaviron in this study may be due to their ability to potentiate the gastric antioxidant defense mechanism resulting in an upregulation of the production and secretion of SOD, CAT, GSH and GST. The increased SOD produced would have scavenged superoxide ions which would be converted by catalase to water. Increased GSH-GST levels has been reported to protect cells from oxidation caused by BPA via mechanisms such as direct removal of oxidants and increasing the activity of glutathione peroxidase resulting in consumption of glutathione and its conversion into ineffective form of disulfate (Sabour, 2019). The collective actions of the increased antioxidant activity would therefore account for the reduced production of BPA-induced free radicals and thus account for the reductions in gastric inflammatory processes (MPO, Il-6, and TNF-α) seen in the *Garcinia kola* and kolaviron treated BPA exposed groups.

Nitrites have been reported to contribute to host defense mechanism against a number of pathogenic microorganisms in the mouth, stomach and skin (Archer, 2002). It has also been observed to increase gastric mucosal blood flow and mucus thickness (Björne *et al.,* 2004). The reduced values in the BPA only treatment group therefore suggests a likely compromise of gastroprotection via mechanisms that maybe associated with reduced mucosal blood flow and mucus thickness. Nitrites in the groups co-exposed to BPA and either *Garcinia kola* or kolaviron was increased compared to BPA only and comparable to controls suggesting a mitigation of gastric injury in these groups (IV and V), respectively.

In conclusion, this study suggests that *Garcinia kola* and its biflavonoid, kolaviron protects the gastric mucosa against Bisphenol A induced impairment by potentiating gastroprotective biochemicals in male Wistar rats. However, in the duration that this study was conducted, BPA induced reduction of gastric mucin was not attenuated by either *Garcinia kola* or kolaviron

Ethical Approval: For this research related to animals, the Applied and Environmental Physiology Unit, Department of Physiology University of Ibadan approved the experimental procedures and protocol, which has complied with all the guidelines laid down by the Animal Care and Use Research Ethics Committee (ACUREC), University of Ibadan and that of the Guide for the Care and Use of Laboratory Animals, 1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA (The Applied and Environmental Unit, Department of Physiology does not issue approval numbers. However, all research studies emanating from this Unit is scrutinised with emphasis on humane care and treatment of animals. Furthermore, the Unit also ensures that all research projects are presented to a larger audience consisting of experts in the field Physiological research. Letters supporting this can be obtained from the Department of Physiology, University of Ibadan if required).

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Niger. J. Physiol. Sci. 39 (June 2024): 65 – 75
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Sixty Minutes Post-Exercise Evaluation of Cardiovascular Responses in Normotensive Students of the University of Maiduguri, Borno State, Northeast Nigeria

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Summary: The study was aimed at providing a pattern of blood pressure decrease after an acute bout of exercise in normotensive subjects. A simple random sampling technique was used to select 138 apparently normotensive subjects (83 males and 55 females; age range 18-30 years), and the mean age was 22.28±0.52 and 19.84±0.28 in males and females, respectively. All participants were indigenes and undergraduate students of the University of Maiduguri, Borno State, Nigeria. The mean BMI was 23.29±3.80 kg/m and 19.07±2.42 kg/m in males and females, respectively. The mean resting SBP (118.65±1.52 and 105.6±2.14 in males and females, respectively), DBP (74.44±1.48 mmHg and 70.04±1.42 mmHg in males and females, respectively), and HR (83.92±2.63 bpm and 87.00±2.76 bpm in males and females, respectively) were recorded on their arrival. The mean SBP (145.28±2.67 mmHg and 123.64±1.97 mmHg in males and females, respectively), DBP (82.68±1.70 mmHg and 75.84±1.36 mmHg in males and females, respectively), and HR (112.96±3.08 bpm and 127.44±4.01 bpm in males and females, respectively) were recorded at 10 minutes of exercise. The study observed a significantly higher ($p < 0.05$) BMI in males than in females. Though BMI was positively associated with SBP at the end of 60 minutes into the recovery in males, the HR was negatively associated with BMI in females at 60 minutes into the exercise recovery. Meanwhile, such association was not seen in other parameters. The mean SBP, DBP, & HR at 10 minutes of exercise were all significantly higher ($p < 0.05$) than the resting state in both males and females. The decrease in the mean SBP, DBP, & HR after exercise was plotted against time for 60 minutes into the exercise recovery. A positive percentage change of SBP (22.56% and 17.18% in males and females, respectively), DBP (11.04% and 8.66% in males and females, respectively) and HR (34.41% and 45.37% in males and females, respectively) was seen at 10 minutes of exercise. A curvilinear pattern of SBP, DBP, and HR decline was observed at the end of 60 minutes. At the 3rd minute, the SBP in both males and females was already on baseline; DBP in males was slightly above baseline with a 2% positive change, while SBP in females was slightly below baseline. The SBP of male and female participants after the 3rd minute fell below baseline and remained persistently negative till the end of 60 minutes, with the female curve being far away from the baseline compared to male. The DBP in both males and females was on the baseline at the 14th and 15th minutes, respectively; the curve fell below baseline and remained persistent, but very close to the baseline. The curvilinear pattern of HR in both males and females showed a positive percentage but was above the baseline in both males and females. The study concluded that a typical curvilinear pattern of SBP crossed the baseline at the 3rd minute in both males and females, the magnitude of which is higher in females than in males; the DBP crosses at the 14th and 16th minutes and remained close to the baseline. Meanwhile, HR remained above the baseline. It is therefore suggested that SBP should be taken as a standard pattern for BP decrease during exercise..

Keywords*: Post exercise, systolic and diastolic blood pressure, Heart rate, Normotensive, BMI, University of Maiduguri*

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DOI: https://doi.org/10.54548/njps.v39i1.9

INTRODUCTION

Physical activity is recommended for both prevention and diagnosis of cardiovascular diseases. It has also been applied in the management of high blood pressure in particular (Guidelines committee 2003; Pescatello *et al*, 2004). During exercise a dramatic increase in systolic and diastolic blood pressure have been reported and the extent of these increase depend on the time and intensity of the exercise (Stone *et al*., 1991). Immediately following a period of dynamic exercise, blood pressure decreases (Ressl *et al*., 1977). The duration for the decrease in systolic blood pressure and diastolic blood pressure may be as much as 30 minutes after exercise at 30 and 18mmHg in normotensive subjects and 45 and 24mmHg in hypertensive subjects

(Wilcox *et al*.,1982). Therefore the optimal gold of interest is the importance of studying cardiovascular recovery from exercise. This recovery may not be simply returning to pre-excercise period rather it could be seen as dynamic period where many physiological changes can occur. Thus, it could be argued that recovery from exercise should also be seen as important as exercise stimulus. As already described by luttreel and Halliwill (2009), the recovery from exercise can be viewed as a vulnerable period in which individuals are at heightened risk for adverse events or window of opportunity for adaptation in which training can be potentially augmented. Furthermore, it may also provide insight into when the cardiovascular system recovers.

The cardiovascular system after exercise exists in a physiological state which differ from rest and exercise. During dynamic exercise systolic blood pressure increase linearly with increasing rates of work reaching a peak value (Plateau) of 200mmHg and 240mmHg and negative drift in normotensive persons. This increase is brought about by the increase in cardiac output and would have even be higher if not for the fact that resistance decreases thereby partially offsetting the increased cardiac output and as a result of reseting the arterial baroreflex to a higher point. However, diastolic blood pressure does not change or change so little that it has no physiological significance. The mechanism adjusts blood pressure, heart rate and systemic vascular resistance is being reported to be mediated reflexly by modulating sympathetic and parasympathetic tone to the heart and vessels on the bases of the mechanical and metabolic status of working muscles (Nobrega *et al*.,2014; Crisafulli, 2017)

It is obvious that cardiovascular responses could be viewed at two stages; during exercise and after exercise, the mechanism which are totally different from that obtained during resting stage. The blood pressure and heart rate takes a shorter duration to reach a peak, averagely the first 1-2 minutes of exercise whereas the recovery from exercise take several hours as recognised early in Hill's initial observation of blood pressure responses following aerobic exercise (Romero *et al*.,2017). The longer duration for the cardiovascular system to return to pre-exercise state stimulate the use of exercise in the management of high blood pressure. More so excercise testing, a way of challenging cardiovascular to physical stress, could reveal abnormalities that are not present at rest and can be used to predict adequacy of cardiac function (Gisler *et al*., 2007). Even in hospital setting, application of exercise testing has been proved to be effective in the diagnosis of some underlying CVS problems with focus on post-exercise recovery. Several reports have revealed that blood pressure decrease below normal level in normotensives without giving a specific patterns of how the decrease was obtained. Therefore evaluation of this pattern is necessary and may also serve as a standard border line between the normal and abnormal responses.

MATERIALS AND METHODS

Participants: A total number of 138 Participants (83 males and 55 females) were recruited for this study, after satisfying the inclusion criteria. This population sampling was based on a previous study that prevalence of normotensives is 90.6% between the ages of 18 to 30 years residing in Maiduguri, Borno state, Nigeria (Aliyu *et al*., 2015). Based on this finding, we assumed that the prevalence of normotensive subjects between that age range, should be applied to the students of the University of Maiduguri. The participants were selected amongst the undergraduate students of the University of Maiduguri, using a simple random sampling technique to participate, to determine Systolic blood pressure, Diastolic blood pressure, Heart rate and Body Mass Index.

All participants recruited for this study were non-athletes, non-smoking students of the university of Maiduguri with no history of cardiovascular and respiratory diseases, absence of use of any medications, including birth control pills. The female participants were not neither pregnant nor menstruating at the time of the study. The procedures of the study were explained to the participants and their verbal and written consents were obtained before participation. Approval was taken from the Health Research Ethics Committee of the University of Maiduguri.

Procedures: The criteria for selection for the participants were based on their responses to Questionnaire. Each participant was asked not to participate in any competitive sports or medication 48 hours prior to the appointment day for the exercise, other than daily normal activities. The venue of the data collection was at the Physiology Laboratory, Department of Human Physiology, University of Maiduguri.

On arrival at the venue, each participant was allowed to rest for at least 30 minutes to obtain BP and HR resting values. Measurements of height and weight was first carried out using a stadiometer (Sz-200, Suzhou Sike Machinery Equipment Co., Ltd, Suzhou Jiangsu China) and digital weighing scale (Vins Medical, England) respectively, before the commencement of the exercise testing. The Body Mass Index (BMI) was calculated from the values obtained from height and weight using the following formula,

$$BMI = \frac{Weight(Kg)}{Height(m^2)}$$

Systolic blood pressure, Diastolic blood pressure and heart rate measurements: Resting Systolic blood pressure, Diastolic blood pressure and heart rate was measured thrice and average was recorded before the onset of the exercise. A standard digital automated BP device (Omron, HEM-7120-E) was used while the subject was in a sitting position, to measure the Systolic blood pressure, Diastolic blood pressure and Heart rate. Duracell batteries was used for the device, which was renewed after each session with a subject.

Training protocol: Bruce *et al* (1976) protocol was employed in carrying out the exercise performance. Briefly, the participants were subjected to 10 minutes exercise on a stationary cycle ergometer, fixed at a speed of 10.5mph. This was in line with previous research work (Myers, *et al*., 1994) that maximal exercise capacity could be reached at approximately 10 minutes.

At the end of the 10 minutes exercise session, the Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured immediately. After that, this was followed by SBP, DBP and HR measurements at 2 minutes and 3 minutes into recovery. Subsequently, the SBP, DBP and HR were taken at intervals

of 2 minutes for a duration of 1 hour to get the pattern or curve of decrease of SBP, DBP and HR after exercise.

Data Analysis: The data obtained were analysed using Statistical Package for Social Sciences (SPSS) version 16. All results were expressed as Mean ± SEM, Independent t-test was used to compare between the means of SBP, DBP and HR response to exercise between male and female groups and analysis of Variance (ANOVA) was used to calculate difference between the mean. Mean percentage increase in SBP, DBP, and HR in male and female was compared immediately after 10 minutes of exercise. A correlation analysis was used to establish a level of association between BMI and SBP, DBP and HR immediately after 10 minutes of exercise and 60 minutes into exercise recovery, Significant level was taken at P< 0.05. The mean percentage change of SBP, DBP and HR for male and female were plotted against time of exercise recovery.

Calculation of percentage change: The following formular was use d to calculate the percentage change:

$$\frac{New\ value - original\ value}{original\ value} \times 100\%$$

If the result is positive it indicates that BP and HR is above resting value, and if the result is zero, the BP and HR is on resting value. However, if the result is negative, BP and HR is below resting value (Madhuri, 2022)

RESULTS

Anthropometric indices and Baseline Values of SBP, DBP and HR of the recruited participants: The participants recruited into this exercise were 150, comprising 91males and 59females, they were satisfied with the inclusion criteria of the study. The age range of the participants is between 18-30years with their mean age of 22.28±0.52 years and 19.84±0.28 years for male and female respectively. During the course of the exercise, we observed that only 138 subjects (83males and 55females) were eligible to be included. This was based on abnormal response that was recorded in 12 of our participants (8 males and 4 females). Further investigation from these 12 participants confirmed that they actually participated in one or more forms of exercise. The mean weight (kg), height (m) and BMI (kg/m2) were 64.38±2.26kg, 1.73±0.01m and 23.29±3.80kg/m2 in male, while in female were 50.44±1.43kg, 1.58±0.01m and 19.07±2.42kg/m2 respectively. The recorded mean systolic blood pressure (mmHg), diastolic blood pressure (mmHg) and heart rate (bpm) at the onset of the exercise were 118.65±1.52mmHg, 74.44±1.48mmHg and 83.92±2.63bpm respectively in male while in female were 105.60±2.14mmHg, 70.04±1.42mmHg & 87.00±2.76bpm respectively (Table 1a).

Table 1a:
Anthropometric indices and Baseline values of SBP, DBP, and HR of recruited participants

Variables	Male (n=83) Mean ± SEM	Female (n=55) Mean ± SEM
Age (years)	22.28±0.52	19.84±0.28
Weight (Kg)	64.38±2.26	50.44±1.43
Height (m)	1.73±0.01	1.58±0.01
BMI (Kg/m^2)	23.29±3.80	19.07±2.42
Systolic (mmHg)	118.82±0.89	105.56±1.37
Diastolic (mmHg)	74.60±0.81	69.93±0.97
Heart Rate (Bpm)	84.15±1.43	87.20±1.80

The values are presented as Mean±SEM

Baseline Mean values of Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), and Heart rate (HR) and at 10minutes of exercise in male and female: The effect of 10 minutes exercise showed that the baseline mean Systolic Blood Pressure, Diastolic Blood Pressure and Heart Rate increased from 118.65±1.52mmHg, 74.44±1.48mmHg and 83.92±2.63bpm respectively to 145.28±2.67mmHg, 82.68±1.70mmHg and 112.96±3.08bpm respectively in male. On the other hand, the same observations were made in female, the mean Systolic Blood Pressure, Diastolic Blood Pressure and Heart Rate from 105.60±2.14mmHg, 70.04±1.42mmHg and 87.00±2.76bpm respectively to 123.64±1.97mmHg, 75.84±1.36mmHg and 127.44±4.01bpm respectively (Table 1b).

Table 1b:
Baseline Mean values of Systolic Blood Pressure, Diastolic Blood Pressure, & Heart rate and after 10minutes of exercise in male and female

Parameters	Systolic Blood Pressure (mmHg)		Diastolic Blood Pressure (mmHg)		Heart Rate (bpm)	
Gender	Base line	After 10mins	Base line	After 10mins	Base line	After 10mins
Male (n=83)	118.82±0.89	145.63±1.54	74.60±0.81	82.92±0.92	84.15±1.43	113.10±1.62
Female (n=55)	105.56±1.37	123.69±1.30	69.93±0.97	75.98±0.90	87.20±1.80	126.76±2.62

The values are presented as Mean±SEM

Mean percentage increase in Systolic Blood Pressure, Diastolic Blood Pressure, & Heart Rate in Male and Female immediately after 10minutes exercise: Table 2 shows the mean percentage increase in Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and Heart Rate (HR) in both male and female immediately after 10minutes of exercise. It was observed that the mean percentage increase in SBP (22.9 ±0.12%) and DBP (11.4±0.07%) was significantly higher (P<0.05) in male than in female (17.6 ± 0.083%) and (8.66±0.07). However, contrary to what was observed in male SBP and DBP, the mean percentage increase in female Heart Rate (46.8 ± 0.22%) was significantly higher than the HR (35.6 ± 0.148%) in male.

Level of association between Basal Metabolic Index (BMI) and Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and Heart Rate (HR) at 10minutes and 60minutes into the exercise recovery: Table 3 shows the level of association between mean BMI,

SBP, DBP and HR at 10 minutes of exercise and 60 minutes recovery into the exercise. No significant association between BMI and increase in SBP in both male and female, which means that the increase in SBP observed at 10 minutes exercise does not associate with BMI. However, at 60 minutes into the exercise recovery, positive association between BMI and decrease in SBP in male was recorded, though the recorded increase in DBP at 10 minutes exercise and decrease in DBP at 60 minutes into the recovery of exercise shows no significant (P<0.05) association in both male and female. Meanwhile HR, at 10 minutes of exercise showed no significant association in both male and female while significant association at 60 minutes of recovery in female was observed, this association was not observed in male.

Effect of Exercise on Systolic Blood Pressure (SBP) Systolic Blood Pressure (SBP) during one-hour after exercise in male: Figure 2a shows SBP baseline, and at 10 minutes of exercise followed by gradual decrease with time for a duration of 60 minutes in male. It was noted that the mean SBP at rest significantly rise (p<0.05) from 118.65±1.52mmHg to 145.28±2.67mmHg at 10 minutes of exercise. This mean value (SBP 145.28±2.67mmHg) was significantly decreased to 124.04±2.26mmHg at 2 minutes, the calculated percentage change at 2 minutes into the exercise recovery with respect to the resting SBP was positive (4.70%), while at 3rd and 4th minute the percentage changes were essentially negative with -0.40% and -0.66% respectively.

Table 2:
Mean percentage increase in Systolic Blood Pressure, Diastolic Blood Pressure, & Heart Rate in Male and Female immediately after 10minutes exercise

	Percentage Increase (%)		
Variables	**SBP (mmHg)**	**DBP (mmHg)**	**HR (bpm)**
Male (n=83)	22.56 ±0.12	11.14 ± 0.07	34.41 ± 0.14
Female (n=55)	17.18 ± 0.08	8.66 ± 0.07	45.37 ± 0.22
Mean difference	5.30	2.48	-10.96
P-value	P<0.001	P<0.001	P<0.001

The values are presented as Mean ± SEM, values of P<0.05 are statistically significant between compared groups.

Table 3:
Level of association between BMI and Systolic Blood Pressure, Diastolic Blood Pressure and Heart Rate immediately after 10 minutes and 60 minutes after exercise

Variable	SBP after 10 mins exercise	SBP after 60 mins exercise	DBP after 10 mins exercise	DBP after 60 mins exercise	HR after 10 mins exercise	HR after 60 mins exercise
Male	-0.0360^{NS}	0.2661*	-0.0628^{NS}	-0.1295^{NS}	0.0222^{NS}	-0.0260^{NS}
Female	0.2629^{NS}	0.0826^{NS}	0.1025^{NS}	-0.1071^{NS}	0.0721^{NS}	-0.3433

Note: *, NS are Significant and not significant respectively (p<0.05

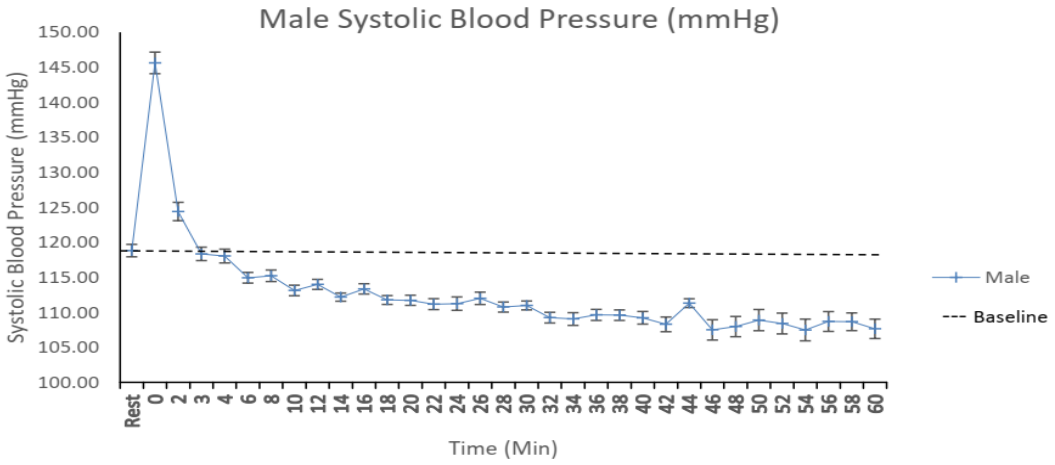

Figure 2a:
Graphical presentation showing male SBP pattern one hour Post Exercise

There after the curve showed a gradual increase in negativity except at 16th and 44th minutes into the exercise recovery when a sharp decrease negativity change was recorded. At the end of the 60th minute the negative percentage change was high (-9.41%).

Systolic Blood Pressure (SBP) during one-hour after exercise in female: Figure 2b show SBP baseline and at 10 minutes of exercise followed by gradual decrease with time for a duration of 60 minutes in female. The mean SBP significantly rise (P<0.05) from 105.6±2.14mmHg at rest to 123.64±1.97mmHg at 10 minutes exercise. The mean rise in the SBP suddenly decrease to 110.12±1.99mmHg at 2 minutes, the calculated percentage change at 2 minutes into the exercise recovery was positive (4.36%) with respect to resting SBP while at 3rd and 4th minutes the percentage changes were negative. The curve thereafter showed a gradually increased negativity except at 22nd, 32nd, 45th minutes into the exercise recovery, was less negative with respect to the resting SBP. The study also noted that the percentage changes of SBP at the end of 60 minutes was -7.02%.

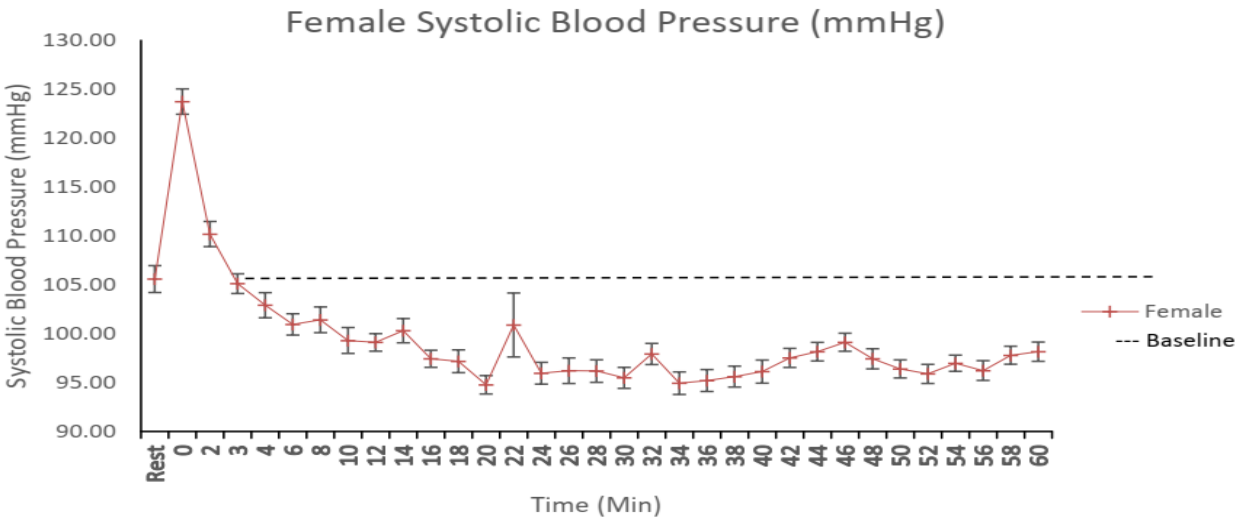

Figure 2b:
Graphical presentation showing Female SBP pattern one hour Post Exercise

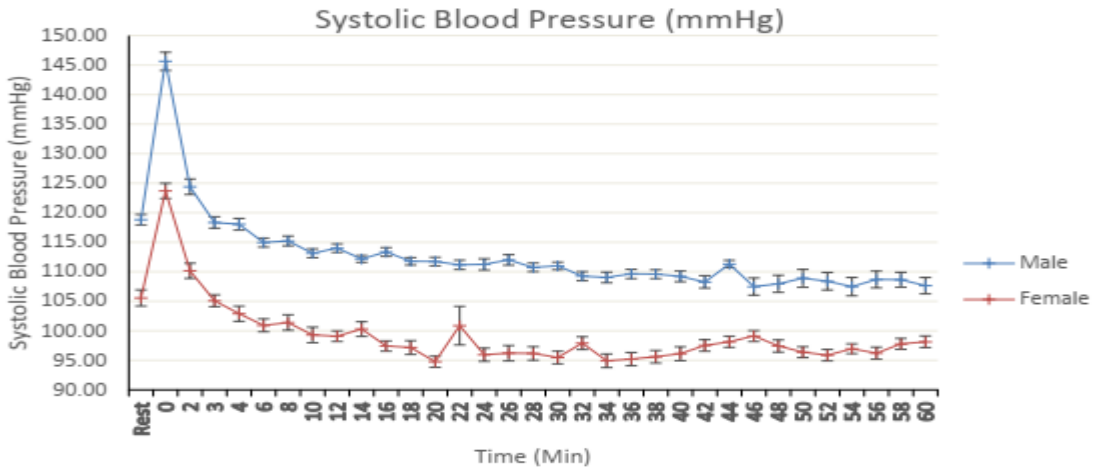

Figure 2c:
Graphical presentation showing comparison between patterns of SBP Decrease in male and female Post exercise

Comparison between patterns of Systolic Blood Pressure (SBP) decrease after 10 minutes of exercise in male and female: The graph shows a high positive mean SBP changes in male compared to female (22.05% and 17.18%) respectively. The mean positive percentage change of SBP was also noticed to be higher in male than in female at 2 minutes into the exercise recovery in respect to the resting SBP. Both curve (male and female) showed zero negative percentage at 3rd minute into the exercise recovery. The zero percentage change was gradually followed by increased negativity with male recorded highest negative change compared to female.

Effect of Exercise on Diastolic Blood Pressure (DBP)
Diastolic Blood Pressure (DBP) during one-hour after 10 minutes of exercise in male: Figure 3a show DBP baseline, and at 10 minutes of exercise followed by gradual decrease with time in male. An initial significant ($p<0.05$) increase of DBP (74.44±1.48mmHg) at rest to (82.68±1.70mmHg) at 10 minutes of exercise, representing a positive percentage change of 11.14% with respect to the resting DBP. At 2 minutes into the exercise recovery the percentage change was also positive (4.20%), thereafter there was a gradual positive percentage decrease and at 14th minute into the exercise recovery the percentage change was zero. The zero percentage was accompanied by gradual decrease of negative percentage changes and at 60 minutes into the exercise recovery the percentage change was -4.44%.

Diastolic Blood Pressure (DBP) during one-hour after 10 minutes of exercise in female: Figure 3b show DBP baseline and at 10 minutes of exercise followed by gradual decrease with time in female. The female DBP showed a statistically significant ($p<0.05$) increase at 10 minutes of exercise from (70.04±1.4mmHg) to (75.84±1.36mmHg), representing 8.66% positive percentage change, with respect to resting DBP. Then the result also recorded a decrease positive percentage change at 2 minutes (5.25%), however at 3rd minutes of recovery a negative (-1.30%) percentage change was noticed. A rebound positive percentage change was seen at 4th, 6th, 8th, 10th, 12thand 14th minutes into the exercise recovery. Whereas at 16th minutes into the recovery the percentage change was negative which was accompanied with gradual increase in negative percentage change till the end of the 60 minutes into the exercise recovery (-6.21%) as showed in figure 3b.

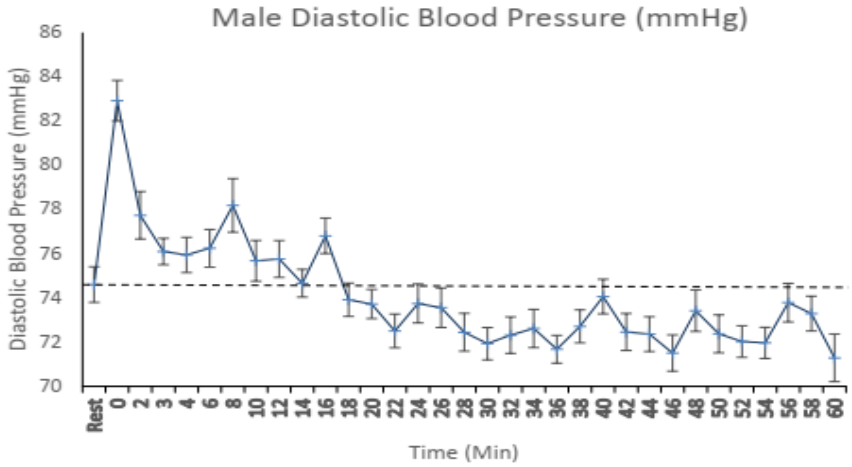

Figure 3a:
Graphical presentation showing male DBP pattern one hour Post Exercise

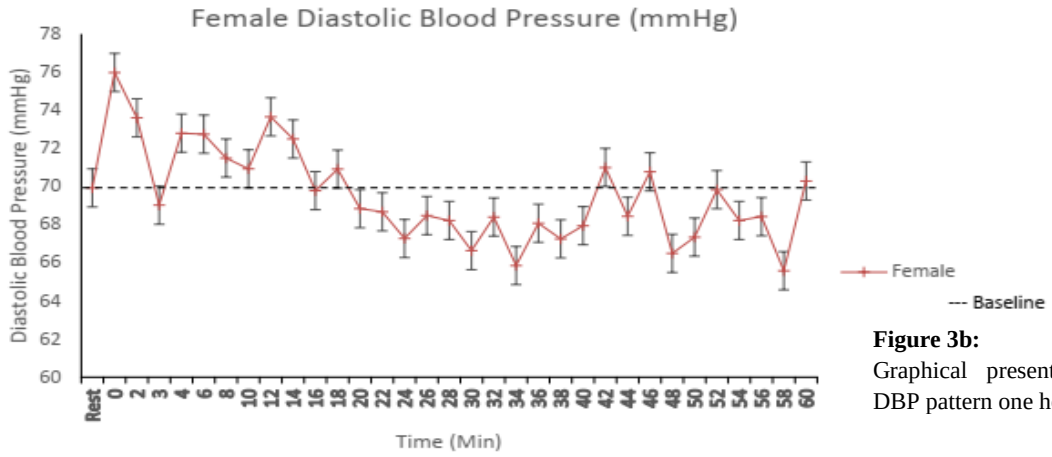

Figure 3b:
Graphical presentation showing Female DBP pattern one hour Post Exercise

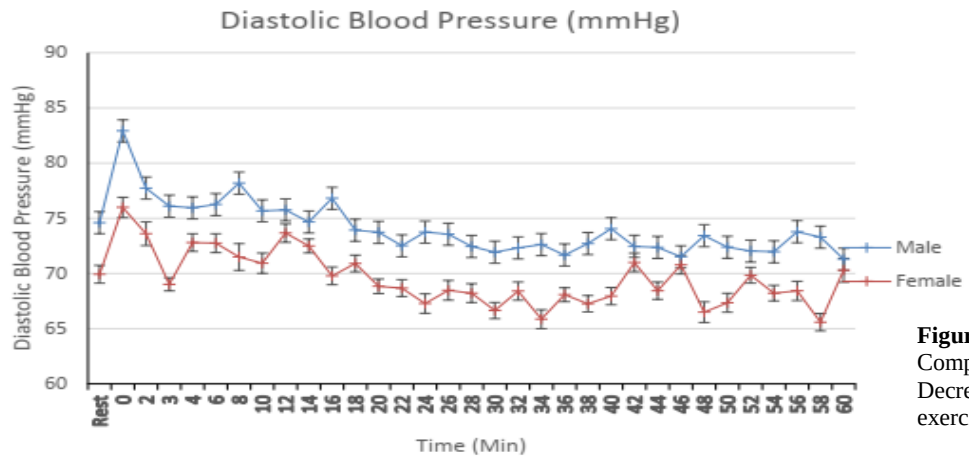

Figure 3c:
Comparison between patterns of DBP Decrease between male and female Post exercise

Comparison between patterns of Diastolic Blood Pressure (DBP) decrease after 10 minutes of exercise in male and female: Figure 3c show the comparison of the pattern of DBP decrease in male and female during 60 minutes after 10 minutes of exercise. A higher positive percentage changes of DBP in male (11.4%) compared to female (8.66%) was recorded at the end of 10 minutes of exercise in respect to the initial DBP. While a gradual decrease in positive percentage changes till percentage changes was negative at 14th minute into the recovery of exercise in male, the female counterpart showed a negative change at 3rd minute into the recovery followed by rebound positive change that terminated by negative changes at 16th minutes into recovery. The result also noticed the percentage negative change failed to return to zero percentage change (baseline) before exercise in both male and female.

Effect of Exercise on Heart Rate (HR)

Heart Rate (HR) during One-Hour after 10 minutes of exercise in male: Figure 4a show HR baseline and at 10 minutes of exercise followed by gradual decrease with time in male. At 10 minutes of exercise, the HR significantly ($p<0.05$) rise from resting 83.92±2.63bpm to 112.96±3.08bpm. The calculated positive percentage change was 34.41% in respect to the initial resting state. The heart rate gradually declined with time but fail to return to resting HR at the end of sixty minutes.

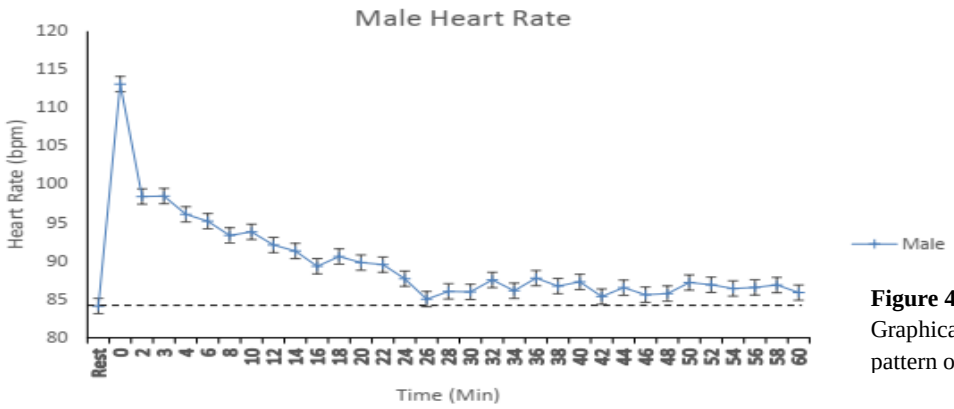

Figure 4a:
Graphical presentation of Male Heart Rate pattern one hour Post Exercise

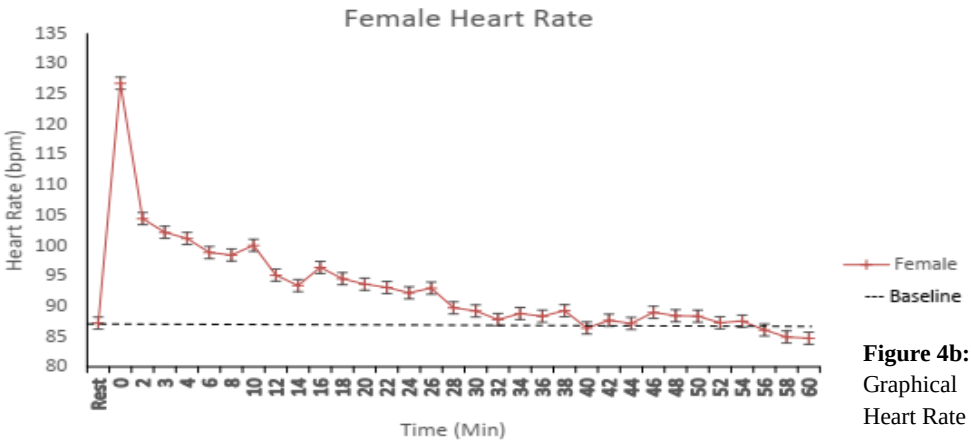

Figure 4b:
Graphical presentation showing Female Heart Rate pattern one hour Post Exercise

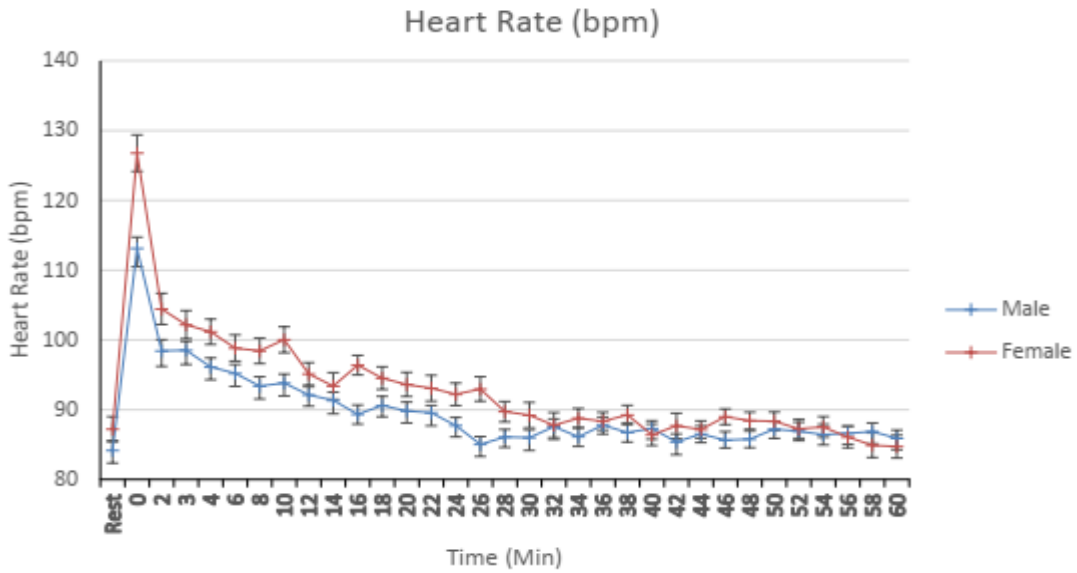

Figure 4c:
Graphical presentation showing comparison between patterns of Heart Rate Decrease between Male and Female Post exercise

Heart Rate (HR) during One-Hour after 10minutes of exercise in female: Figure 4b show HR baseline and at 10 minutes of exercise followed by gradual decrease with time in female. At 10 minutes, there was a significant ($p<0.05$) rise of HR from resting (87.00±2.76bpm) to 127.44±4.01bpm, representing a positive percentage change of 45.37%. Following this sharp increase, the heart rate gradually declined with time, and a negative percentage change was noticed at 40th minutes indicating that baseline of the HR was reached at that time into exercise recovery. Further negative change was also recorded with time and remained negative till end of sixty minutes in respect of resting HR level

Comparison between patterns of decrease of Heart Rate (HR) after 10minutes of exercise in male and female: A comparison of graphic representation in male and female showed a sharp rise with female having a higher percentage increase of 45.37%, while male have 34.41%. The comparison also showed that 19.77% positive percentage

change was recorded at 2 minutes into recovery in female, only 16.98 percent was seen in male. A gradual decline in positive percentage change was recorded in both male and female however the female showed a negative percentage change at 40 minutes into recovery in respect to resting HR. Meanwhile, the female also showed a slight negative change below resting HR level the male counterpart shows slight positive change above resting HR.

DISCUSSION

Evaluation of aerobic dynamic exercise testing of normotensive, non-athlete and apparently healthy participants studying at the University of Maiduguri, for the purpose of determination of pattern curve that could be used as a standard, and equally represent a normal Cardiovascular response to exercise challenges for diagnosis. Therefore, pre-exercise and post-exercise baseline and other parameters influencing cardiovascular dynamic change in health and disease condition were studied carefully following ten minutes (10 minutes) of aerobic exercise. This study showed the mean age of male and female subjects age range between eighteen years (18years) to thirty years (30 years) were 22.28±0.52 and 19.84±0.28 respectively, indicating female subjects attending University of Maiduguri after 20years were fewer compared to male. This finding support the culture of marrying off young female before the age of 20years, and exclusion of married female may explain the discrepancy of the mean age of male and female in this study.

The recorded body mass index in both subjects show a significantly higher BMI in male compared to the female counterpart. Though no association between BMI and mean systolic blood pressure recorded from both male and female age range 18-30years at 10minutes of exercise, positive association of BMI and decrease in SBP seen in male at end of sixty minutes (60 minutes) into the recovery. Meanwhile, such association was not recorded in female. This study supports the general view that both systolic and diastolic blood pressure were higher in male compared to the female (Afrifa-anane *et al*., 2015), which could probably be the reason why prevalence of hypertension is higher among men than women (Cappuccio *et al*., 2004). This discrepancy could also be seen as supportive of genetic, endocrine and body composition differences between sexes (Bassareo and Crisafulli., 2020). Interestingly, the level of association between BMI and increased DBP at 10 minutes, and its decrease at 60 minutes of exercise recovery was not seen in both male and female indicating DBP is less considered in exercise testing. More so, the study also recorded no significant association between BMI and increase HR at 10 minutes of exercise in both sexes. However, at 60 minutes into exercise recovery while male showed no significant association, female show a negative significant association in HR at end of sixty minutes (60 minutes) of exercise recovery, the phenomenon attributed to a greater parasympathetic activity towards the heart as well as less sympathetic input to vascular regulation in women than in men (Raemakers *et al*; 1998; Fisher *et al*; 2013). This could probably be the reason why female have a significant high resting heart rate as it was observed in this study.

A sudden rise in Systolic blood pressure was recorded at the end of ten minutes (10 minutes) of exercise in both male and female. This phenomenon is consistent with other researchers work that dynamic exercise like in our case is characterized by an increase in mean arterial blood pressure notwithstanding the marked systemic vascular resistance reduction due to the metabolic induced vasodilation (Crisafulli *et al*; 2006). A neural mechanism that is responsible for the fine haemodynamic regulation that guarantees blood supply to exercising muscle and avoids excessive mean arterial blood pressure could be implicated. This neural mechanism includes: - 1. Central command 2. exercise pressure reflex and 3. arterial baroreflex, were identified by Green and Paterson (2008) and Nobrega *et al* (2014) as causes of increased blood pressure during exercise. Therefore, the increase in systolic blood pressure are brought about by the increase in cardiac output as a result of increased in heart rate. Meanwhile no significant changes in diastolic blood pressure was recorded in both male and female; supporting other research finding of lack of significant changes in diastolic blood pressure, which was attributed to peripheral vasodilatation in active muscle thereby increases blood flow and availability of oxygen and nutrient. (Romero *et al*., 2017)

A high percentage systolic blood pressure increase recorded in male compared with female is consistent with studies of Dimpka *et al*. (2008). They attributed it to blunt sympathetic response and higher vasodilatory state of women in comparison with men (Wheatley *et al*., 2014). In contrast, higher percentage increase of heart rate was seen in female compared to its male counterpart supporting the studies of Astrand *et al*. (1964), which attributed it to lower parasympathetic outflow to female heart to compensate for lower stroke volume, thereby resulting in the higher cardiac output. Whereas, female sexual hormone also exerts relaxing effect on peripheral resistance (Li and Kloner, 1995; Kaur *et al*., 2015). The study also recorded lower diastolic blood pressure in female compared to male counterpart at the end of 10 minutes exercise supporting the evidence of reduced vasoconstriction and lower vascular resistance in female (Bassareo and Crisafulli, 2020).

Sixty minutes monitoring of systolic blood pressure after ten minutes of exercise showed a typical curvilinear pattern. Subsequent to initial rise of SBP from normal resting systolic blood pressure to a peak at ten minutes of exercise approximately 22.9±0.12% in male and 17.6±0.85%in female of increase, a sharp decline was first seen at two minutes into the exercise recovery. A positive percentage change of 4.7% (representing a higher SBP in respect to resting SBP) in male was noticed at two minutes into the exercise recovery, while female SBP showed an initial decline of positive percentage change (4.36%) at 2 minutes. However, both male and female demonstrated a zero negative percentage change at 3 minutes indicating that SBP has returned to baseline value. This finding support the view that attainment of resting value of SBP could be reached within five minutes post-exercise (Taylor and Bellar, 1998). The importance of determining the percentage decline of SBP after aerobic exercise lies on its usefulness in the assessment of cardiovascular responses to physical stress (Dimpka *et al*., 2008). More so, Miyai *et al*. (2002), asserted that the magnitude of an exercise-induced SBP response may present a risk for death from cardiovascular and non-cardiovascular causes, independent of resting blood pressure. Also a number of research work established an

association between delay recoveries of SBP (Taylor and Bella, 1998) and heart rate (Mora *et al*., 2007) to the resting levels and the presence and extent of heart diseases. Subsequent to the attainment of baseline value of SBP, the graph curve showed a linear gradual persistent negative (indicating that the Systolic blood pressure remained below resting value) percentage change, which is more far away from baseline in female compared to male until the end of 60 minutes. The persistent negative percentage change which is an indication of SBP below resting value, corresponds and support the view of post-exercise hypotension as reported by various research work (Cardoso *et al*., 2010; Halliwill *et al*., 2013), a phenomenon attributed to the liberation of vasodilation substance at site of active muscle (Halliwill, 2001). However, the differences observed in SBP responses during 60minutes of recovery in both sexes could be reflection of structured hemodynamic differences between male and female during recovery from dynamic exercise in which female have been reported to exhibit a reduced capacity to vasoconstriction of the arteriolar bed as compared to men (Bassareo and Crisafulli, 2020). Other studies observed that the decrease in pressure during recovery after dynamic effort is greater in female than in male (Carter *et al*; 2001). Meanwhile, other studies (Christou *et al*., 2005) suggested that female have a lower support of autonomic tone necessary to regulate BP as well as a lower effectiveness of the component that regulate the baroreflex. The following conclusion is made: a curvelinear pattern of SBP during 60minutes of recovery was noticed with the decline in SBP crossing the baseline between 3rd and 4th minute and remained persistent below the resting SBP value which is more pronounced in female than male supporting the theory of post-exercise hypotension.

The DBP responses during sixty minutes into the exercise recovery is similar to that obtained in SBP responses in both male and female, in which a curvilinear pattern was also observed. The male subject showed an initial increase from resting DBP of 74.44±1.48mmHg to 82.68±1.70mmHg after ten minutes of exercise representing a positive percentage increase (DBP above resting value) of 11.14%. Following this initial increase at the peak of 10 minutes of exercise, the DBP recorded at 2 minutes into exercise recovery was 4.20% (positive percentage change in respect to resting DBP value). Gradual decline of positive percentage change with time was seen after 2 minutes and at 14th minutes the resting value of DBP reached. The same observation was recorded for the female counterpart, however while at 2 minutes 8.66% positive percentage change recorded, the resting value of DBP was reached at 16th minutes into the exercise recovery. A negative percentage change was thereafter recorded in both sexes and remained negatively persistent to the end of sixty minutes of monitoring (DBP is below resting value), indicating resting value fail to restore with the magnitude not significant, supporting some research work (Sharon and Denise, 2008). Meanwhile, this study also noticed that DBP value in female was at resting level first at 3rd minute into the exercise recovery followed by sudden rebound positive percentage change after 3rd minute, which thereafter returned to resting value at 16th minute. It appears that the sharp drops in DBP in female could be attributed to the phenomenon of female having reduced vasoconstriction and a lower vascular bed resistance in active tissue performing a dynamic aerobic exercise as suggested by a research work especially after exercise (Bassareo and Crisafulli, 2020). Moreover, exhibition of reduced capacity to vasoconstrict the arteriolar bed as compared to men, and the fact that the decrease in blood pressure during recovery after a dynamic effort is greater in females than in males could be attributed to a greater decrease in stroke volume and a lower rise in systemic vascular resistance in women during recovery which usually persist for 5 minutes after the end of exercise (Carter, *et al*., 2001).

Secondly, the lower Systemic Vascular Resistance (SVR) level of women after exercise have been attributed to a possible explanation a) Female sex hormones, by exerting releasing effect on peripheral resistance vessels (Wallin *et al*., 2010) which could be antagonized through myogenic auto regulation that might be probably the cause of high positive percentage change after 3 minutes and b) High sympathetic activity towards the hearts as well as less sympathetic input to the vascular regulation in women than in men (Fisher *et al*., 2013). It is therefore, obvious that DBP response after a dynamic exercise is regulated through vascular tone with female exhibiting a reduced vasoconstriction and a lower vascular resistance in comparison with male.

The resting Heart rate value of female observed in this work was significantly higher compared to the male counterpart, a phenomenon attributed to lower resting vagal tone discharge in female (Girdler *et al*., 1990). It is commonly accepted that during dynamic exercise the cardiovascular system is adjusted through autonomic nervous system which is characterized firstly by vagal withdrawal followed by sympathetic activation (Maciel *et al*., 1986). These two effects accelerate Heart rate to avoid blood pressure drops due to metabolic induced arteriolar vasodilation in the working muscle that could markedly reduce systemic vascular resistance (SVR) as reported by other study (Romero *et al*., 2017). Therefore, increase heart rate recorded at the end of the present study also substantiated the claim. More so, the study records higher positive percentage change in heart rate in female compared to male counterpart indicating that HR in female at end of exercise is still higher than male counterpart, this could also be seen as genetic difference and lower vagal tone in female than in male. The positive percentage change at 2 minutes into the recovery were 16.08% and 19.77% in both male and female respectively, indicating that female still has higher positive percentage change compared to the male. This represent a fast phase of heart recovery from aerobic dynamic exercise which some studies attributed to parasympathetic reactivation (Pecanha *et al*., 2016). The second phase of heart rate recovery in both subjects thought to be due to withdrawal of sympathetic outflow (Seiller *et al*., 2007), showed a gradual positive percentage decline with time with curvilinear pattern. However, while this positive percentage change remained positive till the end of 60 minutes in male, the female heart rate returned to baseline at the end of 40 minutes and remained negative till the end of the 60 minutes, indicating that the heart rate in male remained positive (slightly above resting heart rate value), while female heart rate remained negative (slightly below resting heart rate value). Therefore, it could be seen that heart rate in both male and female failed to return to baseline at the end of 60 minutes.

60-Minutes post-exercise cardiovascular responses

In summary, the pattern of reduction in blood pressure following 10 minutes of dynamic aerobic exercise were not different between both sexes. Meanwhile, systolic blood pressure was significantly reduced at 2nd minute and was at baseline at 3rd minute for both sexes. The curve, thereafter was persistently negative to the end of sixty minutes of recovery, the female curve was more negative (faraway from baseline) than the male. A significant reduction of DBP was also recorded at 2 minutes in both sexes, and was at baseline at 14th minute in male and 16th minute in female. However, interestingly the female DBP first attained baseline at 3rd minute then rebound and reached baseline at 16th minute. The result also demonstrated that DBP in both male and female, though below resting value, but were not far from baseline. The heart rate also showed a significant decrease at 2nd minute, followed by a gradual decline, the curve were slightly above the baseline in both male and female. It seemed that SBP is more reliable in determining cardiovascular response to exercise in both male and female.

The pattern of SBP and DBP decrease after 10 minutes of dynamic aerobic exercise is curvelinear, with SBP cross baseline at 3 minutes in both sexes and remained persistent below the baseline, while the DBP cross the baseline at 14th and 16th minute in male and female respectively. The HR showed the same pattern however, the curve remained above the baseline till the end of 60 minutes.

Recommendation

Based on the findings of this research, further study is recommended to compare Heart rate Variability and Blood Pressure in both athletes and Normotensive subjects.

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Niger. J. Physiol. Sci. 39 (June 2024): 77 – 85
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Full length Research Article

Toxicological assessment and *In-vitro* Antidiabetic Potential of Cyclohexane-methanol Extract and Oil from Seed of *Azadirachta indica*

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Summary: Neem (*Azadirachta indica* A. Juss, Meliaceae) is a popular medicinal plant widely sought for its antipyretic, antimalarial, anti-inflammatory, antidiabetic, and antibacterial properties, among others. Cold-pressed oil from neem seed (NOil) and its cyclohexane-methanol extract (NOHM) were evaluated for their effects on α-amylase and α-glucosidase activities in vitro. Also, NOil (75, 150, and 200 mg/kg) and NOHM (200, 400, and 800 mg/kg) were orally administered to normal experimental rats for 30 days, following which the lipid profile, antioxidant status, and serum and tissue indices of hepatic, renal, and cardiac damage were evaluated. NOHM caused significantly higher ($p<0.05$) α-glucosidase inhibition than NOil. Respectively, the α-amylase and α-glucosidase inhibitory effects of NOil (IC50 = 4.88 ± 0.38 µg/mL and 74.54 ± 25.26 µg/mL) and NOHM (5.00 ± 0.22 µg/mL and 14.17 ± 5.14 µg/mL) were superior to that of acarbose (9.67 ± 0.09 µg/mL and >150 µg/mL). NOHM produced a stronger hypoglycemic effect than NOil. However, no biochemical alteration of toxicological importance was caused by either following subacute administration to animals as the organ-body weight ratio and serum and tissue indicators of organ damage were not adversely altered. The present findings support the safety of NOil and NOHM at the evaluated dosages. The effect of both oil and extract on key carbohydrate-metabolizing enzymes could partly explain the biochemical rationale underlying the popular ethnomedicinal application of the seed in diabetic management..

Keywords*: Neem oil; toxicity; cyclohexane-methanol extract; α-amylase; α-glucosidase; in vitro*

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Manuscript received- December 2023; Accepted: March 2024

DOI: https://doi.org/10.54548/njps.v38i2.10

INTRODUCTION

Plants contain a rich repertoire of complex bioactive phytochemicals that could function additively or synergistically to exhibit therapeutic effects against a wide array of pathological conditions in humans (Attanzio *et al*., 2022). Approximately 80% of the world population relies on medicinal plants as the primary source of healthcare, partly because they are more readily accessible and affordable than most conventional drugs (Bandaranayake 2006; Komolafe *et al*., 2021). A significant part of traditional medicine involves the use of decoctions, infusions, and powders made from parts of plants, including leaves, bark, root, and seeds, to treat diseases. Crude or semi-purified extract of whole or plant parts could be used individually or as mixtures with other whole plants or plant parts (Sasidharan *et al*., 2011). Oils extracted from the seeds of plants have been used for many centuries by rural communities as food and medicine (Padhye *et al*., 2008; Vermaak *et al*., 2011). Many underexploited plant species are now being developed as sources of viable oils with relevant phytocomponents that afford not only nutritional values but promising therapeutic and health-promoting potentials (Varnham 2014). Seed oil extracted using the cold-pressing methods may afford healthier effects since heat and chemical treatment are not applied. Hence, there is no contamination issue like solvent residues (Garavaglia *et al*., 2016). The complexities of the multiple phytoconstituents in crude and impure extracts like seed oils warrant toxicological preassessment in non-human models to identify any adverse effects, safety threshold, or therapeutic margins of such ethnobotanical preparations. Risks associated with using such natural products could thus be identified early and the toxic agent(s) discarded or modified to allow for an extensive evaluation of safer, viable alternatives (Obidike and Salawu 2013; Winston and Maimes 2007). Sub-acute toxicity screening employed repeated dosing of employed repeated dosing of animals with the test substances for a period ranging from weeks to months to expose deleterious changes in organ, haematological, and biochemical indices (Obidike and Salawu 2013).

Neem (*Azadirachta indica* A. Juss, Meliaceae) is a very popular medicinal plant indigenous to the Indian subcontinent (Gupta *et al*., 2004). However, it is now cultivated in other parts of the world. Neem tree parts,

including the leaf, roots, bark, and seed oils, are widely sought for their numerous biological activities (Cesa *et al*., 2019). Over 100 compounds have been isolated and characterized from neem. The compounds have been shown to have antipyretic, antimalarial, interferon-inducing, anti-inflammatory, immunomodulatory, antibacterial, antiviral, antimutagenic, or antioxidant properties are attributed (Cesa *et al*., 2019; Subapriya and Nagini 2005).

Neem seed oil contains mainly triterpenoids, limonoids, and alkaloids (Gupta *et al*. 2004), and has been orally applied to treat leprosy rheumatism, ulcers, and skin diseases (Deng *et al*. 2013). A few studies have revealed the antibacterial, antidiabetic, antifungal, antimalarial, anti-inflammatory, and antiparasitic properties of neem seed oil (Deng *et al*., 2013; Xu *et al*., 2010; Zhang *et al*., 2010). These biological properties have been ascribed to a wide range of biologically active ingredients, including triterpenoids like salannin, nimbin, azadirachtin as well as gedunin, salannin, and quercetin present in the oil (Alzohairy 2016; Cesa *et al*., 2019). In the present study, we investigated whether neem oil and its methanol-cyclohexane extract possess an in vitro inhibitory effect on α-amylase and α-glucosidase, key carbohydrate-metabolizing enzymes relevant to the diabetic phenotype. Also, we evaluated and compared their safety in an animal model following subacute administration.

MATERIALS AND METHODS

Chemicals: Hydrogen peroxide, malonaldehyde bis-(dimethyl acetal) (MDA), thiobarbituric acid (TBA), and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were procured from Sigma Chem., Co. (London, UK). Creatine kinase MB (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), bilirubin, creatinine, uric acid, urea, cholesterol, triglyceride, high density lipoprotein (HDL-c), low density lipoprotein (LDL-c/VLDL-c) assay kits were obtained from Fortress Diagnostics (Antrim BT41 1QS) and MTD Diagnostics (Maddaloni CE, Italy). Other chemicals were of analytical grade, and the water used was glass distilled.

Acquisition of neem oil and preparation of extract: Cold-pressed Neem oil (100%) was purchased from Georgia Organic Solution LLC, Blakely, GA, USA, in January 2015. The Neem oil (100%, 800 mL) was extracted separately with cyclohexane (2 x 200 mL, 6 hours each) and methanol (500 mL, 6 hours) using a laboratory stirrer mixer. Cyclohexane and methanol were removed from the extracts under reduced pressure (rotary evaporator). The extracts were combined to obtain a dark brown resin (119.5 g).

Assay of α-amylase inhibition potential: The method described by (Bernfeld 1951) was employed to determine the α-amylase inhibitory activity of Neem oil (NOil) and its cyclohexane/methanol extract (NOHM). Briefly, 100 uL of NOil or NOHM (0, 20, 50, 75, 100, 120 ug) and 100 µL of reaction buffer (0.02 mol/L sodium phosphate buffer, pH 6.9 with 0.006 mol/L NaCl) containing Swine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/mL) were incubated for 10 minutes at room temperature (25°C). After that, 100 µL of 1% starch solution in reaction buffer was added to the reacting mixture, which was further incubated for 10 minutes at room temperature and stopped with 200 µL of dinitrosalicylic acid colour reagent. After incubation in a boiling water bath for 5 min, the reaction mixture was cooled to room temperature, diluted with distilled water (2 mL). The absorbance was measured at 540 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices). Percentage inhibition of α-amylase activity by the extracts was calculated as follows: % inhibition = [(AbsControl - AbsSamples)/AbsControl] *100

Assay of α-glucosidase inhibition potential: The ability of Neem oil (NOil) and its cyclohexane-methanol extract (NOHM) to inhibit α-glucosidase activity was determined in accordance with the procedure described by (Apostolidis *et al*., 2007). A mixture containing NOil or NOHM (100 µL) at different concentrations (0, 20, 50, 75, 100, 120 µg) and 100 µL of α-glucosidase solution was incubated at 25˚C for 10 min. Thereafter, 50 µL of p-nitrophenyl-α-D-glucopyranoside solution (5 mmol/l in 0.1 mol/l phosphate buffer, pH 6.9) was added. The reacting mixture was further incubated at room temperature for 5 min. Finally, the absorbance was taken at 540 nm using a microplate reader. The α-glucosidase inhibitory activity was expressed as percentage inhibition as follows:
% Inhibition of α - glucosidase activity = [(AbsControl - AbsSamples)/AbsControl] * 100

Animals: Male albino rats (Wistar strain) weighing 180–220 g, obtained from a private breeder and housed in the rodent colony of the Department of Biochemistry, Federal University, Oye-Ekiti, Nigeria, were used for this study. The rats were kept in wire mesh cages and maintained under controlled light cycle (12 h light/12 h dark) and fed with commercial rat chow (Chikum Feeds Nigeria Limited) ad libitum, and liberally supplied with water. Approval of the experimental protocol was granted by the Animal Ethical Committee of the Federal University Oye-Ekiti.

Experimental design: Age-matched rats were assigned into seven groups (n =6) and treated as follows: Group I (Control) Corn oil (1 ml/kg); Group II 75 mg/kg Neem oil cyclohexane-methanol extract (NOHM)**;** Group III 100 mg/kg NOHM**;** Group IV 200 mg/kg NOHM **;** Group V 200 mg/kg Neem oil (NOil)**;** Group VI 400 mg/kg NOil**;** Group VII 800 mg/kg NOil**.** Corn oil (1 ml/kg), NOHM (75-, 100- or 200 mg/kg), or NOil (200-, 400- or 800 mg/kg) were administered by oral gavage to healthy rats once daily for thirty (30) consecutive days. Animals were sacrificed under mild ether anaesthesia 24 h after the last administration. Blood was collected by cardiac puncture for serum preparation, and the major tissues (liver, kidney, heart) were dissected out for biochemical evaluation.

Preparation of serum and tissue homogenates: Serum was prepared from blood collected from the animals by allowing the blood to clot and thereafter centrifuged at 3000 rpm for 15 min. The supernatant (serum) was carefully separated and stored for biochemical analysis. Organ tissues (liver, kidney, heart) harvested from the animals were rinsed in KCl solution (1.5%) and homogenized in aqueous Tris–HCl buffer (50 mM, pH 7.4). Thereafter, tissue homogenates were centrifuged at 10,000 g for 20 min at 4˚C

to obtain the supernatant extracts on which biochemical analyses were carried out.

Assessment of tissue function status: The toxicological status of different tissues was determined by measuring the levels or activities of biochemical indices and markers in the liver (albumin, total/direct bilirubin, AST and ALT), kidney (creatinine, uric acid, and urea), and heart (CK-MB and LDH) homogenate and in the serum using assay kits obtained from Fortress Diagnostics (Antrim BT41 1QS) and MTD Diagnostics (Maddaloni CE, Italy) according to the instructions of the manufacturer.

Assessment of serum lipid profile: Serum lipid profiles (total cholesterol TC, triglyceride TG, high density lipoprotein HDL-c, low density lipoprotein LDL-c and very low-density lipoprotein VLDL-c) were determined by colorimetric assay kits following the manufacturer's (Fortress Diagnostics, Antrim BT41 1QS) instructions.

Assessment of the antioxidant status in tissues: The levels of reduced glutathione (GSH) and extent of membrane peroxidation in the tissues were estimated and used to assess the antioxidant status.

Quantification of tissue membrane lipid peroxidation: Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Varshney and Kale 1990).

Assay of the level of reduced glutathione (GSH)Levels of GSH in experimental animals' tissues using tissue homogenates were estimated as previously described (Manuwa *et al*., 2017). Sulphosalicylic acid (5 %, 150 µL) was added to the supernatant (100 µL), gently mixed, and allowed to stand for 5 min to allow for protein precipitation. Thereafter, the filtrate (50 µL) was added to 200 µL 0.1 M phosphate buffer (pH 7.4), following which Ellman's reagent (25 µL) was introduced. The blank was prepared with 200 µL buffer, 50 µL of diluted precipitating solution (three parts to two parts of distilled water), and 25 µL of Ellman's reagent. The absorbance was measured at 412 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices). The GSH estimates were obtained from a GSH standard curve.

Statistical analysis; In vitro results were calculated from replicate data (n = 3), and values from in vivo analyses are expressed as mean ± SEM of six animals. Statistical evaluation was done using One Way Analysis of Variance (ANOVA) followed by the Newman–Keuls comparison of means. The significance level was set at $p < 0.05$. Graph Pad Prism (ver.5.0a) was used for statistical analysis, graphing, and IC50 determinations.

RESULTS

α-amylase and α-glucosidase inhibitory effects of NOil and NOHM: As shown in Figure 1A, neem oil, NOil produced in vitro α-amylase inhibitory effect (IC50 = 4.88 ± 0.38 µg/mL) that was comparable and statistically the same as that caused by its cyclohexane-methanol extract, NOHM (IC50 =5.00±0.22 µg/mL). The α-amylase inhibitory effect of both was significantly greater ($P<0.001$) than that of the reference drug, acarbose (IC50 =9.67±0.09 µg/mL). Figure 1B revealed that the in vitro α-glucosidase effect of NOHM (IC50 =14.17±5.14 µg/mL) was found to be significantly superior ($P<0.05$) to that of NOil (IC50 =74.54±25.26 µg/mL). Both NOil ($P<0.05$) and NOHM ($P<0.01$) also caused stronger α-glucosidase inhibitory effect than acarbose (IC50= >150 µg/mL).

Organ to body weight ratio and fasting blood glucose levels: NOil and NOHM did not affect the liver/body weight (A), kidney/body weight (B), and heart/body weight (C) ratios of experimental animals after thirty (30) days of treatment. However, fasting blood glucose was significantly reduced ($P<0.05/P<0.001$) in experimental animals treated with all dosages of NOHM (75 mg/kg, 39%; 100 mg/kg, 40%; 200 mg/kg, 20%) and NOil (200 mg/kg, 24%; 400 mg/kg, 22%; 800 mg/kg, 28%) when compared with the normal, untreated control (Figure 2).

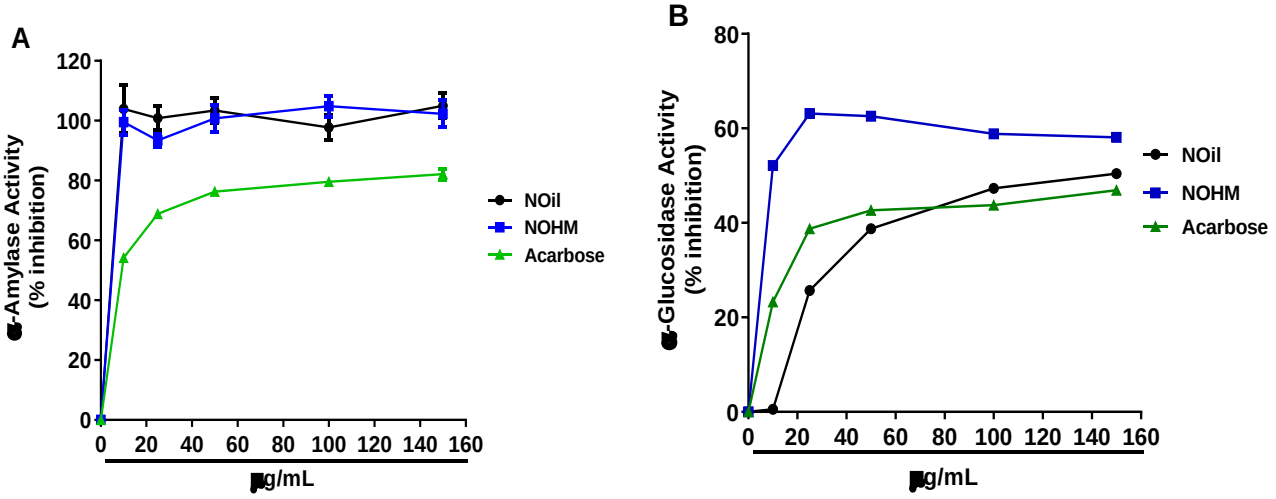

Figure 1:
In vitro inhibition of α-amylase- (A) and α-glucosidase- (B) activities by Neem oil (NOil) and its cyclohexane-methanol extract (NOHM).
† Each value represented mean ± SEM (n = 3)

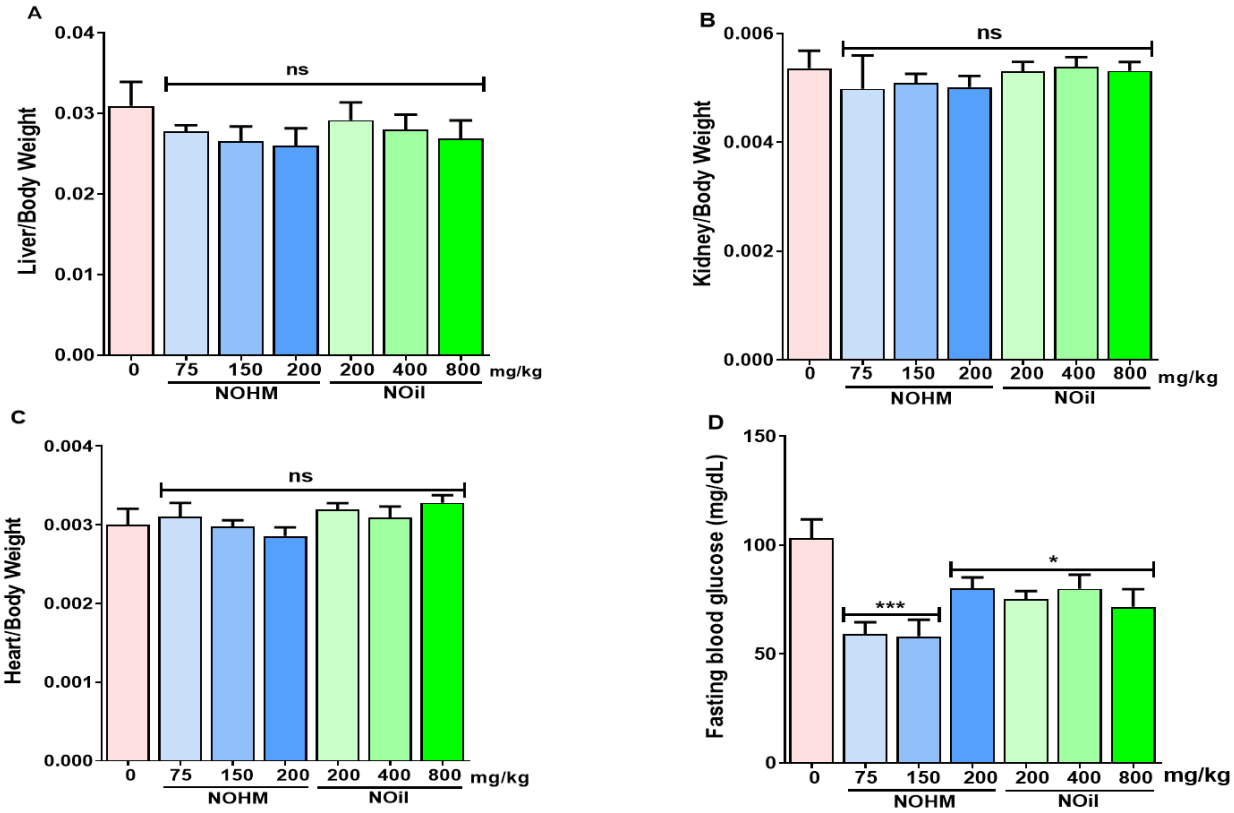

Figure 2:
Influence of Neem oil (NOil) and its cyclohexane-methanol extract (NOHM) on the organ/body weight ratios (A, B, C) and fasting blood sugar (D) of rats.
Values are expressed as mean ± SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control.

Table 1:
Hepatic function markers in the serum and liver of rats treated with Neem oil extract (NOil) and its cyclohexane-methanol extract (NOHM)

Group	ALT		AST		Albumin		Total Bilirubin		Direct Bilirubin	
	Serum (U/l)	Liver (U/mg prot)	Serum (U/l)	Liver (U/mg prot)	Serum (mg/dL)	Liver (g/dL)	Serum (mg/dL)	Liver (mg/dL)	Serum (mg/dL)	Liver (mg/dL)
Control	30.36 ±4.03	3.00 ±0.28	65.63 ±3.15	3.31 ±0.32	3.20 ±0.04	0.048 ±0.002	3.60 ±0.27	5.78 ±0.29	2.2 3±0.14	3.57 ±0.28
NOHM 75	23.58 ±2.86	3.53 ±0.66	73.31 ±7.89	3.92 ±0.33	3.56 ±0.10*	0.063 ±0.007*	4.04 ±0.40	5.61 ±0.25	2.93 ±0.30	3.09 ±0.29
NOHM 100	25.11 ±3.19	2.37 ±0.12	67.64 ±3.48	2.26 ±0.32	3.26 ±0.05	0.044 ±0.002	3.29 ±0.11	6.91 ±0.42	2.97 ±0.26	3.09 ±0.28
NOHM 200	17.36 ±2.82*	2.38 ±0.16	46.45 ±5.81	2.38 ±0.39	3.40 ±0.05	0.033 ±0.002*	3.65 ±0.32	5.60 ±0.47	1.93 ±0.22	3.64 ±0.33
NOil 200	17.24 ±3.45*	3.14 ±0.41	53.84 ±1.19	3.44 ±0.44	3.28 ±0.05	0.061 ±0.004	3.57 ±0.25	5.30 ±0.24	2.20 ±0.22	2.74 ±0.12
NOil 400	19.11 ±2.05*	2.16 ±0.53	35.39 ±1.14**	2.58 ±0.16	3.33 ±0.12	0.055 ±0.003	3.71 ±0.15	5.92 ±0.29	2.53 ±0.25	3.03 ±0.30
NOil 800	16.88 ±0.91*	3.20 ±0.15	52.06 ±8.34	3.28 ±0.49	3.40 ±0.06	0.055 ±0.003	3.42 ±0.16	6.04 ±0.41	2.05 ±0.09	3.49 ±0.25

Values are expressed as mean±SEM (n=6). Values with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control

Biochemical indices of hepatic functions: The biochemical indices of hepatic functions were evaluated in the serum and liver of rats following sub-acute administration of neem oil extract (NOil) and its Cyclohexane-methanol extract (NOHM) (Table 2). The reduction in ALT activity in the serum of experimental animals was statistically significant (P<0.05) following treatment with NOil at 200- (40%), 400- (31%), 800 mg/kg (39%), and NOHM at 200 mg/kg (41%) dosages. However, serum AST activity was significantly (P<0.05; 45%) reduced in rats treated with 400 mg/kg NOil alone. Changes in the hepatic levels of marker enzymes were not significant (P>0.05) for all treatment groups. Except for the increase in serum (11%) and hepatic (32%) bilirubin concentrations of rats treated with 100 mg/kg NOHM and a significant decrease in the hepatic level (30%) following 200 mg/kg treatment, other indices of liver functions typified by total and direct bilirubin were not affected.

Table 2:
Renal function indices in the serum and kidney of rats treated with Neem oil extract (NOil) and its cyclohexane-methanol extract (NOHM)

Group	Uric Acid		Urea		Creatinine	
	Serum (mg/dL)	Kidney (mg/dL)	Serum (mg/dL)	Kidney (mg/dL)	Serum (mg/dL)	Kidney (mg/dL)
Control	6.53±0.8	16.01±0.83	46.30±4.51	19.27±1.54	109.0±4.63	43.89±3.20
NOHM 75	4.37±1.12*	35.06±2.54***	37.12±3.61	17.23±1.87	96.52±3.24	37.72±3.67
NOHM 100	1.91±0.72***	25.79±2.04**	25.16±5.95	17.77±1.04	105.2±13.9	40.87±10.09
NOHM 200	2.22±0.75***	24.48±2.23**	17.45±4.77**	19.63±1.83	109.0±4.63	42.56±12.28
NOil 200	0.88±0.20***	23.57±1.67**	35.46±6.12	18.30±0.84	98.76±2.36	57.97±8.02
NOil 400	1.69±0.17***	13.99±1.22	26.02±2.90	17.79±1.51	101.3±7.55	64.48±3.99
NOil 800	1.17±0.21***	10.03±1.19	30.87±5.94	18.44±1.70	112.9±3.81	20.74±5.46

Values are expressed as mean±SEM (n=6). Values with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control

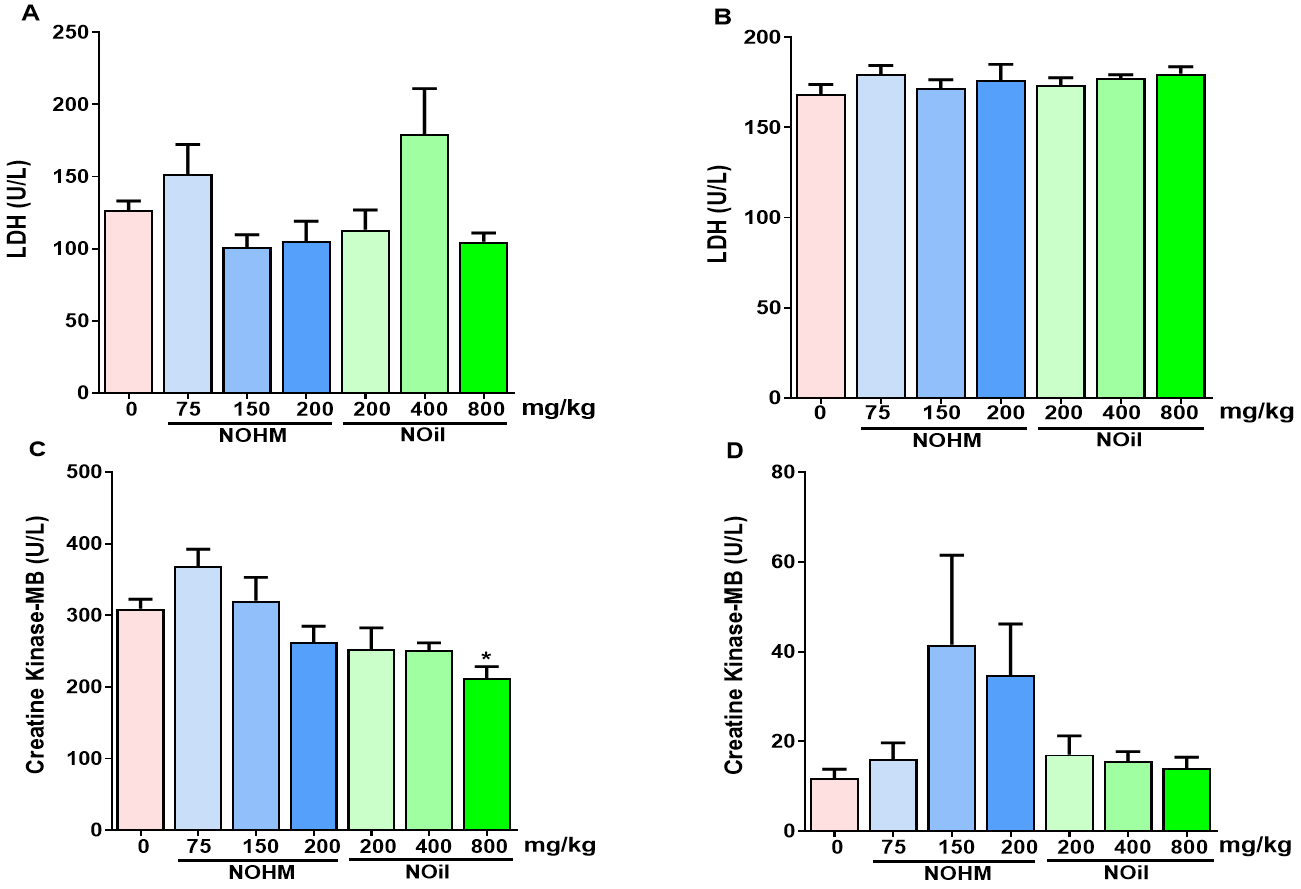

Figure 3:
Serum and cardiac activities of lactate dehydrogenase (A, B) and creatine kinase-MB (C, D) respectively in rats treated with Neem oil extract (NOil) and its cyclohexane-methanol extract (NOHM).
Values are expressed as mean ± SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water; *p<0.05; **p<0.01; ***p<0.001; vs control.

Biochemical indices of cardiac function: Treatment of normal rats with all dosages of NOHM and NOil did not significantly alter the activity of lactate dehydrogenase in the serum or heart tissues (Fig 3). Also, the reduction in serum CKMB activity by NOHM (200 mg/kg; 14%) and NOil (200 mg/kg, 19%; 400 mg/kg, 18%; 800 mg/kg, 30%) was statistically relevant at the highest dosage of NOil (800 mg/kg) but neither extract affected cardiac activity of CKMB.

Serum lipid profile of rats:
Although serum total cholesterol level was unaltered by NOHM (75 mg/kg, 100 mg/kg, 200 mg/kg) and NOil (200 mg/kg, 400 mg/kg, 800 mg/kg) treatment, the levels of triacylglycerols and very-low-density lipoprotein cholesterol (VLDL-c) were significantly decreased by 200 mg/kg NOHM (P<0.05;26%) and NOil at 200 mg/kg (P<0.05;24%), 400 mg/kg (P<0.05; 30%) and 800 mg/kg (P<0.001; 37%) dosages. NOHM at the dosages evaluated

caused significantly (P<0.05) reduced high density lipoprotein-cholesterol (HDL-c) when compared with the normal control. In contrast, neither NOHM nor NOil produced statistically relevant effect on serum low density lipoprotein-cholesterol (LDL-c) in the experimental animals (Fig. 4).

Effect on tissues antioxidant status: The effect of NOHM and NOil on the antioxidant status typified by levels of reduced glutathione (GSH) and the extent of biological membrane peroxidation in tissues of experimental animals are shown in Figure 5. The increases caused by NOHM in GSH levels of analysed tissues were not significant. However, NOil produced a statistically significant increase in GSH level in the hepatic (200 mg/kg; 96%) and cardiac (400 mg/kg; five-folds and 800 mg/kg; 3 folds) tissues compared to normal control. Neem oil and its methanol-cyclohexane extract did not cause membrane peroxidation in treated rats' renal and cardiac tissues. There was, however, a significant reduction (P<0.05) in hepatic membrane peroxidation as a result of NOHM administration to experimental animals at 75 mg/kg (38%), 100 mg/kg (43%), and 100 mg/kg (40%) dosages.

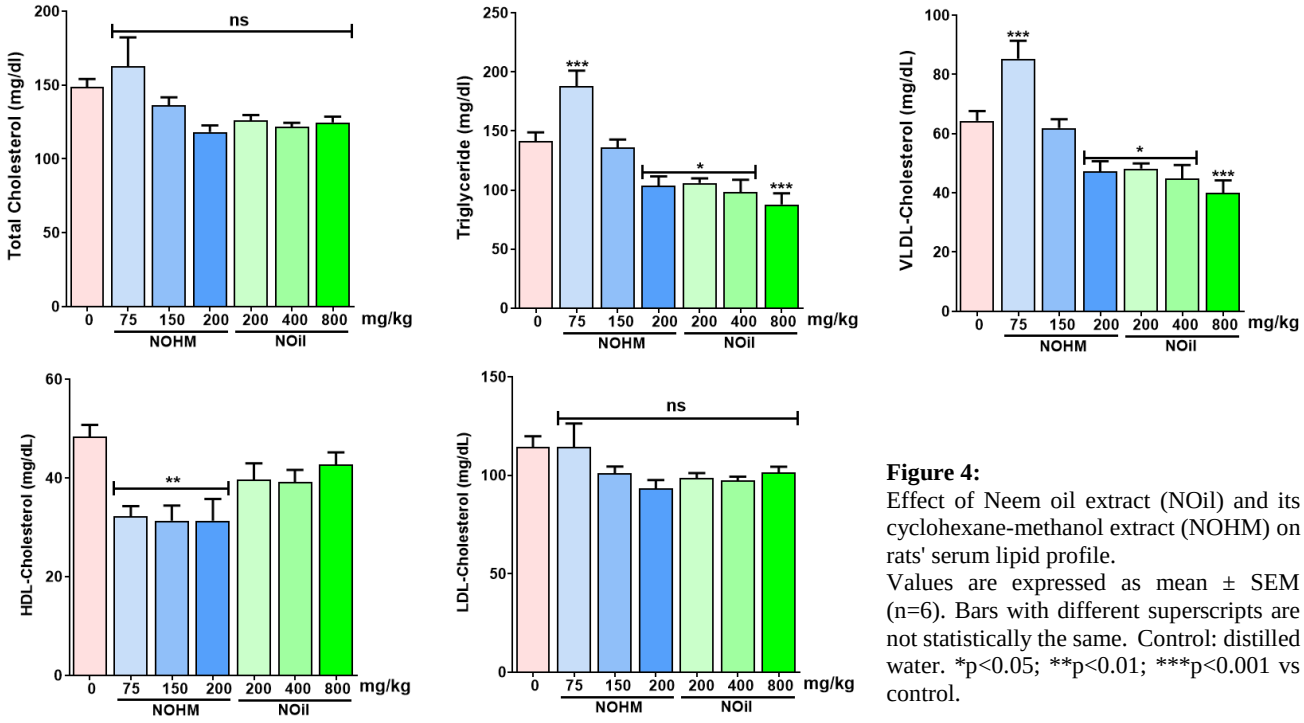

Figure 4:
Effect of Neem oil extract (NOil) and its cyclohexane-methanol extract (NOHM) on rats' serum lipid profile.
Values are expressed as mean ± SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control.

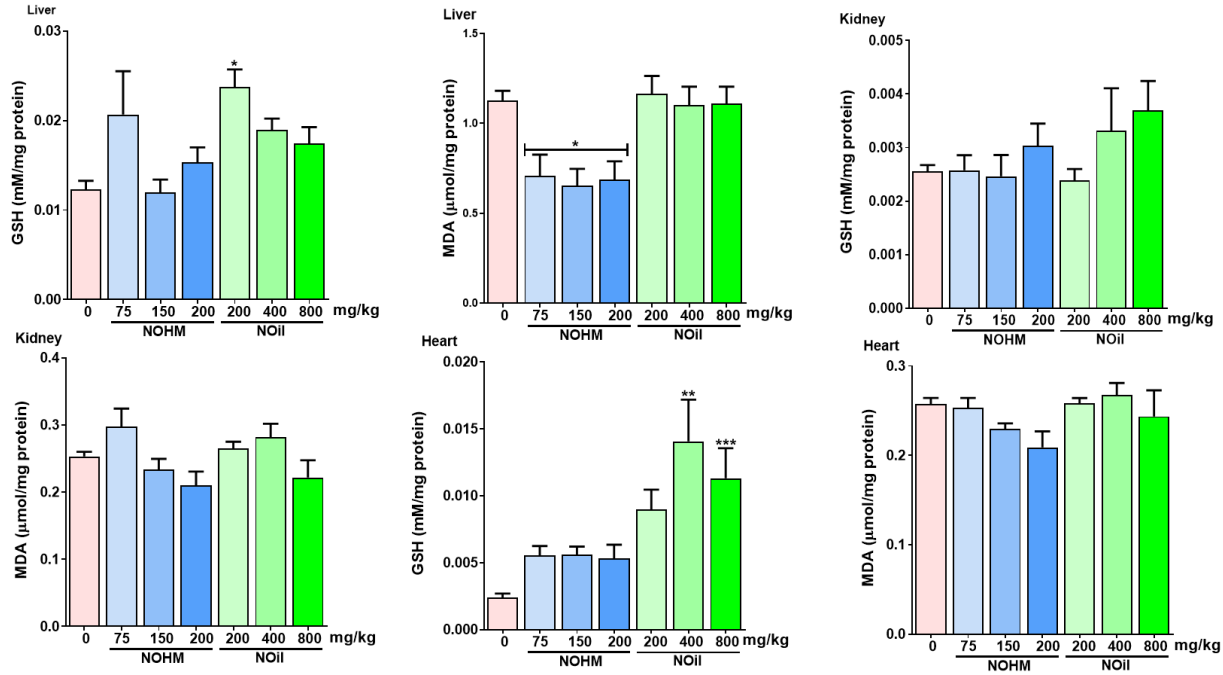

Figure 5:
Tissue antioxidant profile of rats treated with NOil and NOHM
Values are expressed as mean±SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control.

DISCUSSION

In the present study, neem oil and its solvent extract inhibited both α-amylase and α-glucosidase in a dose-dependent manner in vitro. Many compounds with potential inhibitory effects on both enzymes have been characterized from medicinal plants, especially those with traditional use in the treatment of diabetes (Basnet *et al*., 2023; Oboh *et al*., 2014). While α-amylase in the pancreatic juice and saliva facilitates the absorption of glucose by breaking down large insoluble starch molecules to simpler, absorbable ones, α-glucosidase in the mucosal brush border of the small intestine is involved in the digestion of dietary disaccharides into the corresponding monosaccharides (Kazeem *et al*., 2013; Oboh *et al*., 2014). Against this backdrop, inhibition of these duo enzymes represents an important strategy to control and lower postprandial hyperglycemia since this causes the postprandial blood glucose excursion to be considerably reduced (Kazeem *et al*., 2013). The effect of neem oil and its solvent extract on α-glucosidase and α-amylase could be an indication of the possible mechanisms of antidiabetic effect (Ali *et al*., 2002; Chipiti *et al*., 2015; Kazeem *et al*., 2013), especially since both preparations caused significant reductions in the rats' blood glucose levels. As observed for the glucose-metabolizing enzymes, the hypoglycemic effect of the cyclohexane-methanol extract of neem oil was also superior to that of neem oil. Many synthetic drugs, including the oligosaccharide acarbose, act by inhibiting α-amylase and α-glucosidase, and the continuous use of some of these come with side effects such as flatulence and abdominal distention (Li *et al*., 2018; Oboh *et al*., 2014). It is thus desirable to have natural products without the side effects associated with the synthetic ones.

Scientific knowledge of the oral toxicity of herbal products is important to identify safe dose margin, ascertain lethal clinical signs, or selective toxicity to specific tissues in order to prevent indiscriminate use (Olaleye *et al*., 2013). The organ-body weight ratio, including those of major organs (liver, heart, and kidney), is a sensitive indicator of the general health status. The observation that NOil and NOMH produced no effect on these might suggest that normal metabolism and growth of animals were not affected within the treatment period and at the evaluated dosages.

In the present study, biochemical parameters important for toxicity evaluation were evaluated in both the serum and organs (liver, kidney, and heart) of animals to identify possible alterations in hepatic, renal, and cardiac functions by neem oil and its solvent extract. The focus was on these three organs based on their importance to the survival of any organism (Suganthy *et al*., 2018). The liver is involved in metabolism and detoxification of harmful xenobiotics, and the levels of hepatic biomolecules (albumin, total-, direct bilirubin) or the activities of aminotransferases (ALT, AST) have been commonly used to evaluate the hepatic function and health status (Ghosh *et al*., 2019; Olaleye *et al*., 2013). Liver damage typified by cellular necrosis causes the leakage of cellular contents into the extracellular milieu and subsequently into the blood resulting in elevated serum levels of liver function parameters like ALT, AST, and bilirubin (Akinmoladun *et al*., 2015). Albumin, globulin, and total bilirubin are also related to the liver's hepatocellular and secretory functions (Porwal *et al*., 2017). The observations that the serum levels of ALT, AST, and total/direct bilirubin were not increased (but rather decreased for AST, ALT) by neem oil and the neem oil extract coupled with the fact their tissue levels were not significantly reduced by treatment suggest that neem oil and extract were non-toxic to hepatic tissues at the evaluated dosages (Akinmoladun *et al*., 2015; Ghosh *et al*., 2019; Olaleye *et al*., 2013). In terms of assessing renal function, the levels of creatinine, uric acid, and urea are important biochemical indices. An increase in serum creatinine level, a by-product of muscle metabolism that is actively secreted at a constant level by the proximal tubular cells, indicates compromised nephron function and kidney damage. It is directly related to kidney filtration capacity (Ogundipe *et al*., 2017). Urea produced by the liver during amino acid/amino metabolism is transported and excreted by the kidney as one of the components of urine but retained in the organ in case of renal injury or disease. Also, accumulation of uric acid, a by-product of purine nucleotide metabolism, in the blood is an indication of a compromised ability of the kidney to clear waste products (Gowda *et al*., 2010; Ogundipe *et al*., 2017). This investigation revealed that sub-acute administration of either neem oil or its methanol-cyclohexane extract does not negatively impact kidney functions (Porwal *et al*., 2017) since the levels of kidney function markers in the serum were not elevated.

Serum levels of known cardiac marker enzymes such as creatine kinase-MB extract (CK-MB) and LDH and serum lipid profile are used to evaluate cardiac health. While LDH gives a rough estimate of injury to myocardial tissues, CK-MB is more specific and sensitive, being detectable at the early stages of myocardial injury (Komolafe *et al*., 2013a). Both enzymes (CK-MB and LDH) were assessed in serum and cardiac tissues of rats to better correlate events at cellular levels. The non-toxicity of neem oil and its extract to rats' hearts was demonstrated by the insignificant changes in serum and heart enzyme levels. Serious injury to the heart tissues promotes the release of both enzymes into the blood with a concomitant decrease in the heart tissue (Abdel-Baky and Abdel-Rahman 2020; Chen *et al*., 2008; Komolafe *et al*., 2013b).

Lipid profile typified by serum levels of cholesterol, triglyceride, LDL, and HDL is important to higher animals' health because they serve as risk factors of cardiovascular disease (Cromwell and Otvos 2004; Komolafe *et al*., 2013b). Elevated LDL and low concentration of functional HDL is not good for the heart as the condition promotes atherosclerotic progression (Cromwell and Otvos 2004). Although the extracts showed no effect on serum total cholesterol and LDL levels in the present study, rats treated with the cyclohexane-methanol extract of neem oil showed reduced HDL. However, this might not be of toxicological importance since the values are within the reference limits for rats (Ihedioha *et al*., 2013).

The non-enzymatic tripeptide antioxidant, reduced glutathione (GSH), plays the critical role of protecting cells from oxidative damage. GSH levels are reduced under oxidative stress conditions that promote membrane peroxidation (Injac *et al*., 2009; Komolafe *et al*., 2013b). In the present study, membrane lipid peroxidation was quantified by the thiobarbituric acid reactive species (TBARS) contents produced as a result of various free-radical driven propagation of oxidative insult to

polyunsaturated fatty acids (PUFAs) (Injac *et al*., 2009). Neem oil increased GSH contents in the liver and heart tissues but caused no TBARS formation in the organs. On the other hand, rat hepatic membrane peroxidation was reduced by the neem oil extract. Overall, the intervention did not cause oxidative toxicity but instead boosts the antioxidant profiles of the hepatic and extra-hepatic tissues (Komolafe *et al*., 2013a).

In conclusion, neem oil and its cyclohexane-methanol extract both possess strong α-amylase and α-glucosidase inhibitory effects in vitro. The extract is more potent against α-glucosidase than the oil. The effect on key carbohydrate-metabolizing enzymes could partly explain the biochemical rationale underlying the hypoglycemic effect of neem oil as widely reported in folklore medicine. Oral administration of NOil and NOHM up to 800 mg/kg and 200 mg/kg respectively to experimental animals for 30 days appears non-toxic/safe. It does not result in any biochemical alterations of toxicological relevance.

Limitations *Further confirmation of safety through evaluation of chronic toxicity, mutagenicity, or carcinogenicity is still warranted. Information on the effectiveness of the extract and its components to suppress blood glucose levels in diabetic animal model is not provided.*

Funding: *The work was supported by a Tertiary Education Trust Fund (TETFUND) grant provided by the Federal Republic of Nigeria (2016-2017 FUOYE Intervention).*

Credit Author Statement: *BIC Brai: Conceptualization, methodology, funding acquisition, writing-review and editing. BE Amosun: Methodology, investigation. TR Komolafe: Methodology, investigation, writing-review and editing. RO Joseph: Methodology, investigation. K Komolafe: Conceptualization, investigation, writing-original draft. All the authors discussed, edited and approved the final version.*

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Niger. J. Physiol. Sci. 39 (June 2024): 91 – 100
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

The Effects of Consumption of Cooked Beans (*Phaseolus vulgaris*) and Serotonin Precursor Diets on Scopolamine-impaired Memory and Motor Co-ordination in Mice

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Summary: Alzheimer's disease (memory impairment disorder) and motor co-ordination disorders are on the increase worldwide. 5-Hydroxytryphamine (serotonin) is involved in short term memory and motor co-ordination. Whether beans that contain serotonin precursor (tryptophan) can ameliorate memory and motor co-ordination impairment has not been previously ascertained. Therefore, this research was to study the effects of consumption of cooked beans (Phaseolus vulgaris) and serotonin precursor diets on scopolamine-impaired memory and motor co-ordination in mice. Sixty mice were randomly assigned into 6 groups (10 mice per group) namely; Control, Scopolamine only, Scopolamine with 50% cooked beans diet, Scopolamine with serotonin precursor diet, 50% cooked beans diet only and serotonin precursor diet only. Preliminary studies on phytochemical analyses were done before learning/memory and motor co-ordination were also studied. Standard methods were used to study learning/memory and motor coordination. The results showed that preliminary phytochemical screening of cooked beans indicated the presence of tryptophan, flavonoids, alkaloids, and polyphenols (antioxidants). Learning was impaired in Scopolamine only group compared to control and other test groups ($p<0.05$). Memory was also impaired in scopolamine only group compared to all other experimental groups ($p<0.05$). Motor co-ordination was also impaired in scopolamine only group compared to all other groups ($p<0.05$). In conclusion, consumption of beans and serotonin precursor diets improved memory and motor coordination in scopolamine impaired memory and motor co-ordination in mice. The memory and motor co-ordination enhancement observed may be attributed to serotonin synthesized from tryptophan in bean.

Keywords*: Phaseolus vulgaris*, 5-Hydroxytryphamine, Scopolamine, serotonin precursor

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Manuscript received- December 2024; Accepted- February 2024

DOI: https://doi.org/10.54548/njps.v39i1.12

INTRODUCTION

Common bean (*Phaseolus vulgaris*) is a dicotyledonous plant belonging to family Leguminosae (Gepts, 2001). Common bean is one of the major food consumed in our communities. Beans contain: protein, carbohydrates, dietary fibres, minerals, vitamins and many phenolic compounds and is a very nutritious food from many aspects and it is not surprising that nutritionists characterize it as a nearly perfect food (Shansuddin *et al*.,1998., Vanderpoel *et al.*,1990C). It has been reported that beans have anti-carcinogenic, anti-mutagenic (Gref and Eaton (1993), anti-inflammatory, anti-diabetic, hypoglycemic, cardio-protective and antioxidant effects (Bennick *et al.*, 2008). It has also been reported that, beans contain serotonin and its precursor 5-Hydroxytrytophan (5-HTP) (Porta *et al*., 2008). Other chemical compounds present in beans include: saponins, tannins, glycosides, flavonoids etc. Aduema (2016) reported that long term consumption of beans diet improves learning and memory in apparently normal mice.

Notable among the array of chemical constituents present in beans is serotonin which has been reported to influence neurobehavioural actions such as memory, learning, sleep, pain, feeding, sexual and emotional behaviors (Gasbarri and Pompili, 2014., Buhot *et al.*, 2000). Serotonin has been shown to act as neurotransmitter to modulate behaviour in response to changing cues, acting on both neurons and muscles to affect locomotion and learning (Cabaj *et al.*, 2017., Chase and Koelle, 2007).

Scopolamine is a drug of choice in inducing memory impairment in animals including mice. The cognitive dysfunction or memory impairment observed after this drug's usage is analogous to observations in demented patients. Scopolamine is a muscarinic receptor antagonist. It impairs long term potentiation which is responsible for long term memory (Ovsepian *et al.*, 2004). It is also used as anxiogenic agent for evaluation of anxiolytic effects of new drugs. Scopolamine has been shown to impair motor coordination in animals (Hasselmann, 2014).

Owing to the adverse effects of synthetic drugs (Deaton and Nappe, 2021). there is a search for natural remedies which are safer and effective. According to World Health Organization statistical report, 80% of the world's population presently uses traditional medicine for some aspects of primary health care including mental health (WHO, 2003). Therefore, natural products may provide a new source of beneficial neuropsychotropic drugs (Deaton and Nappe, 2021) provided they are scientifically validated and their mechanisms properly established.

Since beans contain serotonin that can potentially modulate behaviour in response to changing cues, it is, therefore, conceivable that the consumption of beans may affect learning and memory as well as motor coordination and balance. Hence, this present study investigated the effects of consumption of common beans and serotonin precursor on learning/memory and motor coordination on scopolamine impaired memory and motor coordination in CD1 mice.

MATERIALS AND METHODS

Preparation of experimental diets: Preparation of beans diet: Twenty (20) cups of beans were bought from Marian market, a local market in Calabar, Nigeria. The beans were cooked, air dried and grounded into powdered form using an electric blender. The powdered form weighed 1,560g. One kilogram of powdered cooked beans was mixed with one kilogram of normal rodent chow making 50:50(w/w) % of beans diet. The constituent was blended in a bending machine for uniform mixture.

Preparation of serotonin precursor diet: Serotonin precursor (5-Hydroxytryptophan) used for this study was obtained from Sigma Aldrich, Germany. The estimation and preparation of the powdered 5-Hydroxytryptophan content of cooked beans was according to the method of Feldman and Lee (1985) and modified by Mosienko *et al*., (2012). The serotonin precursor diet was prepared by mixing 1.15 g of the precursor in 98.85 g of the feed so that the amount of 5 HTP added was equivalent to that contained in every 100 g of cooked beans fed by the mice. An electric blender was used to blend the mixture forming the serotonin precursor diet.

Experimental animals and design: Sixty adults CD1 mice weighing between 17 – 26 g obtained from the animal house of Physiology Department, University of Calabar, Nigeria, were used for this study. They were randomly assigned into 6 groups of 10 mice each, namely; control, scopolamine only, 50% cooked beans + scopolamine, serotonin precursor diet + scopolamine, 50% cooked beans only and 5HT precursor only groups respectively. The mice in the control group were fed normal rodent chow and normal saline intraperitoneally (1ml/kg bodyweight). Group 2 mice were fed normal rodent chow and Scopolamine intraperitoneally (1mg/kg bodyweight). Group 3 mice were fed cooked beans diet and scopolamine intraperitoneally (1mg/kg bodyweight). Group 4 mice were fed serotonin precursor diet and scopolamine intraperitoneally (1mg/kg bodyweight). Group 5 mice were fed cooked beans diet and Scopolamine intraperitoneally (1mg/kg bodyweight). Group 6 mice were fed serotonin precursor diet and normal saline intraperitoneally (1mg/kg bodyweight). Scopolamine was administered once daily for the first week. In the subsequent weeks, Scopolamine was administered once every two days. The feeding and behavioural tests lasted for four weeks.

The experimental animals were kept in pathogen-free and well–ventilated housing unit at room temperature of (28 $\pm$ 2^{o}C) and humidity (85 $\pm$ 5^{o}C). The housing rooms were illuminated on a 12-hour light-dark cycle. The animals were allowed access to water and food ad libitum. Approval for the use of the animals was obtained from the College Ethical Committee of University of Calabar, Nigeria on the use of experimental animals and it was in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guide lines (EEC Directive of 1986; 86/609/EEC).

Behavioural Protocols

Morris water maze for learning and memory: A Morris water maze modified for mice was used to study learning and memory in this study (Parlor *et al*., 1996), which is smaller than the maze developed for rats. The water maze is constructed out of a circular polypropylene pool (Canadian tire "Pelican" pool) that measures 110cm in diameter and 20cm in depth. The pool was filled to a depth of 14-cm (0.5-cm over the platform) with room-temperature tap water, which was made opaque with the addition of 100mL of non-toxic white liquid tempura paint. The water was left to sit overnight in order to attain room temperature (22 ± 1°C).

The pool was divided into four quadrants: Northwest, Northeast, Southwest and Southeast. Boundaries of these quadrants were marked on the edges of the pool with masking tape and labelled: North, South, East and West. A Plexiglas cylinder (13.75 cm x 9 cm diameter) was used as the escape platform in the maze. The cylinder was been filled with cement to weigh it down in the pool. The platform had a removable red and yellow striped top (3 cm x 9 cm in diameter) with a colorful flag erected in the centre. For visible platform tests the level of the water in the pool was adjusted to 0.5-cm below the surface of the striped top, thus creating a visible escape platform, or to 0.5-cm above the white cylinder (with the striped top removed), creating a hidden escape platform.

The pool was located in a room measuring 5.2 x 2.4 m. Several posters were placed on the walls of the room to act as visual cues. There was also furniture in the room (sink, table, chairs) that provides visual cues. During testing, the room was dimly lit with diffuse white light (30 lux). The performance of the animals in the water maze was recorded using a video camera-based computer tracking system (Water maze, Actimetrics) on an IBM PC computer, with the camera fixed to the ceiling 2.1m above the pool.

In our paradigm, testing in the water maze lasted for 8 days: days 1-3 was acquisition training, 4-6 reversal training, day 7 probe trial and day 8 is visible- platform days respectively. Acquisition and reversal training were with the hidden platform (water is 0.5-cm above platform). During reversal, the platform was moved to the opposite side of the maze. During the probe trial, there was no escape platform so that visuo-spatial memory can be assessed. On the

visible-platform day the platform was moved to another quadrant of the pool and the visible top is added to the platform. This assesses basic visual ability and motivation to locate the platform. Each day, the mouse was removed from its home cage and was placed in a clean holding cage without woodchip bedding. Paper towel was torn into strips and placed in the bottom of the holding cages to allow the mice to dry more quickly. This paper towels were replaced when it became wet. Mice were run in squads of 4-6 with 5-minutes between each trial (inter-trial interval) for each mouse (Iivonen *et al.,* 2003).

During acquisition training, the platform was placed in the centre of the Northeast quadrant. Each mouse received 4 trials per day. In each trial, the mouse was given a maximum of 60-sec to locate the escape platform. The starting positions of the mice were predetermined using a Latin square design, which prevents the repetition of starting location sequences on back-to-back test days. Possible start positions were at the boundaries of the quadrants (e.g, West, North, East or South). For each trial, each mouse was removed from its holding cage using a small, clean 500-mL plastic container to minimize handling stress. The animal is then placed into the water at the appropriate start position. The mouse was then permitted to explore the pool and to search for the hidden escape platform for 60-sec. When the animal located the platform, the timer was stopped (manually) and the mouse was allowed to stay on the platform. Once on the platform, the mice were permitted to view the extra-maze environment for 10-sec. If the mouse does not find the platform during the allotted time, the animal was guided onto the platform using the plastic container. The next mouse is then placed in the pool and the same procedure followed. Each animal completes 4 trials per day over 3 days, for 12 trials of acquisition training, each trial from a different one of the 4 start locations.

Reversal training began on day 4. The invisible platform was moved to the opposite quadrant (Southwest quadrant), and mice are again assigned to appropriate start positions. The same procedures as in acquisition training were carried out during reversal training. Each of the animals completes 4 trials per day for 3 days for a total of 12 trials of reversal training. A probe trial was conducted on day 7 to assess visuo-spatial memory. At this time, there was no escape platform in the maze. Each mouse was placed in the pool from one of the four possible start positions and allowed to explore the pool for 60-sec, during which the time spent in each quadrant of the maze is recorded. When the 60-sec is complete the mouse is scooped up using the container and placed in a holding cage to dry before being returned to its home cage.

The visible platform task was conducted on day 8. The visible platform was placed in a new location within the Northwest quadrant of the pool. The same procedures as in acquisition and reversal training are carried out and mice complete 4 trials. During acquisition, reversal and visual training, the following behaviours were measured: swim latency (time to find and mount the escape platform), swim distance, proximity to the platform.

During the probe trial, the measures recorded were: frequency of entries into each quadrant (Northeast, Northwest, Southeast and Southwest), duration of time spent in each quadrant, the number of times the mouse crosses the location of the platform during reversal training (annulus reversal crossing), the number of times the mouse crosses the location of the platform during acquisition training (annulus acquisition crossing), the duration and frequency of thigmotaxic behaviour (9 cm corridor width) and proximity to the platform location.

The novel object recognition task for memory: The novel object recognition task (NORT) was originally designed for rats as a test of declarative memory, after it was discovered that rats will spend more time investigating a new object than a familiar one (Ennaceur and Delacour, 1988). It has since been validated as a test of recognition memory in mice (Brown *et al*., 1999; Podhorna and Brown, 2002; and Sik *et al*., 2003). This test which has many variants is based on a spontaneous behaviour in animals an unconditioned preference for novel objects. The modified method of Brown *et al.* (2002) was used in this study.

Prior to testing all mice were allowed to familiarize with the apparatus for 5 minutes before the test commences. Mice were carried to the test room in their home cages. They were moved from their home cages to the testing apparatus and back using a small container. After each 5 minutes trial, the mice were returned to their cages and the apparatus was cleaned with 70 % ethyl alcohol and permitted to dry between trials.

The behaviours scored during the Open Field (NORT) include: Approaches to Each Object: directing the nose to the object at a distance of < 1 cm and/or touching it with the nose and Time Spent with Each Object: sniffing or climbing the object.

Beam walking for determination of motor coordination: motor coordination was assessed using the beam walking test. The beam walking apparatus was used to test motor coordination and balance. The beam walking test is more sensitive than the mouse rotarod in determining motor coordination deficits (Stanley *et al.,*2005). The beam has a length of 100 cm, a width of 2 cm and is elevated to a height of 40 cm. The beam is marked at 5 cm and 1 cm intervals. It is composed of wood and is coated with black paint. The animals were carried to the test room in their home cage. The mouse was removed from its home cage and placed at one end of the balance beam. After the mouse had secured its grip on the beam, the trial began. The maximum length of the trial is two minutes. The mouse was tested under white light, during the dark phase. The beam was cleaned with 70% ethanol and permitted to dry between each trial. Behaviour scored were**:** Distance travelled, Foot Slips, Number of turns and Latency to fall.

Statistical Analysis Data obtained were presented as mean ± SEM. Experimental data were analyzed using analysis of variance (ANOVA) followed by a post hoc test (Least Square Difference (LSD) test) to determine significant difference between means. Th e analysis was done with an SPSS 18 statistical package. The mean values were considered significant at $p<0.05$.

RESULTS

Phytochemical screening: Preliminary results of the phytochemical analysis of beans showed the presence of large quantities of flavonoids, alkaloids and polyphenols that are antioxidants. The result is presented in Table 1.

Behaviours scored in Morris water maze

Comparison of swim latency during the acquisition training: Figure 1 shows the swim latencies during the acquisition training in the Morris water maze (Days 1, 2 and 3) between the control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans and 5HT precursor diet. The swim latencies in day 1 were 59.67 ± 0.223, 57.85 ± 1.365, 57.14 ± 1.825, 56.31 ± 1.621, 57.90 ± 0.903 and 58.55 ± 1.270 seconds for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans and 5HT precursor diet groups respectively. The result showed that, there was no significant difference between the experimental groups compared to control in day 1. However, in day 2 the swim latency of 50% cooked beans (38.26 ± 3.534 sec) was significantly shorter compared to Control and other experimental groups ($p<0.05$). In acquisition day 3 the swim latency of the scopolamine only group was significantly longer (47.41 ± 3.088 sec) compared to control and other experimental groups.

Comparison of swim latency during the reversal training: Figure 2 represents the swim latency curves during the reversal training in the Morris water maze (Day 4, 5 and 6) between the control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet. The result shown that, in reversal training day 1 the swim latency (39.70 ± 3.822) of the 50% cooked beans diet was significantly shorter compared to control and other experimental groups ($p<0.05$). Similar trend was observed in reversal training day 2. In reversal training day 3 the results for the swim latencies were 32.04 ± 2.895, 49.22 ± 2.111, 16.17 ± 2.902, 19.06 ± 3.482, 15.78 ± 2.241 and 18.29 ± 3.211 seconds for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively. The scopolamine only group was significantly longer compared to control and other experimental groups ($p<0.05$). The result also showed that, the swim latencies of the experimental groups fed with serotonin precursor and cooked beans diets were significantly shorter latency compared to control ($p<0.05$)

Table 1:
Phytochemical analysis of cooked beans

S/N	Chemical Compound	Component Value
1	Alkaloids	+ +
2	Phlobatannin	+
3	Saponins	+ +
4	Flavonoids	+ + +
5	Tannins	+ +
6	Hydroxymethyl Anthraquinone	_
7	Cardiac Glycoside	+
8	Polyphenol	+ + +
9	Reducing Sugars	+ +
10	Anthraquinone	_

Key:
+ + + = *highly present*
+ + = *moderately present*
+ = *slightly present*
- = *absent*

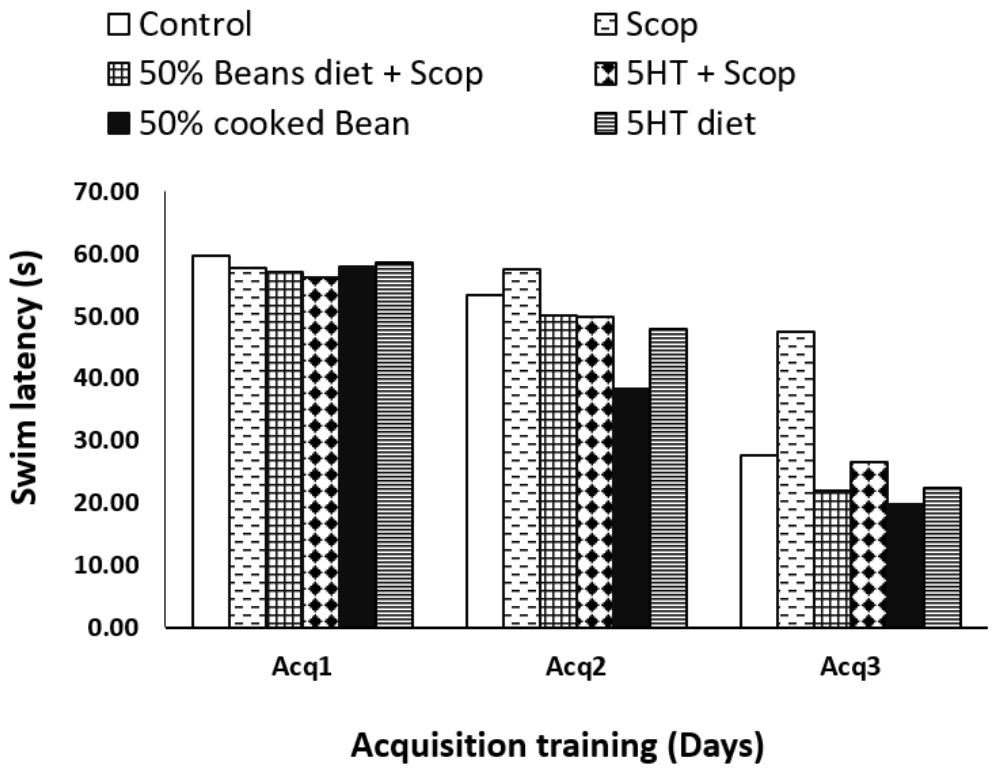

Figure 1:
Comparison of acquisition training of control and test groups in Morris water maze.
Value are expressed as mean ± SEM, n = 10.

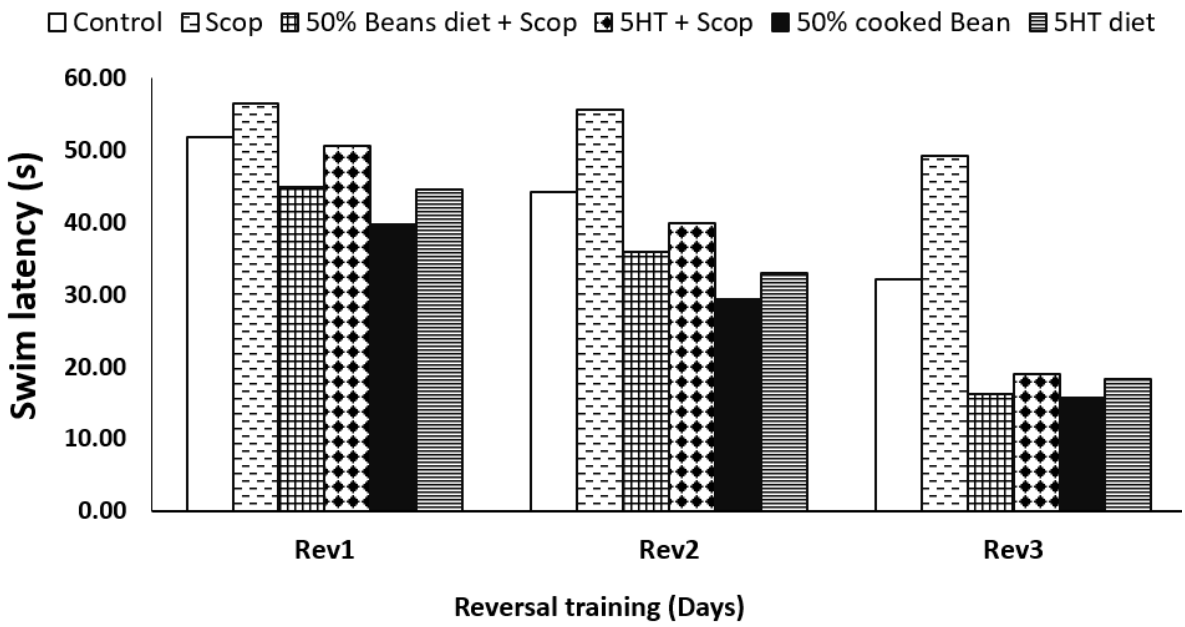

Figure 2:
Comparison of reversal training of control and test groups in Morris water maze.

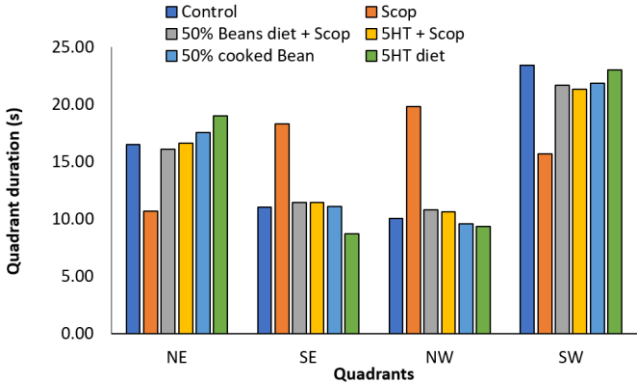

Figure 3:
Comparison of quadrant duration during probe trial test of control and test groups in Morris water maze.

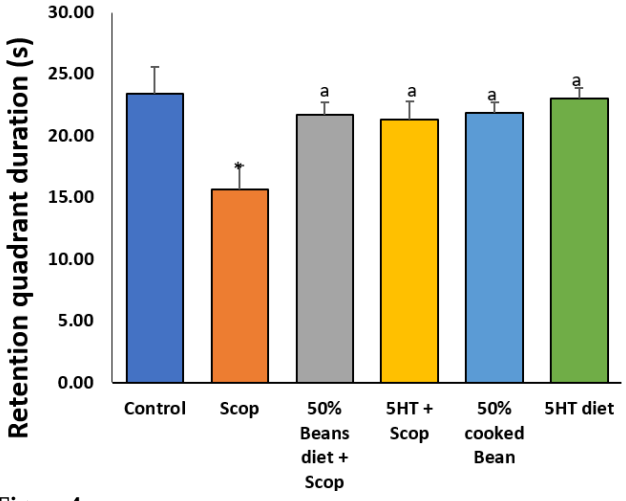

Figure 4:
Comparison of Retention quadrant duration of control and test groups in Morris water maze.
Value are expressed as mean ± SEM, n = 10.
** = p<0.05 vs control; a = p<0.05 vs scopolamine*

Comparison of quadrant duration: Figure 3 represents comparison of the quadrant duration during probe trial test in the Morris water maze. The mean duration in each quadrant were: northeast (16.51 ± 1.341, 10.69 ± 1.035, 16.08 ± 0.653, 16.61 ± 0.679, 17.53 ± 0.667 and 18.96 ± 0.749 for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively), southeast (11.042 ± 1.598, 18.30 ± 1.429, 11.43 ± 1.004, 11.44 ± 0.858, 11.08 ± 0.637 and 8.69 ± 1.019 for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively), northwest (10.048 ± 1.093, 19.80 ± 2.406, 10.81 ± 0.659, 10.62 ± 0.932, 9.55 ± 0.845 and 9.32 ± 0.817 for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively) and southwest (23.40 ± 2.171, 15.68 ± 2.389, 21.67 ± 1.061, 21.32 ± 1.493, 21.82 ± 0.859 and 23. 01 ± 0.825 for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively). The result showed that, the control, cooked beans and serotonin precursor diets groups expressed significant preference to the northeast and southwest quadrants compared to the group treated with scopolamine only ($p<0.05$).

Duration of quadrant retention: Figure 4 represents comparison of the quadrant duration retention during the probe trial in the Morris water maze test between the Control, scopolamine only, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet having durations: 23.40 ± 2.17, 15.68 ± 1.88, 21.67 ± 1.06, 21.32 ± 1.49, 21.3 ± 0.85 and 23.02 ± 0.82 seconds respectively. The result shown significantly reduced quadrant retention compared to control and other experimental groups ($p<0.05$). There was no significant difference between other experimental groups compared to control.

Annulus acquisition frequency: The annulus acquisition crossing during the probe trial task frequencies were 2.26 ± 0.176, 1.20 ± 0.313, 2.20 ± 0.133, 1.89 ± 0.209, 2.56 ± 0.156 and 2.44 ± 0.193 for Control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively. The result shows that the annulus acquisition crossing for the scopolamine only group was significantly reduced compared to control and other experimental groups ($p<0.05$). There was no significant difference between experimental groups compared to control. The result is illustrated in figure 5.

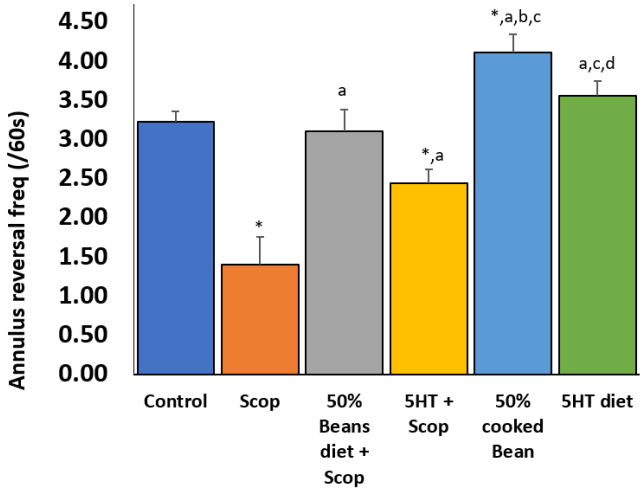

Figure 5:
Comparison of annulus reversal frequency of control and test groups in Morris water maze.
Value are expressed as mean ± SEM, n = 10.
** = p<0.05 vs control; a = p<0.05 vs scopolamine*
b = p<0.05 vs 50% cooked beans + scopolamine
c = p<0.05 vs 5HT precursor + scopolamine
d = p<0.05 vs 50% cooked beans

Annulus reversal crossing frequency: The annulus reversal crossing during the probe trial task frequencies were 3.22 ± 0.133, 1.40 ± 0.30, 3.10 ± 0.276, 2.44 ± 0.175, 4.11 ± 0.230 and 3.56 ± 0.193 for Control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively. The result shown that, the annulus reversal crossing for the scopolamine only group was significantly reduced compared to control and other experimental groups ($p<0.05$). The 50% cooked beans only group also shown a significant increase in reversal crossing compared to control and other experimental groups ($p<0.05$). The result is illustrated in Figure 6.

Comparison of swim latencies on the visible platform test: The swim latencies during visible platform test for Control, scopolamine only, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet, were 15.73 ± 2.461, 37.76 ± 2.360, 10.95 ± 1.755, 14.83 ± 1.566, 8.09 ± 0.794 and 12.64 ± 0.719 seconds respectively. From the result, the swim latency for the scopolamine only group was significantly longer compared to control and other experimental groups ($p<0.05$). The 50% cooked beans only group, showed significantly shortest latency during the visible test compared to control and other experimental groups ($p<0.05$). see Figure 6 below.

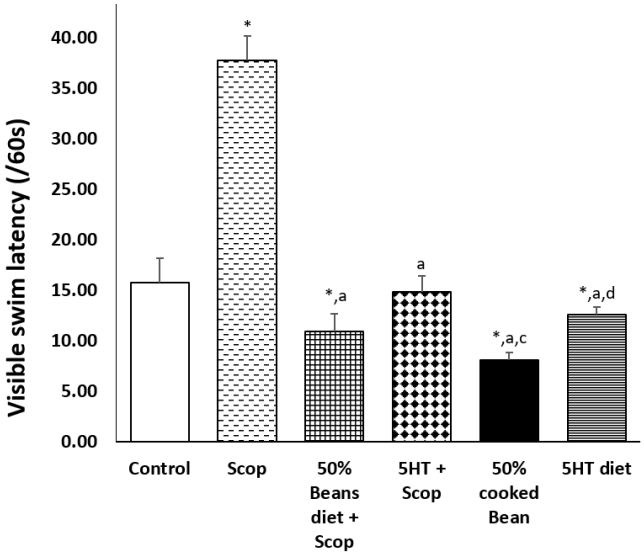

Figure 6:
Comparison of visible swim latency of control and test groups in Morris water maze.
Value are expressed as mean ± SEM, n = 10.
** = p<0.05 vs control; a = p<0.05 vs scopolamine*
b = p<0.05 vs 50% cooked beans + scopolamine
c = p<0.05 vs 5HT precursor + scopolamine
d = p<0.05 vs 50% cooked beans

Behaviours scored in novel object recognition test

Comparison of the habituation index for short term memory: Figure 7 represents the mean habituation index for short term memory for the Control, scopolamine only, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet are 49.14 ± 3.05, -4.30 ± 2.40, 52.98 ± 4.57, 48.31 ± 4.03, 57.61 ± 3.13 and 54.44 ± 4.14 respectively. The habituation index for short term memory of the Scopolamine only group was significantly lower compared to control and other experimental groups ($p<0.05$). There was no significant difference between the other experimental groups compared to control.

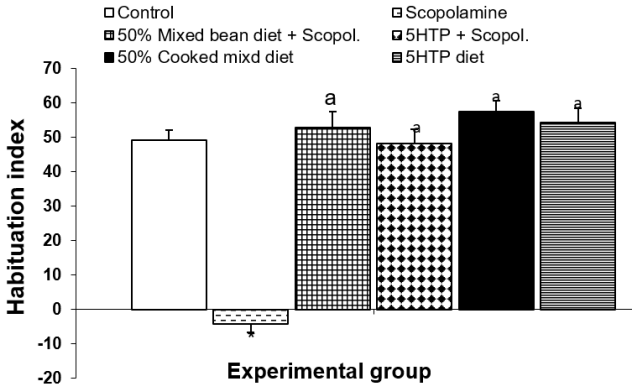

Figure 7:
Comparison of habituation index for short term memory during the NORT in control and test groups
Value are expressed as mean ± SEM, n = 10.
* = p<0.05 vs control; a = p<0.05 vs scopolamine

Comparison of the habituation index for long term memory: Figure 8 represents the mean habituation index for long term memory for the Control, scopolamine only, 50% cooked beans +scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet (7.64 ± 1.86, -8.17 ± 2.91, 5.27 ± 3.82, -1.94 ± 1.19, 13.00 ± 1.09 and 11.89 ± 2.07) respectively. The habituation index for long term memory of the Scopolamine only group was significantly lower compared to control and other experimental groups ($p<0.05$). The mean values of the groups fed with cooked beans only and serotonin precursor diet only were significantly higher compared to control ($p<0.05$).

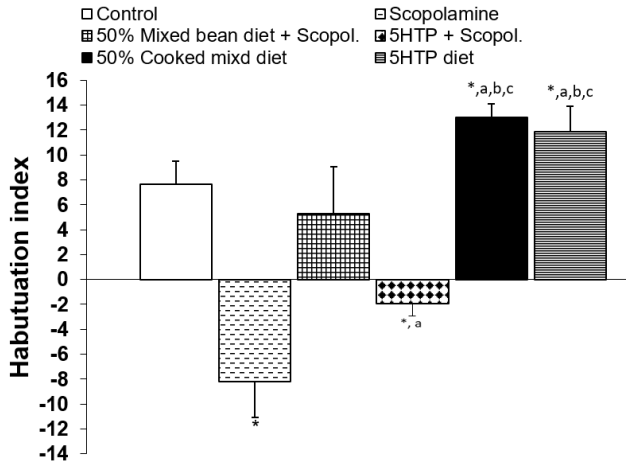

Figure 8:
Comparison of Habituation index for long term memory during the NORT in control, 50% cooked beans, 5HT precursor and scopolamine treated groups
Value are expressed as mean ± SEM, n = 10.
* = p<0.05 vs control; a = p<0.05 vs scopolamine
b =50% cooked mixed diet; c = p<0.05 vs 50% mixed bean diet+Scopol

Comparison of the discrimination index for short term memory: Figure 9 represents the mean discrimination index for short term memory for the Control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet (0.51 ± 0.06, -0.18 ± 0.03, 069 ± 0.15, 0.52 ± 0.01, 0.64 ± 0.14 and 0.52 ± 0.12) respectively. The mean discrimination index for short term memory of the Scopolamine only group was significantly lower compared to the control and other experimental groups ($p<0.05$).

Comparison of the discrimination index for long term memory: The mean discrimination index for long term memory for the Control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet (0.12 ± 0.03, -0.13 ± 0.03, 0.03 ± 0.02, -0.08 ± 0.02, 0.14 ± 0.01 and 0.10 ± 0.01) respectively. The mean discrimination index for long term memory of the Scopolamine only group was significantly lower compared to the control and other experimental groups ($p<0.05$). The group fed with 50% cooked beans only had longer discrimination index compared to control and other experimental groups ($p<0.05$). The group treated with 50% cooked beans diet + scopolamine and serotonin precursor diets + scopolamine had significantly decreased discrimination index compared to control ($p<0.05$).

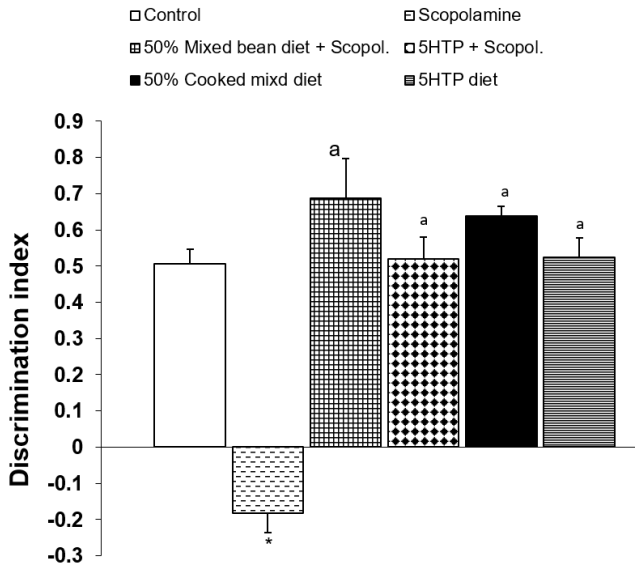

Figure. 9:
Comparison of discriminative index for short term memory during the NORT in control and test groups
Value are expressed as mean ± SEM, n = 10.
* = p<0.05 vs control; a = p<0.05 vs scopolamine

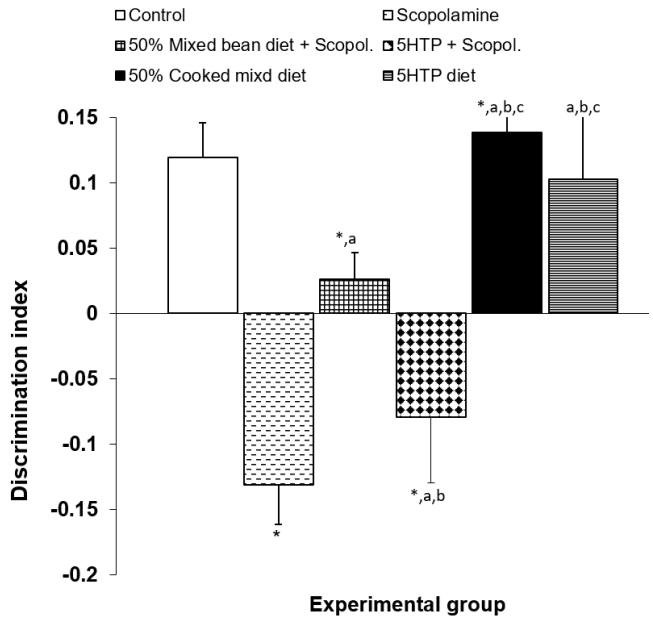

Figure. 10:
Comparison of Discriminative index for long term memory during the NORT in control and test groups
Value are expressed as mean ± SEM, n = 10.
* = p<0.05 vs control; a = p<0.05 vs scopolamine
b = 50% cooked mixed diet
c = p<0.05 vs 50% mixed bean diet + Scopolamine

Behaviours scored in beam walking

Line crosses: Figure 11 represent the comparison of mean line crosses for control, scopolamine only, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet in beam walking (429.89 ± 19.57, 232.20 ± 20.36, 476.80 ± 30.76, 387.78 ±

29.18, 471.11 ± 14.55 and 393.33 ± 18.23) respectively. The line crosses of the scopolamine only administered group was significantly decreased compared to control and other experimental groups ($p<0.05$). The result also showed that, groups fed with cooked beans diet shown a significant increased line crosses compared to control and serotonin precursor diet fed groups ($p<0.05$). There was no significant difference between the serotonin precursor diet groups compared to control.

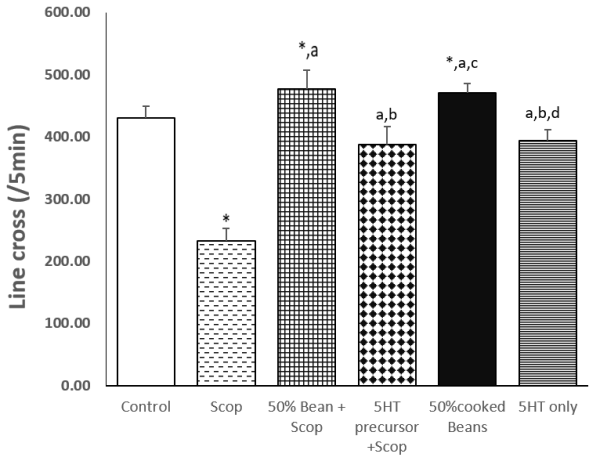

Figure 11:
Comparison line cross of control and test groups in walking beam
Value are expressed as mean ± SEM, n = 10.
** = p<0.05 vs control; a = p<0.05 vs scopolamine*
b = p<0.05 vs 50% cooked beans + scopolamine
c = p<0.05 vs 5HT precursor + scopolamine
d = p<0.05 vs 50% cooked beans

Foot Slips: The mean frequencies of foot slips of the different experimental groups was recorded as follows: 1.33 ± 0.47, 13.00 ± 0.98, 2.30 ± 0.50, 4.22 ± 0.32, 1.00 ± 0.37 and 1.67.050 for control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet respectively. The result indicated that, the number of foot slips in the group treated with scopolamine only was significantly increased compared to control and other treatment groups ($p<0.05$). There was no significant difference between 50% cooked beans + scopolamine, 50% cooked beans, and 5HT precursor diet compared to control. The foot slips in 5HT precursor diet + scopolamine group was significantly increased compared to control ($p<0.05$; Figure 12).

Reversals: Figure 13 below represent the comparison of mean reversals of control, scopolamine, 50% cooked beans + scopolamine, 5HT precursor diet + scopolamine, 50% cooked beans, and 5HT precursor diet in beam walking were 4.89 ± 0.39, 7.70 ± 0.42, 4.70 ± 0.63, 4.67 ± 0.50, 4.33 ± 0.17 and 4.33 ± 0.24 respectively. The results showed that there was a significant increase in mean number of reversals made by the scopolamine group compared to control and other treatment groups ($p<0.05$). there was no significant difference between the treatment groups compared to control.

DISCUSSION

The effects of consumption of cooked beans (*Phaseolus vulgaris*) and serotonin precursor diets on scopolamine-impaired memory and motor co-ordination in mice were studied. The effects of serotonin precursor (5-Hydroxytryptophan) diet were also compared with cooked beans diet because 5-Hydroxytryptophan is one of the constituents of beans. Preliminary phytochemical screening of beans was done before parameters for learning/memory and motor coordination were also studied. The Morris water maze and Novel object recognition tests were used to estimate learning and memory, and beam walking test was used to study motor co-ordination and balance.

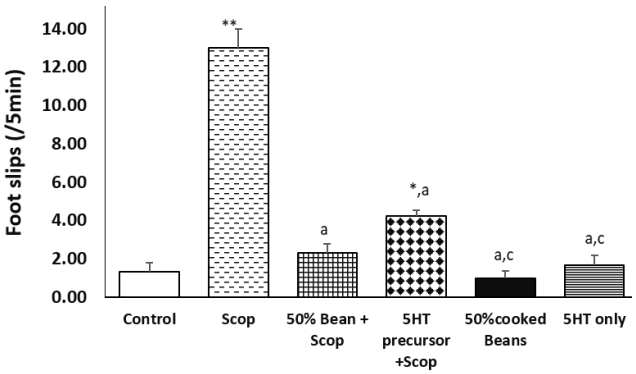

Figure 12:
Comparison of foot slips of control and test groups in walking beam.
Values are expressed as mean ± SEM, n = 10.
*** = p<0.001 vs control; *= p<0.05 vs control*
a=p<0.001 vs scopolamine
c=p<0.05 vs 5HT precursor + scopolamine

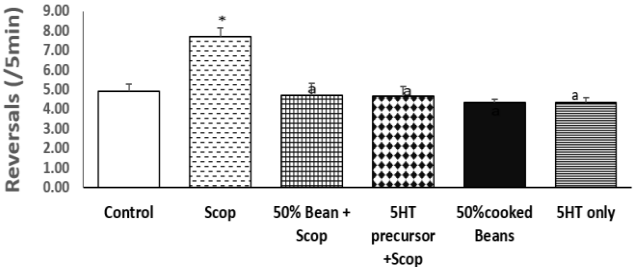

Figure 13:
Comparison of reversals of control and test groups in walking beam
Values are expressed as mean ± SEM, n = 10.
*** = p<0.001 vs control; *= p<0.05 vs control*
a=p<0.001 vs scopolamine
c=p<0.05 vs 5HT precursor + scopolamine

Preliminary results of the phytochemical analysis of beans showed the presence of large quantities of flavonoids, alkaloids and polyphenols that are antioxidants. Flavonoids have been found to improve memory and motor coordination in mice. The results of this study show that the groups fed cooked beans and serotonin precursor diets shown improved learning and memory as well as motor co-ordination compared to scopolamine only treated group. This observation is consistent with the earlier study carried out by Samira *et al.,* (2015) who reported that, antioxidants improved motor co-ordination in mice with impaired memory and motor deficit.

Following the consumption of cooked beans and serotonin precursor diets, swim latencies for the first three days during acquisition training showed that, the swim latencies of all the groups fed cooked beans and serotonin

precursor diets were significantly lower compared to control and the scopolamine only treated group.

The same trend was observed during the reversal training days. This means that these groups of mice were able to locate the hidden escape platform faster and so, learned faster compared to control and scopolamine only treated groups that consumed normal rodent chow. The scopolamine only group had poorer learning curve.

Visuo-spatial memory was assessed during the probe trial in the Morris water maze task. Mice fed cooked beans and serotonin precursor diets and the control group spent significantly more time exploring the retention quadrants compared to the group treated with scopolamine only. This showed that they had improved memory compared to scopolamine only treated group of mice.

The cued version of the Morris water maze assesses cued learning and visual integrity of the animals tested. Impairments in performance in the hidden platform model may be due to some brain lesions or drugs which may affect the motivation to escape, or sensorymotor factors rather than spatial learning. The swim latencies were also used for the comparisons. Shorter swim latencies in the visible platform task indicate improved cued learning. Longer swim latencies indicate poor cued learning. The mice fed both cooked beans as well as serotonin precursor diets had significantly shorter swim latencies compared to the control and scopolamine only treated group. This means that consumption of cooked beans and serotonin precursor diets improved learning process and visual integrity in mice.

Beans are rich in vitamin B6 and contain tryptophan (Portas *et al*., 2000). In significant measures Tryptophan hydroxylase converts tryptophan into 5-HTP which in turn is converted into serotonin (5-HT) by the enzyme aromatic acid decarboxylase that uses vitamin B6 as co-enzyme. Serotonin is a neurotransmitter that is known to improve learning and memory as well as cognitive functions (Portas *et al*., 2000; Walther *et al*., 2003). Furthermore, the property of beans diet improving learning and memory is further enhanced by the presence of mineral compounds such as glutamic acid (Yehuda *et al*., 1996), magnesium, potassium, phosphorus and calcium, etc. which are known to enhance memory and learning.

From the results obtained, the habituation index for short term memory for the Scopolamine only treated group was lower compared to other experimental groups, whereas the values from the groups fed cooked beans and serotonin precursor diets groups appeared higher. Decrease in habituation depicts deficit in associative learning (Bolivar, 2010). The Scopolamine only treated group was also observed to have decreased long term habituation index, thus confirming their memory deficits. The long term habituation index for the animals fed cooked beans and serotonin precursor diet appeared to be higher than that of the Scopolamine only treated group. This increased habituation could be interpreted as improvement in the memory of these animals (Blackford *et al.*, 2013).

short term memory capabilities of group of mice fed cooked be beans diet was the highest as shown by the significant increase in intrasession habituation. However, the animals fed serotonin precursor diet had improved long term memory as shown by the significant increase in intersession habituation index.

From the results obtained, the discrimination index for short term memory of the Scopolamine only treated group was significantly lower compared to control and other experimental groups, while the value for other treated test groups showed no significant difference compared to control. However, the result of discrimination index for long term memory showed that the values for the animals fed cooked beans and serotonin precursor diets were observed to be more positive compared to the Scopolamine only treated group, with that of the 50% cooked beans diet group showing the highest. The increased positivity of these groups indicates more time spent exploring the novel object and thus implied improvement in recognition memory. These results depict that, cooked beans diet improved both short- and long-term memory of the mice whereas, serotonin precursor diet improves short term memory. The observed memory enhancement following cooked beans diet consumption may be due to the involvement of serotonin present in cooked beans diet.

The results obtained from beam walking showed that, the groups of mice fed cooked beans and serotonin precursor diets showed better motor coordination compared to scopolamine only treated group. The scopolamine only treated group had a poor motor co-ordination compared to control. It is possible that scopolamine suppressed motor centers in the brain as indicated in the results showing decreased line crossings, a higher number of foot slips and greater number of reversals. The result also shows that the group of mice fed cooked beans diets had higher significant motor co-ordination compared to control and other experimental groups. This is because, decreased frequency of food slips and increase line crosses indicate a higher level of maneuverability in the beam, thus indicating better motor learning ability.

The results of this study shows that the groups of mice fed with cooked beans as well as serotonin precursor diets had improved motor co-ordination compared to control and scopolamine only treated group. This observation is consistent with the earlier study carried out by Samira *et al.*, (2015). Who reported that, antioxidants improved motor co-ordination in mice with impaired memory and motor deficit. The effects of consumption of cooked beans (*Phaseolus vulgaris*) and serotonin precursor diets on scopolamine-impaired memory and motor co-ordination in mice were studied. Preliminary studies on phytochemical analysis of the beans were studied before learning and memory as well as motor coordination. Preliminary results of the phytochemical analysis of beans showed the presence of large quantities of flavonoids, alkaloids and polyphenols that are antioxidants. Learning was impaired in animals treated with scopolamine only compared with control and other test groups ($p<0.05$). Memory was also impaired in the group of animals treated with scopolamine only compared to control and other experimental groups.

Motor coordination was impaired in animals treated with scopolamine only compared to control and other experimental groups($p<0.05$). Consumption of beans as well as serotonin precursor diets improved learning and memory as well as motor coordination.

In conclusion, consumption of cooked beans and serotonin precursor diets improved learning and memory as well as motor coordination. The learning and memory enhancement observed may be attributed to antioxidants and

serotonin precursor (tryptophan) present in cooked beans diet and the motor coordination enhancement may also be due to antioxidants present also in cooked beans.

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Niger. J. Physiol. Sci. 39 (June 2024): 101 – 109
www.njps.physiologicalsociety.com

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Full length Research Article

Efficacy of Aqueous Extract of *Talinum Triangulare* on the Microanatomy of the Hippocampus and Short-Term Memory of Scopolamine Hydrobromide-Induced Alzheimer's Type Cognitive Dysfunction Rats

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Summary: The study aimed at elucidating the potency of aqueous extract of *Talinum Triangulare* on the hippocampal neurons, astrocytes as well as assessing short term memory of scopolamine-induced Alzheimer's type rats. Fifty-four Wistar rats (180-200g) were used for the study; thirty experimental rats were randomly grouped into five, each containing six rats designated A, B, C, D and E while twenty-four rats were used to establish 50% lethal dose (LD50). Alzheimer's type cognitive dysfunction was intraperitoneally (ip) induced with scopolamine hydrobromide (1mg/kg, ip) for seven days in groups B-E prior to the oral administration of the aqueous extract (875 and 1750mg/kg) and donepezil (1mg/kg), followed by the novel object recognition test, histological and GFAP staining processes. Results revealed atrophied pyramidal cells, hyperchromatic, numerous glial cells with pale cytoplasmic inclusions and astrogliosis in groups B, C, and E while group D showed ameliorative potentials compared to group A. Also, short term memory was significantly higher in group D compared to groups B, C and E. In conclusion, aqueous extract of *Talinium triangulare* leaves reduced the potentials of scopolamine hydrobromide by restoring abnormal neurons, hence, enhancing cognitive memory in the rats used in the present study.

Keywords*: Alzheimer's disease, Astrogliosis, Atrophy, Rats, Scopolaminne hydrobromide. Talinium triangulare leaves*

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Manuscript received- February, 2023; Accepted- December, 2023

DOI: https://doi.org/10.54548/njps.v39i1.13

INTRODUCTION

Cognitive alteration is a very serious global health issue with neuropsychiatric and neuro-degradative ailments such as Alzheimer's disease (AD) which are debilitating in nature (Commenges *et al*., 2000). Neuroprotection is the process and relative mechanisms that protect the central nervous system upon neuron injury which may be as a result of vehement (stroke or trauma) or habitual nervous tissue ailments for example, AD (Kummar *et al*., 2006). Alzheimer's ailment is a habitual neuro-degrading malady defined analytically by mental and recall loss, with extracellular amyloid plaques and intracellular neurofibriliary entangles as it classical ensign pathologies (Zheng *et al*., 2007 and Lee *et al*., 2001). In addition, short-term and long-term memory among others is affected.

The universal incidence of nervous ailment from 60 years and above ranged from 5 to 7% (Prince *et al*., 2013). Akter *et al*.,(2012) inferred that AD is the most habitual kind of dementia followed by arteriovenous dementia. Moreover, there is scanty knowledge on the frequency of dementia and its sub-types in the sub-Saharan Africa (Paddick *et al*., 2013; Prince *et al*., 2013; Ferri *et al*., 2005) with extensive hints on public health policies. While certain reports disclosed minimal happenings of dementia in sub-Saharan Africa (Prince *et al*., 2013; Paraiso *et al*., 2011; Yusuf *et al*., 2011), others disclosed happenings rates analogized with those in the Western countries (Paddick *et al*., 2013; Guerchet *et al*., 2009). The incidence of AD in the Yoruba Africans was 2 to 3 times low analogized compared to the African Americans (Hendrie *et al*., 1995). Studies also disclosed an apparent contrast in AD happenings between males and females in which two thirds of AD cases were females (Alzheimer's Association, 2017). In 2010, the impact on health care budget revealed that dementia cost $ 800,000,000,000.00 and may hit $1,000,000,000,000.00 by 2018 worldwide (Prince *et al*., 2016), which is far more than what most developing countries faced with economic strife can afford. Also, there is also emotional as well as physical stress brought to the family of the AD patient (Sugimoto *et al*., 2000).

In the past 20 years, nutritional neuroscience came up with significant contributions to our understanding of the relation between nutrition and cognitive physiology (Gillette *et al*., 2007), where medicinal herbs such as *Talinum Triangulare* as a choice of preventing AD became supreme to multiple researchers. *Talinum Triangulare* popularly referred to as water leaf, is a common plant in our locality and grows in maximum part of Nigeria. This herb is known to contain antioxidants, antidiabetic, antibacterial, anti-inflammatory and antifungal qualities (Nkosi *et al*., 2006). The plant is documented to possess neuroprotective effects as AD influence largely the cerebral cortex and the hippocampus with loss of mass and atrophy as the disease advances (Apostilova *et al*., 2012). Moreover, neurodegenerative disease is ascribed to oxidative weight caused by the formation of disenthrall oxidants resulting to cell destruction by changing large molecules in the cell (Lim *et al*., 2001). The antioxidants in the cell and that from the plants such as *Talinum Triangulare* helps in neutralizing the extra disenthrall radicals, protect the cell from poisons and also interpose to obviate disease (Pharm-Huy *et al*., 2008). The disenthrall oxidant searching attribute of *Talinum Triangulare*, due to the elevated amount of polyphenols (Oboha *et al*., 2012) may provide a safe preference; hence, the need to investigate the efficacy of aqueous extract of *Talinum Triangulare* leaves using cognitive dysfunction rats in the present study.

MATERIALS AND METHODS

Animal breeding: In line with the principles of laboratory animal care (NIH publication NO. 85-23, revised 1985) including specific national laws applied, ethical approval (Approval No.: FAREC-FBMS 042ANA3719) was gotten from the Faculty of Basic Medical Sciences, College of Medical Sciences, University of Calabar, Calabar, Nigeria. A total of fifty four adult Wistar rats (female and male) with lade between 180-200g were obtained from the University of Calabar animal farm. The rats were housed for two weeks at standard conditions of temperature (27oC – 30oC) for acclimatization in animal house located in the Department of Human Anatomy, University of Calabar. Rat chow manufactured by the Agro Feed Mill Nigeria Ltd, Calabar and drinking water were used to feed the animals. The rats were haphazardly divided into five arrays; each array with six rats labelled A, B, C, D and E while the lingering twenty-four rats were used to establish the LD50 after acclimatization.

Plant extract preparation: Fresh water leaves (*Talinum Triangulare*) were bought from the Watt market, Calabar, Cross River State, Nigeria. In the Department of Botany, University of Calabar, the fresh *Talinum Triangulare* was registered (voucher number: HERB/BOT/UCC/332), acknowledged, and authenticated. In order to free debris, the leaves were washed and air-dried in the laboratory. With the use of a mixer grinder (model number Bravo3JARS),the dried samples were merged into powder (1600g) and macerated in 1000 mL of distilled water for 24 hours. With the use of a Whatman No.1 filter paper and chess cloth, the mixture was then filtered. A man-made vacuum (model number F.NR:1508.0271) was then used to concentrate the solution obtained. The syrupy residue was kept in a cool dry place for later use at 40-50°C.

Alzheimer's type cognitive dysfunction induction: The induction of Alzheimer's type cognitive dysfunction was done through ip administration of 1.0mg/kg body weight of scopolamine hydrobromide (SHB) to the rats in arrays B, C, D and E for seven days.

LD_{50} determination: Twenty-four rats were used to establish the LD50. According to the Lorke's method, the LD_{50} of ethanol extract of *Talinum Triangulare* was established to be >7000mg/kg. Using 12.5% (875mg/kg) and 25% (1750mg/kg) of the established LD50 (7000mg/kg body weight of aqueous extract of *Talinum Triangulare*), the dosage of ethanol extract administration was determined.

Donepezil administration and plant extract: Group A(negative control) was given animal chow and water ad libitum; array B received 1.0mg/kg body weight of SHB only to become the positive control; array C was given 1.0mg/kg body weight of SHB and 1.0mg/kg body weight of Donepezil; 1.0mg/kg body weight of SHB and 875mg/kg body weight aqueous wring of Talinum tiangulare was administered to group D and array E was given 1.0mg/kg body weight of SHB and 1750mg/kg body weight aqueous wring of Talinum tiangulare for fourteen days.

Histological method: The animals were immolated with their brain tissues perfused and treated24 hours after the last administration. The whole brain was eviscerated, weighed and fixed in 10% buffered formal saline. This was done to maintain the morphological integrity of the tissue (Williams *et al*., 2006). The hippocampus of the rats was dissected out and used for histological studies using the haematoxylin and eosin (H & E) staining method and observed under light microscope (OMAX: 40X-2500X). During tissue processing, the hippocampal tissue was dehydrated through ascending percentage of alcohol for an hour each. The tissue was cleared in two changes of xylene for an hour each, infiltrated and embedded in molten paraffin wax. The solid tissue blocks in paraffin were mounted in the rotator microtome and sections were cut at six µm. The cut sections were floated in warm water bath, then picked and mount with an albumenized slide. Paraffin slides of the hippocampal tissue were de-waxed of paraffin through two changes of xylene for 5 minutes each, rehydrated through ascending percentage of alcohol and rinsed under ceaseless tap water. For 15 minutes, cut tissues were later dyed with haematoxylin and rinsed under ceaseless tap water for 5 minutes. For a minute, cut tissues were distinguished in acid alcohol, blued and counter stain with Eosin for another 1 minute. Sections were rinsed in tap water, dehydrate and cleared in xylene, allowed to dry with few drops of distyrene plasticizer xylene (DPX) kept on the slide and cover slipped (Avwioro, 2010).

Immunohistochemical procedure: Sections of serial paraffin (5 µm thick) were deparaffinized and dehydrated. For 30 minutes, 0.05% hydrogen peroxide was used to block the endogenous peroxidase activity. At a pH of 7.4for 5 minutes, the slides were washed in phosphate-buffered

saline. In a 0.01M citrate buffer (pH 6) in a microwave for 5 minutes, sections were later placed. At 37oC, in 30 minutes, the slides were incubated in 1% Bovine serum albumin for. At room temperature for 90 minutes, two drops of antibodies {Glial fibrillary acidic protein (GFAP) and the anti-mouse immunoglobulins conjugated to a peroxidase-labelled dextran polymer} were applied to the sections and then incubated in 3,3'-diaminobenzidine for 15 minutes. With Mayer's haematoxylin and observed under light microscope, the slides were counterstained, dehydrated, cleared and mounted with DPX.

Novel Object Recognition Task: The novel object recognition task {(NORT) a relatively high-through-put, big and delicate course for assessing composites for cognition-accentuating action} in rat, is a facile assay for cognitive physiology.

Apparatus: The NORT utilizes the equipment for open field maze. This consists of a square area surrounded by high walls. The size of the area was large (50 x 50 cm). To prevent reflections, the walls were white and the bed of the field marked with blue lines that divide the barony into equivalent diminutive squares which was good for manual scoring} concealed with plexi-glass, with the equipments domiciled on the ground. With red line (centre square), a square barony in the very midpoint of the barony was also drawn. To permit animals to observe and familiarize their environments, the laboratory area was sufficiently but dimly lit while negating stress from bright lights. Two coequal materials of coequal colour, texture and size were gotten and new material, different from the coequal materials also gotten. Rats were picked and returned to their cages at the end of the ten minutes. To prevent odour being used as a cue, methylated spirit was accustomed to absolutely perfect the open field barony. After 3-5 minutes breach, a new rat was innovated into the field and data recorded after each test.

Statistical analysis: Data were analyzed using the ANOVA with the statistical package for social science version 22. A two-way post hoc test used in multiple comparisons with results expressed as mean ± SEM at $p < 0.05$.

RESULTS

Histological results: Sections of the hippocampus in groups B, C, D and E showed atrophied pyramidal cells, hyperchromatic and numerous glial cells with pale cytoplasmic inclusions. Some cell membranes were disrupted, indicating gliosis. Hypervacuolations were also seen (Plate 1 B-E). These effects were more in group B (Plate 1B). whereas group D showed strong ameliorative potentials of aqueous extract of *Talinum Triangulare* (plate 1D).

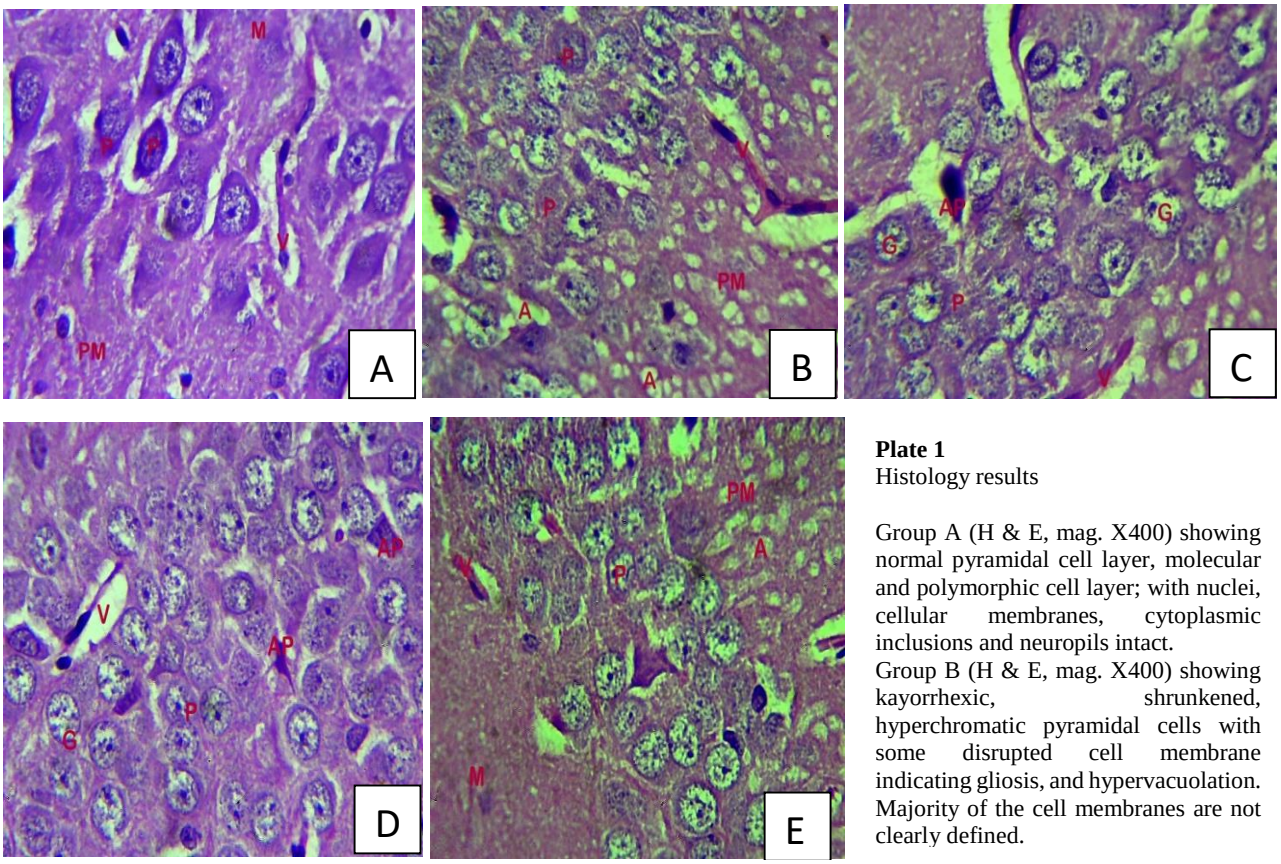

Plate 1
Histology results

Group A (H & E, mag. X400) showing normal pyramidal cell layer, molecular and polymorphic cell layer; with nuclei, cellular membranes, cytoplasmic inclusions and neuropils intact.
Group B (H & E, mag. X400) showing kayorrhexic, shrunkened, hyperchromatic pyramidal cells with some disrupted cell membrane indicating gliosis, and hypervacuolation. Majority of the cell membranes are not clearly defined.
Group C (H & E, mag. X400)showing kayorrhexic and atrophied pyramidal cell with hyperchromatic staining. There are vacuolations in the polymorphic layer filled with lipids. Majority of the cell membranes are not clearly defined.
Group D (H & E, mag. X400) showing mild atrophic, shrunken and hyperchromatic pyramidal cell with marked pale inclusions in the PM layer.
Group E (H & E, mag. X400)displaying kayorrhexic shrunken, hyperchromatic and atrophied pyramidal cells with numerous neuropils. Majority of the membranes are not clearly defined. Polymorphic layer is hypervacuolated, filled with lipids.

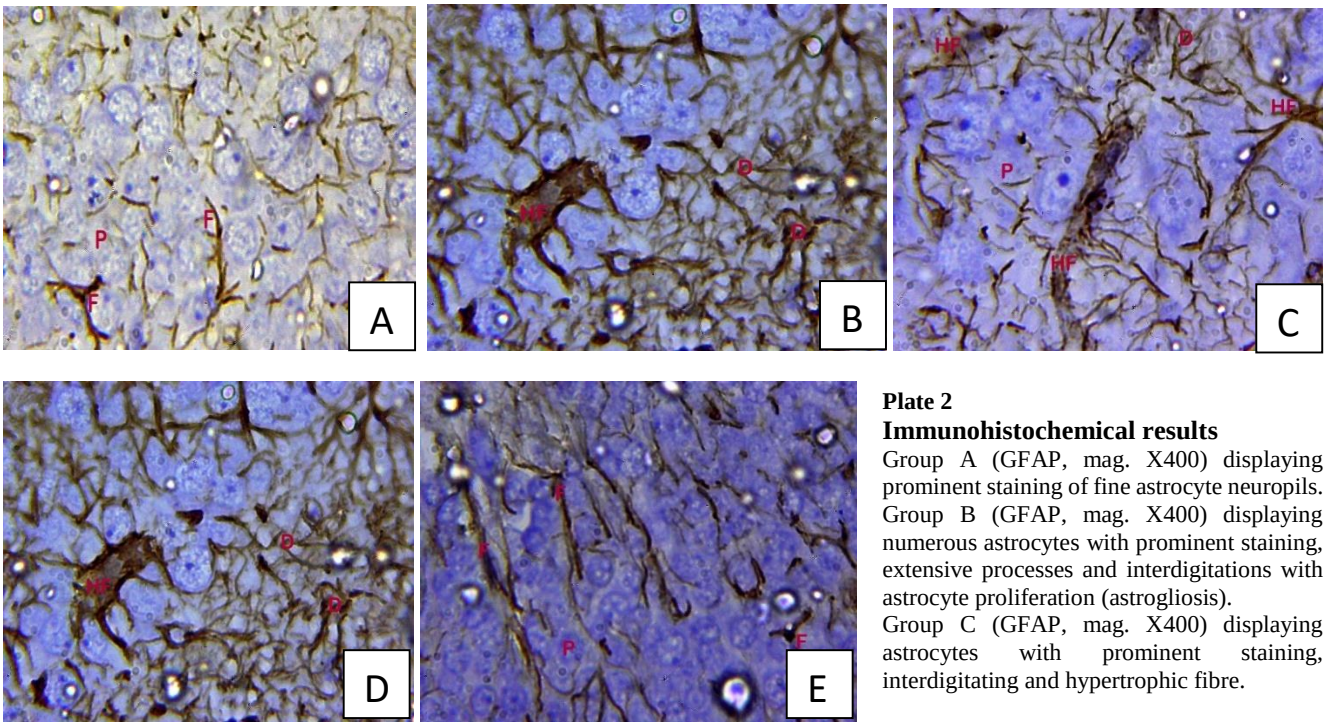

Plate 2
Immunohistochemical results
Group A (GFAP, mag. X400) displaying prominent staining of fine astrocyte neuropils.
Group B (GFAP, mag. X400) displaying numerous astrocytes with prominent staining, extensive processes and interdigitations with astrocyte proliferation (astrogliosis).
Group C (GFAP, mag. X400) displaying astrocytes with prominent staining, interdigitating and hypertrophic fibre.
Group D (GFAP, mag. X400) showing numerous astrocytes with prominent staining, extensive processes and interdigitations and hypertrophic fibre. There is astrocyte proliferation (astrogliosis).
Group E (GFAP, mag. X400) displaying prominent staining of fine astrocyte processes and fibres. There is astrogliosis.

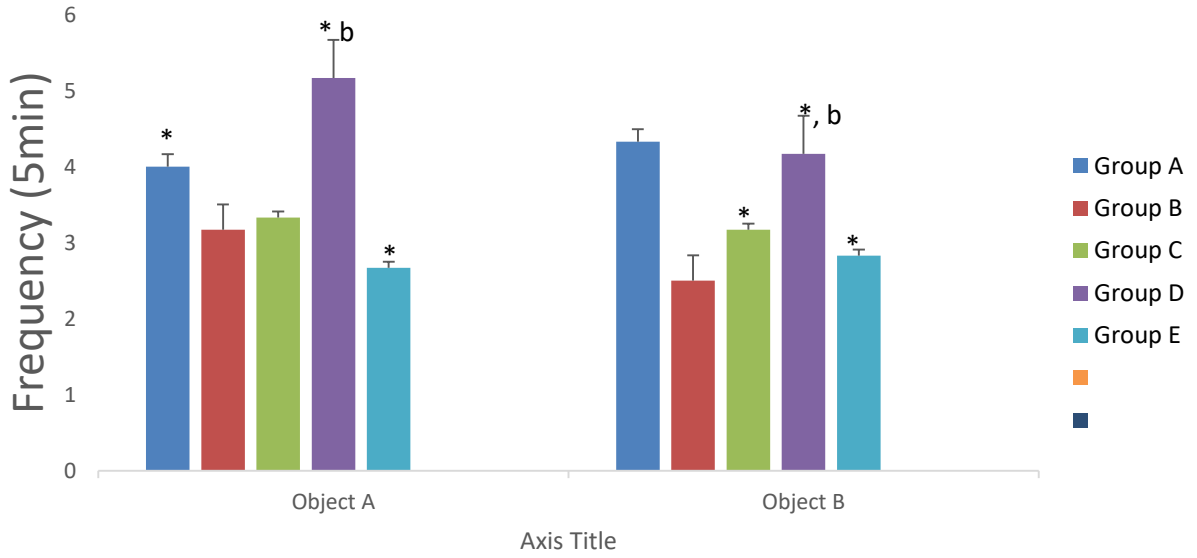

Figure 1:
Comparison of frequency of line crossing in the different experimental groups during the Novel Object Recognition Task
Values are set as mean ± SEM, n=6.
**= meaningfully variant SBB at p<0.05, b= meaningfully variant Donepezil at p<0.05*

Immunohistochemical results: The immunohistochemical sections revealed densely stained numerous astrogliosis with hypertrophied inter-digitizing fibres (Plate 2 B-E) compared to the negative control group (Plate 2A). These reactive astrocytes were less in groups C and E (plates 8 and 10) indicating pyramidal cell repairs.

Novel object recognition task: Neurobehavioral study comparing frequency of line crossing during habituation periods for object A and object B are: 4.00±0.52 and 4.33±0.71 (control group A); 3.17±0.01 and 2.50±0.89 (group B); 3.33±0.42 and 3.17±0.75 (group C); 5.17±1.05 and 4.17±1.01 (group D) and 2.67±0.33 and 2.83±0.31 (group E) (Figure1). In the object A prevalence of probationary test, array B was meaningfully decreased when compared with group A while the group E was meaningfully reduced analogized with the groups A to D. Group D was meaningfully elevated analogized with group A, B, C and E ($p<0.05$). Whereas, the object B prevalence of probationary test showed that group B was meaningfully decrease analogous to the control group at $p<0.05$ (Fig. 1).

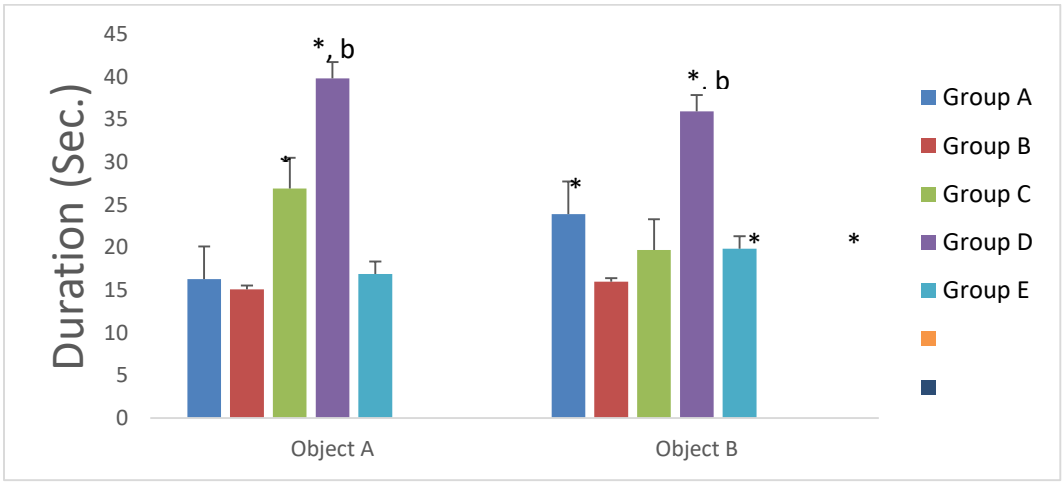

Figure 2:
Frequency of exploration test during habituation period (trial 1, day 1)
Values are seen as mean ± SEM, n=6
**= meaningfully variant control at p<0.05, b= meaningfully variant SHB at p<0.05*

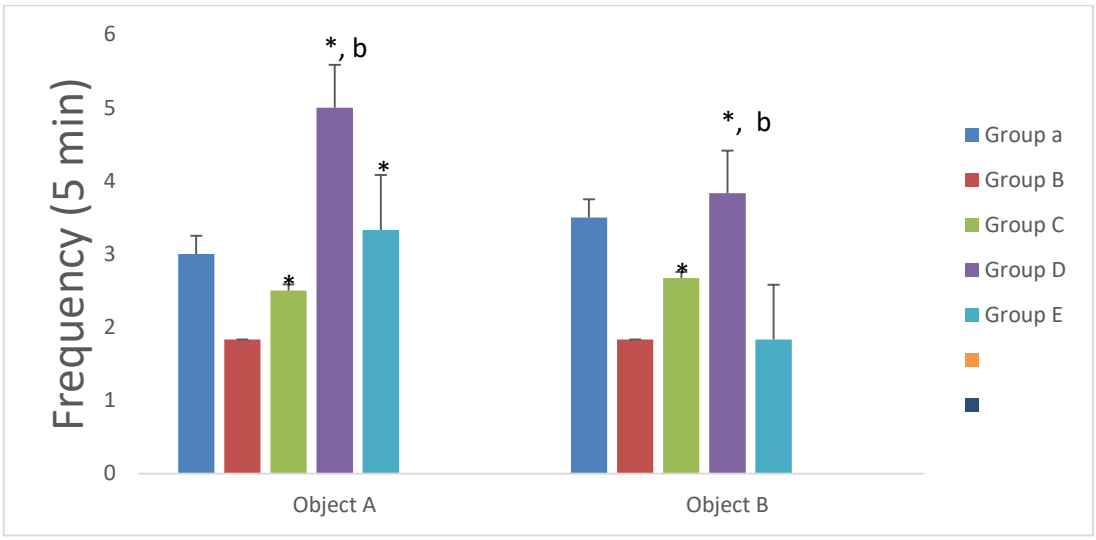

Figure 3:
Frequency of exploration test during the test period (trial 2, day 1)
Data are shown as mean ± SEM, n=6
**= meaningfully variant SHB at p<0.05, b= meaningfully variant Donepezil at p<0.05*

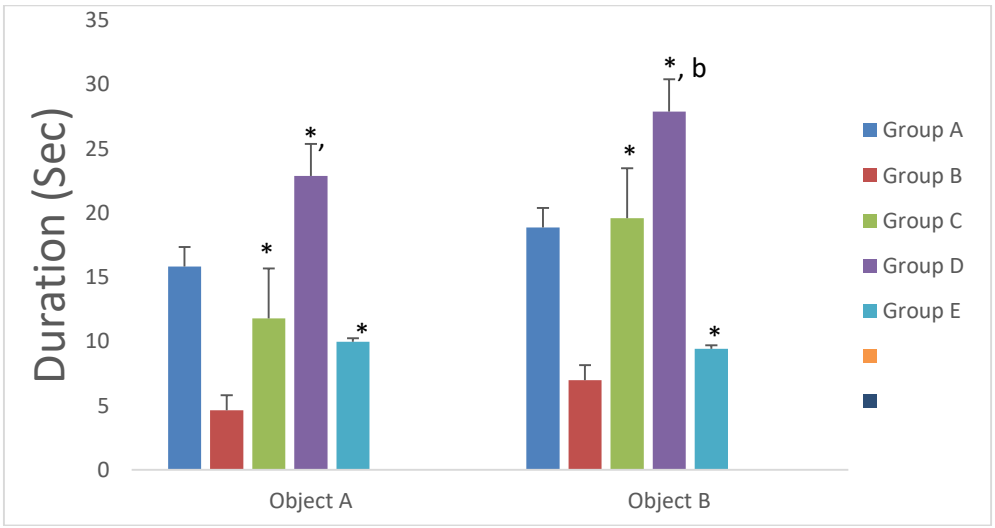

Figure 4:
Duration of exploration in NORT during trial 2, day 1(test period)
**=meaningfully variant SBB at p<0.05, b= meaningfully variant Donepezil at p<0.05*

The mean values from the duration of probationary test for object A and B during habituation period (trial 1, day 1) are: 16.31±3.66 and 23.92±6.14 (control group A); 15.11±5.29 and 15.98±5.40 (group B); 26.92±5.33 and 19.71±4.80 (group C); 39.82±8.88 and 35.96±8.74 (group D) and 16.90±3.59 and 19.85±4.32 (group E). The duration of exploratory test in object A revealed that array D was meaningfully raised comparable to other groups. The duration of probationary test in material B disclosed that array D was meaningfully raised analogous to other groups as shown in figure 2($p<0.05$). The mean values from the prevalence of probationary test for object A and B (trial 2, day 1) are: 3.00±0.58 and 3.50±0.43 (control group A); 1.83±0.70 and 1.83±0.79 (group B); 2.50±0.43 and 2.67±0.21 (group C); 5.00±0.86 and 3.83±0.87 (group D) and 3.33±1.02 and 1.83±0.48 (group E) trial 2 day 1. The prevalence of probationary test for object A in trial 2 day 1 displayed that group D was meaningfully elevated analogous to other arrays ($p<0.05$). The prevalence of probationary test for material B displayed that array D was meaningfully higher compared to array B and C with no meaningful variations between group E and group B (figure 3). The values of the duration of Novel Object Recognition Task for object A and B in trial 2 day 1 are: 15.82±2.57 and 18.86±1.95 (control group A); 4.63±1.78 and 6.97±2.38 (group B); 11.78±2.85 and 19.57±3.59 (group C); 22.86±3.35 and 27.88±7.19 (group D) and 9.96±1.03 and 9.41±2.48 (group E). The fate of period during NORT for object A revealed that array B was meaningfully reduced compared to array A while array D disclosed meaningfully elevation comparable to array A and B. Array E was meaningfully elevated when analogized with group B and meaningfully reduced when compared with array A. The continuance in array C was meaningfully longer analogous to array B (figure 4).The result of continuance of the NORT in material B disclosed that array D was meaningfully elevated when analogous to array B ($p<0.05$) and array E showed no meaningful decline when analogous to array B ($p<0.05$) and group E showed meaningful decrease when analogized with group D. Also, array C showed meaningful elevation compared with array B and group E as shown in figure 4($p<0.05$.

DISCUSSION

In rodents, the use of scopolamine to cause cognitive dysfunction is a recognized process employed to unclog inquiry and the output of aggrandize for Alzheimer's disease (AD) including other ailments (Nitta, 2002). The cognitive blemish conjugated with scopolamine hydrobromide (SHB) is cognate to that in AD. Following intraperitoneal injection of SHB, the cholinergic neurotransmitter is barred, causing cholinergic malfunction and cognitive remodelling in rats (Oh *et al*., 2009). Fan *et al*., (2005)displayed that SHB caused remembrance remodelling, is conjugated with blemished brain oxidative trouble status, this may be ameliorated with the administration of *Telfairia occidental* seeds aqueous extract at different doses (Eru *et al*., 2020a & 2021b). While B-D-glucagon polysaccharide suppresses glial cell aggregation, reduce cell count and sizes and volume in hyperglycaemic rodents (Agbor *et al*., 2022); and Citrus esculentus extract reversed radiation-induced damaged on the ultrastructure of albino Wistar rats' testes (Udoh *et al*., 2020), Averrhoa carambola aqueous fruit extract ameliorates effects of diazepam on rats hippocampus (Anani *et al*., 2020) and Ziziphus jujube fruits extract offer protective influence on oxidative brain damage and reduce the activity of AChE in hypothyroid rat brain tissues (Mohammed, 2021). In the present study, rats with SHB-induced memory remodelling were used to examine the capability of aqueous extract of *Talinum Triangulare* on its hippocampus.

Loss of cellular integrity, degeneration of cells and cellular vacuolations in the hippocampus with atrophic and kayorrhexic cells were observed in group B treated with SHB alone in the present study. These histological changes imply cellular damage which may account for the poor performance observed in the neurobehavioral tests. These features are similar to Eru *et al*., (2022, 2021a, 2021b, 2020a, 2020b); Deb *et al*., (2005) where scopolamine caused obvious impairment of recall in behavioural tests which correlate with the histomorphological alterations in the hippocampal rats. The exact mechanism responsible for this degeneration is presently not clear but may be due to oxidants formation as oxidative troubles cause neurons injury (Zou *et al*., 2015). Reports have it that SHB triggers the induction of oxidants and cause free radical injury (Fan *et al*., 2005: Lin and Beal, 2006). The hippocampus plays a good part in cognition, mood regulation, and response to stress, learning and memory (Balu and Lucki, 2009). Oxidative stress affects the hippocampus mostly (Candelario-Jalil *et al*., 2001). These degenerative changes may lead to hippocampal dysfunction characterized by impaired study and recall (Khakpai *et al*., 2012).

The cytoarchitecture of groups C, D and E showed mild effects analogous to array B treated with SHB. In our study, all the test arrays were able to ameliorate insults inflicted by SHB on pyramidal cells of the hippocampus. A study has it that loss of neurons, gliosis, swollen or destroyed axons and myelin sheath are characteristics of chemically induced neurodegeneration (Cavanagh, 1984). This is true because the neurodegenerative disease caused by SHB in group B showed atrophied pyramidal cells and numerous vacuolations filled with lipids (plate 2). The normal integrity of the soma as well as it processes is very important for normal nervous system. When the soma is injured, different degenerative changes occur because of obstacle in blood flow leading to ischemia and hypoxia, crushing of nerve fibres and injection of toxic substances/chemicals (Abbas and Nelson, 2004). In the hippocampus, the cornus amonis 1 (CA1) and cornus amonis 2 (CA2) subfields are vulnerable to cell injury (George and Schneider, 1998) which is in line with the pyramidal cells in group B, C, D and E that showed atrophied pyramidal cells, loss of plasma membranes and pale staining cytoplasm of the glial cells. The present study also confirmed involvement of pyramidal cells found in the pyramidal layer as the degenerative changes observed in the hippocampus were predominantly evident mostly in the experimental groups that received SHB. These degenerative changes may lead to dysfunction of the hippocampus characterized by inability to establish new memory. Furthermore, the distorted cytoarchitecture of the hippocampus observed was mild in the experimental group C, D, and E analogous to the positive control. The observed output showed dose affiliated format of cellular

restoration with array E having the most enhanced capabilities from the aqueous extract of *Talinum Triangulare* on the hippocampal pyramidal cells which could improve studying and recall. This agrees with David *et al*., (2008) who reported that *Talinum triangulare* has beneficial outputs on cerebral nerve cells. These ameliorative potentials could be ascribed to the elevated polyphenols (antioxidants) component in the plant which according to Pharm-Hey *et al*., (2008), could help to expunge excess free radicals, avert cell poisons as well as avert more harm from the SHB.

Immunohistochemical staining displayed little GFAP positive astrocytes in the hippocampus of array A rats. In contrast, astrogliosis and hypertrophied fibres were boldly elevated in the hippocampus of the SHB administered array. This corroborates Anderson *et al*., (2016) who documented that certain growth of astrocytes were observed after injury when the reactive reply is to preventive scar within the wound. The reactive astrocyte from other arrays administered with donepezil and *Talinum triangulare* were boldly elevated in the hippocampus when analogized with the negative control and reduce astrogliosis when analogized with the positive control administered with SHB.

The astrocytes (characteristic star-shaped cells in the brain and spinal cord called) have numerous functions including making available comprehensive structural, metabolic and trophic assistance to neurons (Sofroniew and Vinters, 2010; Verkhratsky and Butt, 2013) and regulate regular synaptic transmission (Halassa and Haydon, 2010). In response to damage inflicted on the central nervous system, astrocytes counter from their normal quiescence mode to a so called reactive mode. This headway of reactive gliosis is noticed by structural modifications (hypertrophy), physiological remodelling and absolute elevation in the expression of astrocyte specific intermediate filament and GFAP (Middeldop and Hol, 2011). The reactive astrocytes present in the nervous tissue indicate the early stage of neuronal cell loss (Abbas and Nelson, 2004). A drastic elevation in GFAP-positive astrocytes was seen in SHB administered array analogous to the control animals in the present study. Consequently, the investigation of GFAP is a sign for comprehending neurodegenerative alteration. Gliosis that happens in the scopolamine treated group could be caused by the creation of free radicals. Nerve cells are exactly susceptible to oxidative destruction due to their increased polyunsaturated fatty acid inclusion in cell envelops, increased oxygen devour and asthenic antioxidant aegis (Rego and Oliveira, 2003). Antioxidants may minimize such a reactive gliosis by decreasing the dangerous influence of reactive oxygen species in the central nervous system (Bell *et al*., 1995).

Immunohistochemical result from the present research displayed less expression of GFAP positive reactive astrocyte in group C to E. Reactive astrocytes chaperone all immediate wound and gradual neurological ailment. Reactive astrocytes while aiding cellular brace to beget axons (Anderson *et al*., 2016), can as well impair axon revitalization (Silver and Miller, 2004). When antioxidants are consumed either in diets or medications, it will neutralize reactive oxygen species from the living system and give health benefits. *Talinum Triangulare* leaves have been reported to possess antioxidants and minerals that help brain physiology. The depletion in GFAP expression cells in these experimental arrays may be ascribed to the antioxidant capabilities of the plant aqueous extract used in the present study. Studies have it that medicinal plants are rich source of antioxidant components such as flavonoids, quinones, phenolics, alkaloids and vitamins C which can reduce the rate of occurrence of oxidative stress and related disease (Zhang *et al*., 2011), *Talinum Triangulare* extract contain many of these compounds.

Neurobehavioural parameter revealed that animals in array B administered SHB alone, displayed less prevalence and continuance in examination of new recognition analogous with array A that was fed with animal chow and water ad libitum. This indicates memory impairment as recall loss is generally the precocious signal of Alzheimer's illness. This result is similar to Mugwagwa *et al*., (2015) who reported SHB caused recall impairment in long duration recall novel object recognition performance. In the current research, animals in arrays D and E revealed increased prevalence and continuance of examination of new material. This demonstrates that aqueous extract of *Talinum triangulare* may have memory-aiding activity against scopolamine-induced memory impairment. Our findings support David *et al*., (2008) who reported that *Talinum triangulare* possess favourable results on neurons of the cerebral cortex and may enhance the cognitive ability in Swiss albino rats. Consequently, data collected from NORT displayed that SHB induce studying and recall inhibition. This may be due to oxidative trouble from SHB, evidenced in the histological alterations and astrogliosis in the hippocampus of the Wistar rats.

In conclusion, aqueous extract of *Talinium triangulare* reduced atrophied cells and astrogliosis with enhanced spatial learning and memory in scopolamine induced-Alzheimer type cognitive rats in the present study. Although, the potentials of aqueous extract of *Talinium triangulare* were dose dependent.

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Niger. J. Physiol. Sci. 39 (June 2024): 111 – 118
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Effect of Aqueous Extract of *Hibiscus Sabdariffa* on Cadmium Chloride-Induced Neurotoxicity in Male Wistar Rats

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Summary: This study investigated the neurologic effect of aqueous extract of *Hibiscus Sabdariffa* on cadmium chloride-induced neurotoxicity in Wistar rats. Thirty male Wistar rats were grouped accordingly; Group A: control, Group B-F: CdCl2 0.3ml; *H. Sabdariffa* 1.5ml; CdCl2 + *H. Sabdariffa* 1.0ml; CdCl2 + *H. Sabdariffa* 1.5ml; CdCl2 + *H. Sabdariffa* 2ml, respectively. Gas chromatography coupled to flame ionization detector (GC-FID), total antioxidant capacity (TAC) and mineral analysis of *H. Sabdariffa* was done to reveal the bioactive agents in the plant sample. Oxidative stress, muscle function markers and plasma electrolytes were assayed. Phytochemical screening revealed alkaloids, phytate, anthraquinone and flavonoids. The mineral analysis revealed predominantly, Mn, Ca, K and Fe. The biochemical results were in comparison with control and statistically significant at 95% confidence interval. There was an increase in SOD, CAT, GSH, GR and GPx in groups C-F, MDA decreased in group C, E and F while PC decreased in group C and F. There was a decrease in plasma creatine kinase in groups C to F and increase in AChE in group C. Ca and K levels had no significant change ($P<0.05$). Nitric oxide level had no significant change in group C, E and F, but increased in group D. This study demonstrated that *H. Sabdariffa* has the potential to ameliorate the neurotoxicity caused by CdCl2 in Wistar rats.

Keywords*: Neurotoxicity; Hibiscus sabdariffa; cadmium; neuromuscular function; phytochemicals*

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Manuscript received- February 2024; Accepted- May 2024

DOI: https://doi.org/10.54548/njps.v39i1.14

INTRODUCTION

Cadmium (Cd) is a toxic, nonessential transition metal that is classified as a human carcinogen (Wang and Du 2013; Viaene, 2000). Over the years, many different forms of exposure to cadmium have been identified, with cadmium being present in the environment as a result of many human exploratory activities (Mehrdad *et al*., 2017). The constant sources of cadmium contamination are related to its industrial application as a corrosive reagent, as well as its use as a stabilizer in polyvinyl chloride (PVC) products, color pigments, and Ni-Cd batteries (Genchi, *et al*., 2020). In areas with contaminated soils, house dust is a potential route for cadmium exposure (Janneke, *et al*., 2007). Cadmium produces neuropathological and neurochemical alterations in central nervous system (CNS), leading to cognitive dysfunction (Sola, *et al*., 2022). Over the years, interest in food diversity for improved health and nutrition has increased in favor of plant-based natural food products with high bioactive compounds. *Hibiscus Sabdariffa* is a plant of the Malvaceae family, commonly known as roselle (Salem, *et al*., 2021; Lucresse, *et al*., 2019). Also called karkade, bissap, sobolo or zobo, depending on the region, *H. Sabdariffa* is prepared as a traditional drink in many African countries. The flowers are mainly used, reputed for their thirst-quenching and relaxing properties (Lucresse, *et al*., 2019). *H. Sabdariffa* has been used as a traditional medicine for various ailments, such as hypertension (Ellis, *et al*., 2022; Koch, *et al*., 2019), liver disorders (Ellis, *et al*., 2022), inflammation (Alshami and Ahmed, 2014), diabetes (Ahmed and Abozed, 2015) and cancer (Koch, *et al*., 2019). Due to an increase in prevalence of neurological disorders, there is an urgent need to discover complementary source of medicines to reduce the challenges in availability or cost related to conventional medicines. The aim of this study is to investigate the neurologic effects of aqueous extract of *Hibiscus Sabdariffa* on cadmium chloride-induce neurotoxicity in male Wister rats

MATERIALS AND METHODS

Ethical approval: This study was approved by the Ethical Considerations Committee, Directorate of Research and Human Development, Madonna University, Elele, Rivers State, Nigeria. The reference Number is MUECC/20230148.

Apparatus and Equipment: Surgical latex gloves, oral gavage/cannula, syringe, lithium heparin bottles, cotton wool, micro hematocrit tube. Aluminium cages and bedding, feeding troughs, orogastric tubes, plain sample bottles, test tubes of various sizes, dissecting kit, dissecting board, water bath, medicated soap, desiccator bottle, slides and cover slips and electronic weighing scale.

Chemical agents: Cadmium chloride (CdCl2), normal saline, distilled water, Diethyl ether, Methylated Spirit, Heparin. The concentration of CdCl2 was 2mg/ml.

Feed: The feed used was the pelleted Top Feed®

Plant collection: The calyx of *H. Sabdariffa* used for this study was obtained from the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Madonna University, Elele, Rivers State, Nigeria.

Extraction Procedure: Dried *Hibiscus Sabdariffa* calyces (flowers) were obtained from Madonna University Botanical Garden. A measure quantity of 100g of the calyces was grinded into smaller pieces in order to facilitate extraction and was added to 70cl of distilled water in a ratio of 1:7 and was allowed to boil for 35 minutes. After heating, the mixture was allowed to cool to room temperature and then strained using filter paper to separate the liquid extract from the solid plant material which was then stored in a refrigerator daily after administration.

Phytochemical Screening: The Phytochemical Screening to determine the different bioactive constituents of *H. Sabdariffa* was done by Gas Chromatography Coupled to flame ionization detector (GCFID) using standard procedure by Lee and Harnly (Lee and Harnly, 2005), Total Antioxidant Capacity (TAC) using standard procedure by Narendhirakannan and Rajeswari (Narendhirakannan and Rajeswari, 2010), and mineral analysis was performed by Atomic Absorption Spectroscopy (AAS).

Table 1:
The design of this study

Groups	Treatments
A	Feed and water only
B	$CdCl_2$ 0.3ml
C	*H. Sabdariffa* 1.5ml
D	$CdCl_2$ + *H. Sabdariffa* 1.0ml
E	$CdCl_2$ + *H. Sabdariffa* 1.5ml
F	$CdCl_2$ + *H. Sabdariffa* 2.0ml

Animal protocol: A total of thirty (30) male Wistar rats weighing 150g to 170g were sampled. They were purchased and housed in Madonna University Biomedical Research Animal House, Faculty of Basic Medical Sciences; in aluminum cages and were allowed to acclimatize for 2 weeks, exposed to 12/12 hours light/dark cycle. Feed and water were provided ad libitum. The rats were randomly grouped into 6, with each cage housing five rats. After 2 weeks of acclimatization, administration $CdCl_2$ and *H. Sabdariffa* commenced.

Study duration: After 2 weeks (14 days) of acclimatization, this study lasted for 4 weeks (28 days).

Sample collection: The animals were anaesthetized with diethyl ether before an incision was made in the cranial region, an incision was made in the scalp and the skull was opened after which the brain was carefully removed and placed on ice before homogenization.

Brain Tissue Homogenization: All animals were anaesthetized with chloroform (0.5 ml intraperitoneally) and transcardially perfused with normal Saline (0.9% NaCl) followed by 4% Paraformaldehyde (PFA) in phosphate buffer (PB; 0.1 M pH 7.4). The brains were removed from the skull and placed in normal saline for biochemical analysis. Oxidative stress markers and electrolytes were assayed.

Biochemical analysis

Assay for oxidative stress markers: The oxidative stress markers tested for include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH), protein carbonyl (PC) and malondialdehyde (MDA).

Assay for Superoxide dismutase: Using method by Rani, *et al*., 2013. The SOD assay measures the activity of superoxide dismutase (SOD) by inhibiting the autoxidation of adrenaline. Superoxide anions produced during adrenaline oxidation can turn it into adrenochrome, but SOD prevents this reaction by converting superoxide anions into hydrogen peroxide. The amount of adrenochrome formed is directly proportional to the SOD activity (Rani, *et al*., 2013).

Assay for Catalase: Using the method by Chin-Chan, *et al*., 2015. The assay of Catalase (CAT) activity is based on the measurement of the decomposition of hydrogen peroxide (H2O2) by CAT. CAT catalyzes the breakdown of H2O2 into water and molecular oxygen. The rate of decomposition of H2O2 can be measured spectrophotometrically by monitoring the decrease in absorbance at a suitable wavelength, directly indicating the CAT activity (Chin-Chan, *et al*., 2015).

Assay for Glutathione Peroxidase: Using the method by Saez and Nuria Están-Capell, 2014. The assay for Glutathione Peroxidase (GPx) activity is based on the measurement of the rate of glutathione (GSH) oxidation by hydrogen peroxide (H2O2) in the presence of GPx. GPx catalyzes the reduction of H2O2 and organic hydroperoxides using GSH as a cofactor, regenerating GSH in the process. The decrease in absorbance resulting from the oxidation of GSH can be measured spectrophotometrically and is directly related to GPx activity (Saez and Nuria Están-Capell, 2014).

Assay for Glutathione reductase: This was based on the method by Chatterjee, *et al*. 2009. Glutathione reductase together with its co-factor, NADPH, catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione wherein the oxidation of NADPH to NADP+ is monitored as a decrease in absorbance at 340 nm. This rate of decrease in absorbance is directly proportional to the

glutathione reductase activity in the sample because the enzyme is present at rate limiting concentrations (Chatterjee, *et al*. 2009).

Assay for Reduced Glutathione: Using Satish and Pal, 2015 method. The reduced form of glutathione comprised in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable yellow colour when 5,5 dithiobis-2-nitrobenzoic acid (Ellman's reagent) is added to sulfhydryl- compounds. The chromorphoric product resulting from the reaction of Ellman's reagent with the reduced GSH is measured at 412nm (Satish and Pal, 2015).

Assay for Malondialdehyde: This protocol is based on Esterbauer and Cheeseman, 1990 method and can be adapted depending on specific requirements and sample types. The assay of Malondialdehyde (MDA) is based on the reaction of MDA with thiobarbituric acid (TBA) under acidic conditions to form a chromophore that can be measured spectrophotometrically. MDA is a reactive carbonyl compound and a product of lipid peroxidation, making it a commonly used marker for oxidative stress and lipid damage (Esterbauer and Cheeseman, 1990).

Assay for Protein carbonyl: This protocol is based on Chong, *et al*. 2017 method. The assay for protein carbonyl content involves the detection of carbonyl groups that are formed on proteins through oxidative damage. Carbonyl groups are reacted with a suitable reagent, such as 2,4-dinitrophenylhydrazine (DNPH), to form stable protein hydrazones. The hydrazones are then measured spectrophotometrically, and the amount of protein carbonyl content is quantified (Chong, *et al*., 2017).

Assay for Muscle function markers

Assay for Creatine Kinase (CK): Using Shackebaei, *et al*. 2010 method. Creatine kinase (CK), present in the sample, catalyses the transfer of a high energy phosphate group from creatine phosphate to ADP. The ATP produced in this reaction is subsequently used to phosphorylate glucose to produce glucose-6-phosphate (G-6-P) in the presence of hexokinase. G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) with the concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate reduced (NADPH). The rate of formation of NADPH is monitored at 340 nm and is proportional to the activity of CK in the sample (Shackebaei, *et al*. 2010).

Assay for Acetylcholinesterase: Using Méndez-Armenta and Rios 2007 method. It is based on the reaction between thiols and chromogenic 5,5'- dithiobis-2-nitrobenzoic acid (DTNB) as it measures the formation of the yellow ion of 5-thio-2-nitrobenzoic acid (TNB) (Méndez-Armenta and Rios, 2007).

Assay for Nitric oxide: Using the method by Moncada and Higgs, 1993. The assay is a widely used colorimetric method to indirectly measure nitric oxide levels by detecting its stable reaction product, nitrite (NO2-) (Moncada and Higgs, 1993).

Electrolyte Assay: Calcium is measured using a Potentiometer. This method determines the potential difference that develops between the inner and outer phases of an ion selective electrode. The electrode is made of a selectively permeable material to calcium ion. The potential is measured by comparing it to the potential of reference electrode. Since the reference electrode has a constant potential, the voltage difference between the two electrodes is attributed to the concentration of ionized calcium in the sample 25. Determination of Serum Potassium Concentration were measured using Centronic GmbH kit via turbidimetric determination method by Hillmann et. al., 1967 and Tietz, 1976.

Statistical analysis: Data was expressed as mean ± SEM and ($P<0.05$) was considered statistically significant. Data collected from this study was analyzed using One-Way analysis of variance (ANOVA) and Post Hoc analysis with the aid of IBM®SPSS Version 21.0.

RESULTS

Phytochemical Analysis of *H. Sabdariffa:* Table 2 shows the results of phytochemical analysis using GC-FID. The concentrations of alkaloid, phytate, anthraquinone and flavonoids in *H. sabdariffa* are 140.512 ug/ml, 231.13 ug/ml, 191.230 ug/ml and 240.124 ppm. The results indicate that flavonoid is the most abundant phytochemical in *Hibiscus sabdariffa*.

Table 2:
Gas chromatography coupled to flame ionization detector (GC-FID) screening of *H. Sabdariffa*

Component	Concentration	Unit
Anthocyanins	132.101	ug/ml
Polyphenols	127.011	ppm
Tannins	3.421	ug/ml
Alkaloids	140.512*	ug/ml
Oxalates	28.120	ug/ml
Phytate	231.13*	ug/ml
Terpenoids	2.4540	ug/ml
Anthraquinone	191.230*	ug/ml
Flavonoids	240.124*	ppm
Phenols	134.245	ppm
Saponins	10.712	ug/ml
Glycosides	11.261	ug/ml
Steroids	12.120	ppm
Citric acid	12.781	ug/ml
Beta-carotene	15.3470	ug/ml
Malic acid	10.241	ug/ml
Flavonols	7.8931	ppm
Thiamine	2.252	ug/ml
Glycosides	6.452	ug/ml
Ribalinidine	12.4131	ug/ml

Key - * Major phytonutrients in GC-FID analysis of *H. Sabdariffa*

Total Antioxidant Capacity (TAC) of *H. sabdariff* : At 20mg/ml and 40mg/ml the absorbance of *H. Sabdariffa* was 0.58 and 0.71 and that of ascorbic acid, a well-known antioxidant which was used as a standard, was 0.123 and 0.245 respectively. Consequently, an increased concentration of *H. Sabdariffa* (100mg/ml) showed an absorbance of 0.150 indicating a positive correlation in the absorbance of *H. Sabdariffa* and Ascorbic acid. This

indicates that *H. Sabdariffa* has a potential antioxidant activity to ascorbic acid.

Mineral analysis of *H. Sabdariffa*: The results of mineral analysis of *H. Sabdariffa* (Table 4) show that there are high levels of Fe, K, Ca, Mn present in the extract.

Table 3:
Total Antioxidant Capacity of *H. Sabdariffa*

Concentration	Abs. of ascorbic acid	Abs. of *H. Sabdariffa*
20mg/ml	0.123	0.58
40mg/ml	0.245	0.71
60mg/ml	-	0.90
80mg/ml	-	0.120
100mg/ml	-	0.150

Table 4:
Mineral analysis of *H. Sabdariffa*

Mineral	Concentration (mg/kg)
Fe	88.12
K	91.20
Cu	0.10
Ca	102.40
Mg	21.23
Mn	110.61
Zn	0.10

Results from table 5, showed a significant increase (P≤0.05) in SOD in group C, D and F when compared to control and $CdCl_2$ group. There was also a significant decrease (P≤0.05) in SOD levels in Group B when compared to control. In comparison with CdCl group, there was a significant increase in group E. In comparison with group C, SOD levels in group B, D, E and F were significantly decreased. There was a significant increase (P≤0.05) in CAT levels in group C, E and F when compared to the control and $CdCl_2$ group. Also, there was a significant decrease (P≤0.05) in CAT levels in Group B and group D when compared to control. In comparison with Hibiscus group CAT levels in group B, D, E and F were significantly decreased. There was a significant increase (P≤0.05) in GPx levels in group C, E and F when compared to the control and $CdCl_2$ group and also a significant decrease (P≤0.05) in GPx levels in Group B ($CdCl_2$ 0.3ml) and group D when compared to control. In comparison with group C, GPx levels in group B, D, E and F were significantly decreased. The results also showed a significant increase (P≤0.05) in GR levels in group C, D, E and F when compared to the control and $CdCl_2$ group and a significant decrease (P≤0.05) in GR levels in Group B when compared to control. In comparison with group C, GR levels in group B, D, E and F were significantly decreased (P≤0.05).

Table 5 also showed a significant increase (P≤0.05) in GSH levels in group C, E and F when compared to control and $CdCl_2$ group and also a significant decrease (P≤0.05) in GSH levels in Group B and Group D when compared to control. In comparison with Hibiscus group, GSH levels in group B, D, E and F were significantly decreased(P≤0.05). There was a significant decrease (P≤0.05) in MDA levels in group C, E and F when compared to control and $CdCl_2$ group and also a significant increase in MDA level was also seen in group D when compared to control and a significant increase (P≤0.05) in MDA levels in Group B when compared to control. In comparison with Hibiscus group, MDA levels in group B, D, E and F were significantly increased (P≤0.05). Results showed a significant decrease (P≤0.05) in PC levels in group C and F when compared to the control and $CdCl_2$ group. There was also a significant decrease in PC level as seen in group D and E when compared to the $CdCl_2$ group and a significant increase (P≤0.05) in PC levels in Group B and group D when compared to control. In comparison with Hibiscus group, PC levels in group B, D, E and F were significantly increased (P≤0.05).

Results from Table 6 showed a significant decrease (P≤0.05) in Ca^{2+} levels in group B ($CdCl_2$ 0.3ml) when compared to control and Hibiscus group. In comparison with $CdCl_2$ group, Ca^{2+} levels in group C, D, E and F were significantly increased (P≤0.05). The results also showed a significant decrease (P≤0.05) in K^+ levels in group B ($CdCl_2$ 0.3ml) when compared to the control and Hibiscus group. In comparison with $CdCl_2$ group, K^+ levels in group C, D, E and F were significantly increased (P≤0.05). There was a significant increase (P≤0.05) in NO levels in group B ($CdCl_2$ 0.3ml) when compared to the control and Hibiscus group. There was a significant increase (P≤0.05) in NO levels in Group D when compared to control and Hibiscus group. In comparison with $CdCl_2$ group, NO levels in group C, D, E and F were significantly decreased (P≤0.05).

Table 5:
Changes in brain stress markers in response to treatments

Groups	SOD (U/g)	CAT (U/g)	GPx (U/g)	GR (U/g)	GSH (U/g)	MDA (mmol/g)	PC (mmol/g)
Control	12.00 ± 0.46^{bc}	2.86 ± 0.08^{bc}	2.26 ± 0.06^{bc}	2.20 ± 0.10^{bc}	1.40 ± 0.08^{bc}	1.16 ± 0.05^{b}	0.40 ± 0.05^{bc}
$CdCl_2$ 0.3ml	1.40 ± 0.10^{ac}	0.18 ± 0.06^{ac}	0.18 ± 0.04^{ac}	0.34 ± 0.12^{ac}	0.14 ± 0.02^{ac}	12.40 ± 1.77^{ac}	5.36 ± 0.09^{ac}
H. sabdariffa 1.5ml	15.14 ± 0.59^{ab}	5.70 ± 0.04^{ab}	8.26 ± 0.17^{ab}	5.82 ± 0.07^{ab}	5.46 ± 0.12^{ab}	0.09 ± 0.03^{b}	0.18 ± 0.09^{ab}
$CdCl_2$ + *H. sabdariffa* 1.0ml	8.46 ± 0.42^{abc}	2.78 ± 0.19^{bc}	1.74 ± 0.02^{abc}	1.34 ± 0.10^{abc}	1.22 ± 0.09^{bc}	1.80 ± 0.35^{b}	0.52 ± 0.06^{bc}
$CdCl_2$ + *H. sabdariffa* 1.5ml	12.34 ± 0.19^{bc}	4.48 ± 0.10^{abc}	3.20 ± 0.18^{abc}	2.62 ± 0.13^{abc}	3.78 ± 0.07^{abc}	0.35 ± 0.21^{b}	0.26 ± 0.04^{bc}
$CdCl_2$ + *H. sabdariffa* 2.0ml	13.94 ± 0.13^{abc}	4.72 ± 0.04^{abc}	4.48 ± 0.12^{abc}	$4.48\pm.18^{abc}$	4.54 ± 0.12^{abc}	0.12 ± 0.02^{b}	0.14 ± 0.02^{abc}
Total	63.28	20.72	20.12	16.80	**16.54**	**15.92**	**6.86**
Average	10.55	3.453	3.353	2.80	**2.76**	**2.65**	**1.14**

Key: [a](P≤0.05)-Significantly different compared to control group, [b] (P<0.05)-Significantly different compared to $CdCl_2$ group [c](P≤0.05) - Significantly different compared to *Hibiscus* group. Data represented as Mean ±SEM. P≤0.05 was considered significantly different.
SOD- superoxide dismutase ; CAT-catalase; GPx-glutathione peroxidase ; GR-glutathione reductase; GSH-reduced glutathione; MDA-malondialdehyde; PC-protein carbonyl

Table 6:
Changes in muscle function markers in response to treatments

Groups	CK-MM(U/L)	AChE (U/L)
Control	22.04±0.57[bc]	32.52±0.54[bc]
$CdCl_2$ 0.3ml	44.04±0.95[ac]	11.90±2.33[ac]
H. Sabdariffa 1.5ml	11.30±0.32[ab]	46.60±0.58[ab]
CdCl2 + *H. Sabdariffa* 1.0ml	19.82±0.45[abc]	10.88±0.69[ac]
CdCl2 + *H. Sabdariffa* 1.5ml	13.74±0.27[abc]	36.38±1.54[bc]
CdCl2 + *H. Sabdariffa* 2.0ml	12.34±0.39[ab]	51.14±1.65[abc]
Total	123.28	189.42
Average	20.55	31.57

Key: [a](P≤0.05)-Significantly different compared to control group, [b] (P<0.05)-Significantly different compared to $CdCl_2$ group [c] (P≤0.05) -Significantly different compared to *Hibiscus* group. Data represented as Mean ±SEM. P≤0.05 was considered significantly different.

Table 7:
Changes in electrolytes in response to treatments

Groups	Ca (mg/dl)	K (meq/L)	NO (µmol/L)
Control	5.06±0.07[b]	4.46±0.09[b]	0.21±0.15[b]
$CdCl_2$ 0.3ml	2.26±0.07[ac]	2.22±0.40[ac]	2.48±0.10[ac]
H. Sabdariffa 1.5ml	5.08±0.12[b]	4.38±0.06[b]	0.02±0.00[b]
$CdCl_2$ + *H. Sabdariffa* 1.0ml	5.14±0.15[b]	4.38±0.11[b]	1.18±0.16[abc]
$CdCl_2$ + *H. Sabdariffa* 1.5ml	5.10±0.08[b]	4.44±0.10[b]	0.0±0.00[b]
$CdCl_2$ + *H. Sabdariffa* 2.0ml	4.92±0.06[b]	4.62±0.06[b]	0.02±00[b]
Total	27.56	24.5	3.91
Average	4.59	4.08	0.65

Key: [a](P≤0.05)-Significantly different compared to control group, [b] (P<0.05)-Significantly different compared to $CdCl_2$ group [c] (P≤0.05) -Significantly different compared to *Hibiscus* group. Data represented as Mean ±SEM. P≤0.05 was considered significantly different.

DISCUSSION

The outcome of this study showed an increase in antioxidant levels, as indicated by the positive changes in biomarkers such as SOD, CAT, GPx, GSH, and GR which lead to a decrease in lipid peroxidation and subsequently lower MDA levels. This suggests that the aqueous extract of *H. Sabdariffa* may have a protective effect against cadmium chloride-induced oxidative stress modifications in the brain (Olasehinde, *et al*., 2022). This effect of *H. Sabdariffa* could be attributed to several antioxidant compounds it contains.

Previous studies have shown that the extracts of the *H. Sabdariffa* plant have lipid-lowering and antidiabetic effects due to their high levels of bioactive compounds (Da-Costa-Rocha, 2014). The effect of *H. Sabdariffa* in the present study may add to the list of its potential therapeutic applications in complementary and alternative medicine. Calcium is important in the contraction and expansion of blood vessels, nerve impulse transmission, muscle contraction and hormone production (Kamel, 2017). In muscle cells, nerve endings release calcium ions, which bind to activator proteins. The activator proteins initiate the complex process of muscle contraction and allow your muscles to move (Kamel, 2017; Zheng, *et al*., 1991). Calcium channels are proteins that allow calcium ions to enter or leave nerve cells. Calcium channels are involved in various types of neurotransmissions, such as excitatory, inhibitory, modulatory, and synaptic plasticity (Brown, *et al*., 2019). The Group administered (cadmium only) CdCl2 show a significant decrease in calcium levels which can cause increased risk of calcium deficiency, which in turn reduces calcium release from bone tissues and increases the risk of osteoporosis (Mahdi, *et al*., 2021; Nordberg, *et al*., 2018). *H. Sabdariffa* treated groups showed no significant change in calcium levels compared to control, by reducing oxidative stress, the extract might have helped maintain calcium balance. *H. Sabdariffa* contains various phytochemicals that can act as metal chelators, such as anthocyanins, flavonoids, phenolic acids, and organic acids (Farag, *et al*., 2015), The extract's chelating properties might help sequester cadmium, preventing its interaction with calcium and maintaining calcium levels. Potassium is important in maintaining fluid balance, regulating nerve and muscle activity, and supporting cardiovascular health, neuronal excitability, and synaptic transmission (Kamel, 2017). The Group administered (cadmium only) CdCl2 showed a significant decrease, which can reduce the uptake of potassium by the cells, leading to lower intracellular potassium concentration and higher extracellular potassium concentration. This can affect the membrane potential of the cells (Christer, 2019) and can impair the function of sodium-potassium pumps, which are proteins that transport sodium and potassium ions across the cell membrane to maintain the membrane potential (Kamel, 2017). Acetylcholinesterase is an enzyme that breaks down a chemical called acetylcholine, which is a neurotransmitter that carries signals between nerve cells and muscle cells. Acetylcholinesterase is important for regulating the activity of acetylcholine and preventing its accumulation in the synapses, which are the gaps between nerve cells (Trang, 2023). Acetylcholinesterase is found in various parts of the body, such as the blood, the brain, the neuromuscular junctions, and other organs. Acetylcholinesterase has various functions, such as controlling muscle contraction and relaxation, modulating learning and memory, and influencing sleep and arousal (Trang, 2023; Ramesh, *et al*., 2013). The Group administered (cadmium only) CdCl2 show a significant decrease in AChE levels, in a similar study cadmium exposure caused a decrease in acetylcholinesterase activity in the blood, brain, and heart (Nunes *et al*., 2014). These effects may be related to oxidative stress and inflammation induced by cadmium. *H. Sabdariffa* treated groups showed significant increase in AChE levels compared to control, Cadmium-induced oxidative stress can negatively impact acetylcholine receptors and neurotransmission (Nunes, *et al*., 2014; Pretto, *et al*., 2009). The antioxidant properties of the extract might have mitigated this oxidative damage, resulting in the preservation of AChE levels. CK-MM is a type of creatine kinase (CK) enzyme that is mostly found in the skeletal muscle. CK is a protein that helps your body's cells do their jobs. CK-MM is the most common form of CK in the body, and it is involved in muscle contraction and relaxation. CK-MM levels can rise when you have muscle damage or disease, such as muscular dystrophy or rhabdomyolis (Salvatore, *et al*., 2016; Washington and Gerald, 2012). The Group administered (cadmium only) CdCl2 show a significant increase in CK-MM levels, in a similar study cadmium exposure caused an increase in CK-MM activity

in the serum and skeletal muscle, indicating muscle damage and dysfunction (Yang, 2015). These effects may be related to oxidative stress and inflammation induced by cadmium. *H. Sabdariffa* treated groups showed significant decrease in CK-MM levels, The *H. Sabdariffa* extract might possess antioxidant and anti-inflammatory properties that mitigate oxidative stress and inflammation induced by cadmium exposure, The extract might have metal-chelating properties that reduce cadmium-induced damage to muscle cells, thereby preventing CK-MM release (Da-Costa-Rocha, 2014; Bizzozero, 2009). Protein carbonyl is a biomarker of oxidative stress (Fernando, *et al*., 2016). Carbonylation is the most common protein modification that takes place as consequence of severe oxidative stress 50. The Group administered (cadmium only) CdCl2 show a significant increase in PC levels, this may be due to oxidative stress resulting to oxidation of proteins (Fernando, *et al*., 2016). *H. Sabdariffa* treated groups showed significant decrease in PC levels, The *H. Sabdariffa* extract might contain antioxidants that can scavenge reactive oxygen species (ROS) generated by cadmium exposure. This would prevent the oxidation of proteins and the formation of protein carbonyls (Farag, 2015; Pretto, *et al*., 2009). The extract could have metal-chelating properties that sequester cadmium ions. By reducing cadmium levels, the extract might indirectly prevent protein oxidation. Nitric oxide is a colorless gas that has many important roles in the human body. It is produced by various cells and tissues, such as the endothelial cells that line the blood vessels, the nerve cells that transmit signals, and the immune cells that fight infections. Nitric oxide helps to regulate blood pressure, blood flow, nerve transmission, and inflammation. It also has some effects on memory, learning, and sleep. Nitric oxide is a free radical, which means it has an unpaired electron that makes it very reactive. This can be beneficial or harmful, depending on the amount and location of nitric oxide in the body (Nui, *et al*., 2023; Singh, *et al*., 2019). The Group administered (cadmium only) CdCl2 show a significant increase in NO levels, by increasing the degradation of nitric oxide by enhancing the activity of reactive oxygen species (ROS), which are molecules that can cause cellular damage by reacting with lipids, proteins, and DNA. ROS can react with nitric oxide and form peroxynitrite, which is a highly toxic compound that can damage cellular components (Nui, *et al*., 2023; Singh, *et al*., 2019; Robbins and Grisham, 1997). *H. Sabdariffa* treated groups showed no significant change in NO levels compared to control, The extract contains antioxidants (Da-Costa-Rocha, 2014) that scavenge reactive oxygen species (ROS) generated by cadmium exposure. Reduced ROS levels would lead to lower peroxynitrite formation.

In conclusion, the findings of the study indicate that cadmium chloride triggers neurotoxic manifestations by disrupting balance in stress, muscle function markers and electrolyte balance. However, rats' treatment with aqueous extract of *Hibiscus Sabdariffa* may ameliorate this effect. This suggests that *H. Sabdariffa* can effectively act as a natural remedy to against neurotoxicity.

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Niger. J. Physiol. Sci. 39 (June 2024): 111 – 118
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Effect of Aqueous Extract of *Hibiscus Sabdariffa* on Cadmium Chloride-Induced Neurotoxicity in Male Wistar Rats

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Summary: This study investigated the neurologic effect of aqueous extract of *Hibiscus Sabdariffa* on cadmium chloride-induced neurotoxicity in Wistar rats. Thirty male Wistar rats were grouped accordingly; Group A: control, Group B-F: CdCl2 0.3ml; *H. Sabdariffa* 1.5ml; CdCl2 + *H. Sabdariffa* 1.0ml; CdCl2 + *H. Sabdariffa* 1.5ml; CdCl2 + *H. Sabdariffa* 2ml, respectively. Gas chromatography coupled to flame ionization detector (GC-FID), total antioxidant capacity (TAC) and mineral analysis of *H. Sabdariffa* was done to reveal the bioactive agents in the plant sample. Oxidative stress, muscle function markers and plasma electrolytes were assayed. Phytochemical screening revealed alkaloids, phytate, anthraquinone and flavonoids. The mineral analysis revealed predominantly, Mn, Ca, K and Fe. The biochemical results were in comparison with control and statistically significant at 95% confidence interval. There was an increase in SOD, CAT, GSH, GR and GPx in groups C-F, MDA decreased in group C, E and F while PC decreased in group C and F. There was a decrease in plasma creatine kinase in groups C to F and increase in AChE in group C. Ca and K levels had no significant change ($P<0.05$). Nitric oxide level had no significant change in group C, E and F, but increased in group D. This study demonstrated that *H. Sabdariffa* has the potential to ameliorate the neurotoxicity caused by CdCl2 in Wistar rats.

Keywords*: Neurotoxicity; Hibiscus sabdariffa; cadmium; neuromuscular function; phytochemicals*

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Manuscript received- February 2024; Accepted- May 2024

DOI: https://doi.org/10.54548/njps.v39i1.14

INTRODUCTION

Cadmium (Cd) is a toxic, nonessential transition metal that is classified as a human carcinogen (Wang and Du 2013; Viaene, 2000). Over the years, many different forms of exposure to cadmium have been identified, with cadmium being present in the environment as a result of many human exploratory activities (Mehrdad *et al*., 2017). The constant sources of cadmium contamination are related to its industrial application as a corrosive reagent, as well as its use as a stabilizer in polyvinyl chloride (PVC) products, color pigments, and Ni-Cd batteries (Genchi, *et al*., 2020). In areas with contaminated soils, house dust is a potential route for cadmium exposure (Janneke, *et al*., 2007). Cadmium produces neuropathological and neurochemical alterations in central nervous system (CNS), leading to cognitive dysfunction (Sola, *et al*., 2022). Over the years, interest in food diversity for improved health and nutrition has increased in favor of plant-based natural food products with high bioactive compounds. *Hibiscus Sabdariffa* is a plant of the Malvaceae family, commonly known as roselle (Salem, *et al*., 2021; Lucresse, *et al*., 2019). Also called karkade, bissap, sobolo or zobo, depending on the region, *H. Sabdariffa* is prepared as a traditional drink in many African countries. The flowers are mainly used, reputed for their thirst-quenching and relaxing properties (Lucresse, *et al*., 2019). *H. Sabdariffa* has been used as a traditional medicine for various ailments, such as hypertension (Ellis, *et al*., 2022; Koch, *et al*., 2019), liver disorders (Ellis, *et al*., 2022), inflammation (Alshami and Ahmed, 2014), diabetes (Ahmed and Abozed, 2015) and cancer (Koch, *et al*., 2019). Due to an increase in prevalence of neurological disorders, there is an urgent need to discover complementary source of medicines to reduce the challenges in availability or cost related to conventional medicines. The aim of this study is to investigate the neurologic effects of aqueous extract of *Hibiscus Sabdariffa* on cadmium chloride-induce neurotoxicity in male Wister rats

MATERIALS AND METHODS

Ethical approval: This study was approved by the Ethical Considerations Committee, Directorate of Research and Human Development, Madonna University, Elele, Rivers State, Nigeria. The reference Number is MUECC/20230148.

Apparatus and Equipment: Surgical latex gloves, oral gavage/cannula, syringe, lithium heparin bottles, cotton wool, micro hematocrit tube. Aluminium cages and bedding, feeding troughs, orogastric tubes, plain sample bottles, test tubes of various sizes, dissecting kit, dissecting board, water bath, medicated soap, desiccator bottle, slides and cover slips and electronic weighing scale.

Chemical agents: Cadmium chloride (CdCl2), normal saline, distilled water, Diethyl ether, Methylated Spirit, Heparin. The concentration of CdCl2 was 2mg/ml.

Feed: The feed used was the pelleted Top Feed®

Plant collection: The calyx of *H. Sabdariffa* used for this study was obtained from the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Madonna University, Elele, Rivers State, Nigeria.

Extraction Procedure: Dried *Hibiscus Sabdariffa* calyces (flowers) were obtained from Madonna University Botanical Garden. A measure quantity of 100g of the calyces was grinded into smaller pieces in order to facilitate extraction and was added to 70cl of distilled water in a ratio of 1:7 and was allowed to boil for 35 minutes. After heating, the mixture was allowed to cool to room temperature and then strained using filter paper to separate the liquid extract from the solid plant material which was then stored in a refrigerator daily after administration.

Phytochemical Screening: The Phytochemical Screening to determine the different bioactive constituents of *H. Sabdariffa* was done by Gas Chromatography Coupled to flame ionization detector (GCFID) using standard procedure by Lee and Harnly (Lee and Harnly, 2005), Total Antioxidant Capacity (TAC) using standard procedure by Narendhirakannan and Rajeswari (Narendhirakannan and Rajeswari, 2010), and mineral analysis was performed by Atomic Absorption Spectroscopy (AAS).

Table 1:
The design of this study

Groups	Treatments
A	Feed and water only
B	$CdCl_2$ 0.3ml
C	*H. Sabdariffa* 1.5ml
D	$CdCl_2$ + *H. Sabdariffa* 1.0ml
E	$CdCl_2$ + *H. Sabdariffa* 1.5ml
F	$CdCl_2$ + *H. Sabdariffa* 2.0ml

Animal protocol: A total of thirty (30) male Wistar rats weighing 150g to 170g were sampled. They were purchased and housed in Madonna University Biomedical Research Animal House, Faculty of Basic Medical Sciences; in aluminum cages and were allowed to acclimatize for 2 weeks, exposed to 12/12 hours light/dark cycle. Feed and water were provided ad libitum. The rats were randomly grouped into 6, with each cage housing five rats. After 2 weeks of acclimatization, administration $CdCl_2$ and *H. Sabdariffa* commenced.

Study duration: After 2 weeks (14 days) of acclimatization, this study lasted for 4 weeks (28 days).

Sample collection: The animals were anaesthetized with diethyl ether before an incision was made in the cranial region, an incision was made in the scalp and the skull was opened after which the brain was carefully removed and placed on ice before homogenization.

Brain Tissue Homogenization: All animals were anaesthetized with chloroform (0.5 ml intraperitoneally) and transcardially perfused with normal Saline (0.9% NaCl) followed by 4% Paraformaldehyde (PFA) in phosphate buffer (PB; 0.1 M pH 7.4). The brains were removed from the skull and placed in normal saline for biochemical analysis. Oxidative stress markers and electrolytes were assayed.

Biochemical analysis

Assay for oxidative stress markers: The oxidative stress markers tested for include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH), protein carbonyl (PC) and malondialdehyde (MDA).

Assay for Superoxide dismutase: Using method by Rani, *et al*., 2013. The SOD assay measures the activity of superoxide dismutase (SOD) by inhibiting the autoxidation of adrenaline. Superoxide anions produced during adrenaline oxidation can turn it into adrenochrome, but SOD prevents this reaction by converting superoxide anions into hydrogen peroxide. The amount of adrenochrome formed is directly proportional to the SOD activity (Rani, *et al*., 2013).

Assay for Catalase: Using the method by Chin-Chan, *et al*., 2015. The assay of Catalase (CAT) activity is based on the measurement of the decomposition of hydrogen peroxide (H2O2) by CAT. CAT catalyzes the breakdown of H2O2 into water and molecular oxygen. The rate of decomposition of H2O2 can be measured spectrophotometrically by monitoring the decrease in absorbance at a suitable wavelength, directly indicating the CAT activity (Chin-Chan, *et al*., 2015).

Assay for Glutathione Peroxidase: Using the method by Saez and Nuria Están-Capell, 2014. The assay for Glutathione Peroxidase (GPx) activity is based on the measurement of the rate of glutathione (GSH) oxidation by hydrogen peroxide (H2O2) in the presence of GPx. GPx catalyzes the reduction of H2O2 and organic hydroperoxides using GSH as a cofactor, regenerating GSH in the process. The decrease in absorbance resulting from the oxidation of GSH can be measured spectrophotometrically and is directly related to GPx activity (Saez and Nuria Están-Capell, 2014).

Assay for Glutathione reductase: This was based on the method by Chatterjee, *et al*. 2009. Glutathione reductase together with its co-factor, NADPH, catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione wherein the oxidation of NADPH to NADP+ is monitored as a decrease in absorbance at 340 nm. This rate of decrease in absorbance is directly proportional to the

glutathione reductase activity in the sample because the enzyme is present at rate limiting concentrations (Chatterjee, *et al*. 2009).

Assay for Reduced Glutathione: Using Satish and Pal, 2015 method. The reduced form of glutathione comprised in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable yellow colour when 5,5 dithiobis-2-nitrobenzoic acid (Ellman's reagent) is added to sulfhydryl- compounds. The chromorphoric product resulting from the reaction of Ellman's reagent with the reduced GSH is measured at 412nm (Satish and Pal, 2015).

Assay for Malondialdehyde: This protocol is based on Esterbauer and Cheeseman, 1990 method and can be adapted depending on specific requirements and sample types. The assay of Malondialdehyde (MDA) is based on the reaction of MDA with thiobarbituric acid (TBA) under acidic conditions to form a chromophore that can be measured spectrophotometrically. MDA is a reactive carbonyl compound and a product of lipid peroxidation, making it a commonly used marker for oxidative stress and lipid damage (Esterbauer and Cheeseman, 1990).

Assay for Protein carbonyl: This protocol is based on Chong, *et al*. 2017 method. The assay for protein carbonyl content involves the detection of carbonyl groups that are formed on proteins through oxidative damage. Carbonyl groups are reacted with a suitable reagent, such as 2,4-dinitrophenylhydrazine (DNPH), to form stable protein hydrazones. The hydrazones are then measured spectrophotometrically, and the amount of protein carbonyl content is quantified (Chong, *et al*., 2017).

Assay for Muscle function markers

Assay for Creatine Kinase (CK): Using Shackebaei, *et al*. 2010 method. Creatine kinase (CK), present in the sample, catalyses the transfer of a high energy phosphate group from creatine phosphate to ADP. The ATP produced in this reaction is subsequently used to phosphorylate glucose to produce glucose-6-phosphate (G-6-P) in the presence of hexokinase. G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) with the concomitant reduction of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide phosphate reduced (NADPH). The rate of formation of NADPH is monitored at 340 nm and is proportional to the activity of CK in the sample (Shackebaei, *et al*. 2010).

Assay for Acetylcholinesterase: Using Méndez-Armenta and Rios 2007 method. It is based on the reaction between thiols and chromogenic 5,5'- dithiobis-2-nitrobenzoic acid (DTNB) as it measures the formation of the yellow ion of 5-thio-2-nitrobenzoic acid (TNB) (Méndez-Armenta and Rios, 2007).

Assay for Nitric oxide: Using the method by Moncada and Higgs, 1993. The assay is a widely used colorimetric method to indirectly measure nitric oxide levels by detecting its stable reaction product, nitrite (NO2-) (Moncada and Higgs, 1993).

Electrolyte Assay: Calcium is measured using a Potentiometer. This method determines the potential difference that develops between the inner and outer phases of an ion selective electrode. The electrode is made of a selectively permeable material to calcium ion. The potential is measured by comparing it to the potential of reference electrode. Since the reference electrode has a constant potential, the voltage difference between the two electrodes is attributed to the concentration of ionized calcium in the sample 25. Determination of Serum Potassium Concentration were measured using Centronic GmbH kit via turbidimetric determination method by Hillmann et. al., 1967 and Tietz, 1976.

Statistical analysis: Data was expressed as mean ± SEM and ($P<0.05$) was considered statistically significant. Data collected from this study was analyzed using One-Way analysis of variance (ANOVA) and Post Hoc analysis with the aid of IBM®SPSS Version 21.0.

RESULTS

Phytochemical Analysis of *H. Sabdariffa:* Table 2 shows the results of phytochemical analysis using GC-FID. The concentrations of alkaloid, phytate, anthraquinone and flavonoids in *H. sabdariffa* are 140.512 ug/ml, 231.13 ug/ml, 191.230 ug/ml and 240.124 ppm. The results indicate that flavonoid is the most abundant phytochemical in *Hibiscus sabdariffa*.

Table 2:
Gas chromatography coupled to flame ionization detector (GC-FID) screening of *H. Sabdariffa*

Component	Concentration	Unit
Anthocyanins	132.101	ug/ml
Polyphenols	127.011	ppm
Tannins	3.421	ug/ml
Alkaloids	140.512*	ug/ml
Oxalates	28.120	ug/ml
Phytate	231.13*	ug/ml
Terpenoids	2.4540	ug/ml
Anthraquinone	191.230*	ug/ml
Flavonoids	240.124*	ppm
Phenols	134.245	ppm
Saponins	10.712	ug/ml
Glycosides	11.261	ug/ml
Steroids	12.120	ppm
Citric acid	12.781	ug/ml
Beta-carotene	15.3470	ug/ml
Malic acid	10.241	ug/ml
Flavonols	7.8931	ppm
Thiamine	2.252	ug/ml
Glycosides	6.452	ug/ml
Ribalinidine	12.4131	ug/ml

Key - * Major phytonutrients in GC-FID analysis of *H. Sabdariffa*

Total Antioxidant Capacity (TAC) of *H. sabdariff* : At 20mg/ml and 40mg/ml the absorbance of *H. Sabdariffa* was 0.58 and 0.71 and that of ascorbic acid, a well-known antioxidant which was used as a standard, was 0.123 and 0.245 respectively. Consequently, an increased concentration of *H. Sabdariffa* (100mg/ml) showed an absorbance of 0.150 indicating a positive correlation in the absorbance of *H. Sabdariffa* and Ascorbic acid. This

indicates that *H. Sabdariffa* has a potential antioxidant activity to ascorbic acid.

Mineral analysis of *H. Sabdariffa*: The results of mineral analysis of *H. Sabdariffa* (Table 4) show that there are high levels of Fe, K, Ca, Mn present in the extract.

Table 3:
Total Antioxidant Capacity of *H. Sabdariffa*

Concentration	Abs. of ascorbic acid	Abs. of *H. Sabdariffa*
20mg/ml	0.123	0.58
40mg/ml	0.245	0.71
60mg/ml	-	0.90
80mg/ml	-	0.120
100mg/ml	-	0.150

Table 4:
Mineral analysis of *H. Sabdariffa*

Mineral	Concentration (mg/kg)
Fe	88.12
K	91.20
Cu	0.10
Ca	102.40
Mg	21.23
Mn	110.61
Zn	0.10

Results from table 5, showed a significant increase (P≤0.05) in SOD in group C, D and F when compared to control and $CdCl_2$ group. There was also a significant decrease (P≤0.05) in SOD levels in Group B when compared to control. In comparison with CdCl group, there was a significant increase in group E. In comparison with group C, SOD levels in group B, D, E and F were significantly decreased. There was a significant increase (P≤0.05) in CAT levels in group C, E and F when compared to the control and $CdCl_2$ group. Also, there was a significant decrease (P≤0.05) in CAT levels in Group B and group D when compared to control. In comparison with Hibiscus group CAT levels in group B, D, E and F were significantly decreased. There was a significant increase (P≤0.05) in GPx levels in group C, E and F when compared to the control and $CdCl_2$ group and also a significant decrease (P≤0.05) in GPx levels in Group B ($CdCl_2$ 0.3ml) and group D when compared to control. In comparison with group C, GPx levels in group B, D, E and F were significantly decreased. The results also showed a significant increase (P≤0.05) in GR levels in group C, D, E and F when compared to the control and $CdCl_2$ group and a significant decrease (P≤0.05) in GR levels in Group B when compared to control. In comparison with group C, GR levels in group B, D, E and F were significantly decreased (P≤0.05).

Table 5 also showed a significant increase (P≤0.05) in GSH levels in group C, E and F when compared to control and $CdCl_2$ group and also a significant decrease (P≤0.05) in GSH levels in Group B and Group D when compared to control. In comparison with Hibiscus group, GSH levels in group B, D, E and F were significantly decreased(P≤0.05). There was a significant decrease (P≤0.05) in MDA levels in group C, E and F when compared to control and $CdCl_2$ group and also a significant increase in MDA level was also seen in group D when compared to control and a significant increase (P≤0.05) in MDA levels in Group B when compared to control. In comparison with Hibiscus group, MDA levels in group B, D, E and F were significantly increased (P≤0.05). Results showed a significant decrease (P≤0.05) in PC levels in group C and F when compared to the control and $CdCl_2$ group. There was also a significant decrease in PC level as seen in group D and E when compared to the $CdCl_2$ group and a significant increase (P≤0.05) in PC levels in Group B and group D when compared to control. In comparison with Hibiscus group, PC levels in group B, D, E and F were significantly increased (P≤0.05).

Results from Table 6 showed a significant decrease (P≤0.05) in Ca^{2+} levels in group B ($CdCl_2$ 0.3ml) when compared to control and Hibiscus group. In comparison with $CdCl_2$ group, Ca^{2+} levels in group C, D, E and F were significantly increased (P≤0.05). The results also showed a significant decrease (P≤0.05) in K^+ levels in group B ($CdCl_2$ 0.3ml) when compared to the control and Hibiscus group. In comparison with $CdCl_2$ group, K^+ levels in group C, D, E and F were significantly increased (P≤0.05). There was a significant increase (P≤0.05) in NO levels in group B ($CdCl_2$ 0.3ml) when compared to the control and Hibiscus group. There was a significant increase (P≤0.05) in NO levels in Group D when compared to control and Hibiscus group. In comparison with $CdCl_2$ group, NO levels in group C, D, E and F were significantly decreased (P≤0.05).

Table 5:
Changes in brain stress markers in response to treatments

Groups	SOD (U/g)	CAT (U/g)	GPx (U/g)	GR (U/g)	GSH (U/g)	MDA (mmol/g)	PC (mmol/g)
Control	12.00 ± 0.46^{bc}	2.86 ± 0.08^{bc}	2.26 ± 0.06^{bc}	2.20 ± 0.10^{bc}	1.40 ± 0.08^{bc}	1.16 ± 0.05^{b}	0.40 ± 0.05^{bc}
$CdCl_2$ 0.3ml	1.40 ± 0.10^{ac}	0.18 ± 0.06^{ac}	0.18 ± 0.04^{ac}	0.34 ± 0.12^{ac}	0.14 ± 0.02^{ac}	12.40 ± 1.77^{ac}	5.36 ± 0.09^{ac}
H. sabdariffa 1.5ml	15.14 ± 0.59^{ab}	5.70 ± 0.04^{ab}	8.26 ± 0.17^{ab}	5.82 ± 0.07^{ab}	5.46 ± 0.12^{ab}	0.09 ± 0.03^{b}	0.18 ± 0.09^{ab}
$CdCl_2$ + *H. sabdariffa* 1.0ml	8.46 ± 0.42^{abc}	2.78 ± 0.19^{bc}	1.74 ± 0.02^{abc}	1.34 ± 0.10^{abc}	1.22 ± 0.09^{bc}	1.80 ± 0.35^{b}	0.52 ± 0.06^{bc}
$CdCl_2$ + *H. sabdariffa* 1.5ml	12.34 ± 0.19^{bc}	4.48 ± 0.10^{abc}	3.20 ± 0.18^{abc}	2.62 ± 0.13^{abc}	3.78 ± 0.07^{abc}	0.35 ± 0.21^{b}	0.26 ± 0.04^{bc}
$CdCl_2$ + *H. sabdariffa* 2.0ml	13.94 ± 0.13^{abc}	4.72 ± 0.04^{abc}	4.48 ± 0.12^{abc}	$4.48\pm.18^{abc}$	4.54 ± 0.12^{abc}	0.12 ± 0.02^{b}	0.14 ± 0.02^{abc}
Total	63.28	20.72	20.12	16.80	**16.54**	**15.92**	**6.86**
Average	10.55	3.453	3.353	2.80	**2.76**	**2.65**	**1.14**

Key: [a](P≤0.05)-Significantly different compared to control group, [b] (P<0.05)-Significantly different compared to $CdCl_2$ group [c](P≤0.05) - Significantly different compared to *Hibiscus* group. Data represented as Mean ±SEM. P≤0.05 was considered significantly different.
SOD- superoxide dismutase ; CAT-catalase; GPx-glutathione peroxidase ; GR-glutathione reductase; GSH-reduced glutathione; MDA-malondialdehyde; PC-protein carbonyl

Table 6:
Changes in muscle function markers in response to treatments

Groups	CK-MM(U/L)	AChE (U/L)
Control	22.04±0.57[bc]	32.52±0.54[bc]
$CdCl_2$ 0.3ml	44.04±0.95[ac]	11.90±2.33[ac]
H. Sabdariffa 1.5ml	11.30±0.32[ab]	46.60±0.58[ab]
CdCl2 + *H. Sabdariffa* 1.0ml	19.82±0.45[abc]	10.88±0.69[ac]
CdCl2 + *H. Sabdariffa* 1.5ml	13.74±0.27[abc]	36.38±1.54[bc]
CdCl2 + *H. Sabdariffa* 2.0ml	12.34±0.39[ab]	51.14±1.65[abc]
Total	123.28	189.42
Average	20.55	31.57

Key: [a](P≤0.05)-Significantly different compared to control group, [b] (P<0.05)-Significantly different compared to $CdCl_2$ group [c] (P≤0.05) -Significantly different compared to *Hibiscus* group. Data represented as Mean ±SEM. P≤0.05 was considered significantly different.

Table 7:
Changes in electrolytes in response to treatments

Groups	Ca (mg/dl)	K (meq/L)	NO (µmol/L)
Control	5.06±0.07[b]	4.46±0.09[b]	0.21±0.15[b]
$CdCl_2$ 0.3ml	2.26±0.07[ac]	2.22±0.40[ac]	2.48±0.10[ac]
H. Sabdariffa 1.5ml	5.08±0.12[b]	4.38±0.06[b]	0.02±0.00[b]
$CdCl_2$ + *H. Sabdariffa* 1.0ml	5.14±0.15[b]	4.38±0.11[b]	1.18±0.16[abc]
$CdCl_2$ + *H. Sabdariffa* 1.5ml	5.10±0.08[b]	4.44±0.10[b]	0.0±0.00[b]
$CdCl_2$ + *H. Sabdariffa* 2.0ml	4.92±0.06[b]	4.62±0.06[b]	0.02±00[b]
Total	27.56	24.5	3.91
Average	4.59	4.08	0.65

Key: [a](P≤0.05)-Significantly different compared to control group, [b] (P<0.05)-Significantly different compared to $CdCl_2$ group [c] (P≤0.05) -Significantly different compared to *Hibiscus* group. Data represented as Mean ±SEM. P≤0.05 was considered significantly different.

DISCUSSION

The outcome of this study showed an increase in antioxidant levels, as indicated by the positive changes in biomarkers such as SOD, CAT, GPx, GSH, and GR which lead to a decrease in lipid peroxidation and subsequently lower MDA levels. This suggests that the aqueous extract of *H. Sabdariffa* may have a protective effect against cadmium chloride-induced oxidative stress modifications in the brain (Olasehinde, *et al*., 2022). This effect of *H. Sabdariffa* could be attributed to several antioxidant compounds it contains.

Previous studies have shown that the extracts of the *H. Sabdariffa* plant have lipid-lowering and antidiabetic effects due to their high levels of bioactive compounds (Da-Costa-Rocha, 2014). The effect of *H. Sabdariffa* in the present study may add to the list of its potential therapeutic applications in complementary and alternative medicine. Calcium is important in the contraction and expansion of blood vessels, nerve impulse transmission, muscle contraction and hormone production (Kamel, 2017). In muscle cells, nerve endings release calcium ions, which bind to activator proteins. The activator proteins initiate the complex process of muscle contraction and allow your muscles to move (Kamel, 2017; Zheng, *et al*., 1991). Calcium channels are proteins that allow calcium ions to enter or leave nerve cells. Calcium channels are involved in various types of neurotransmissions, such as excitatory, inhibitory, modulatory, and synaptic plasticity (Brown, *et al*., 2019). The Group administered (cadmium only) CdCl2 show a significant decrease in calcium levels which can cause increased risk of calcium deficiency, which in turn reduces calcium release from bone tissues and increases the risk of osteoporosis (Mahdi, *et al*., 2021; Nordberg, *et al*., 2018). *H. Sabdariffa* treated groups showed no significant change in calcium levels compared to control, by reducing oxidative stress, the extract might have helped maintain calcium balance. *H. Sabdariffa* contains various phytochemicals that can act as metal chelators, such as anthocyanins, flavonoids, phenolic acids, and organic acids (Farag, *et al*., 2015), The extract's chelating properties might help sequester cadmium, preventing its interaction with calcium and maintaining calcium levels. Potassium is important in maintaining fluid balance, regulating nerve and muscle activity, and supporting cardiovascular health, neuronal excitability, and synaptic transmission (Kamel, 2017). The Group administered (cadmium only) CdCl2 showed a significant decrease, which can reduce the uptake of potassium by the cells, leading to lower intracellular potassium concentration and higher extracellular potassium concentration. This can affect the membrane potential of the cells (Christer, 2019) and can impair the function of sodium-potassium pumps, which are proteins that transport sodium and potassium ions across the cell membrane to maintain the membrane potential (Kamel, 2017). Acetylcholinesterase is an enzyme that breaks down a chemical called acetylcholine, which is a neurotransmitter that carries signals between nerve cells and muscle cells. Acetylcholinesterase is important for regulating the activity of acetylcholine and preventing its accumulation in the synapses, which are the gaps between nerve cells (Trang, 2023). Acetylcholinesterase is found in various parts of the body, such as the blood, the brain, the neuromuscular junctions, and other organs. Acetylcholinesterase has various functions, such as controlling muscle contraction and relaxation, modulating learning and memory, and influencing sleep and arousal (Trang, 2023; Ramesh, *et al*., 2013). The Group administered (cadmium only) CdCl2 show a significant decrease in AChE levels, in a similar study cadmium exposure caused a decrease in acetylcholinesterase activity in the blood, brain, and heart (Nunes *et al*., 2014). These effects may be related to oxidative stress and inflammation induced by cadmium. *H. Sabdariffa* treated groups showed significant increase in AChE levels compared to control, Cadmium-induced oxidative stress can negatively impact acetylcholine receptors and neurotransmission (Nunes, *et al*., 2014; Pretto, *et al*., 2009). The antioxidant properties of the extract might have mitigated this oxidative damage, resulting in the preservation of AChE levels. CK-MM is a type of creatine kinase (CK) enzyme that is mostly found in the skeletal muscle. CK is a protein that helps your body's cells do their jobs. CK-MM is the most common form of CK in the body, and it is involved in muscle contraction and relaxation. CK-MM levels can rise when you have muscle damage or disease, such as muscular dystrophy or rhabdomyolis (Salvatore, *et al*., 2016; Washington and Gerald, 2012). The Group administered (cadmium only) CdCl2 show a significant increase in CK-MM levels, in a similar study cadmium exposure caused an increase in CK-MM activity

in the serum and skeletal muscle, indicating muscle damage and dysfunction (Yang, 2015). These effects may be related to oxidative stress and inflammation induced by cadmium. *H. Sabdariffa* treated groups showed significant decrease in CK-MM levels, The *H. Sabdariffa* extract might possess antioxidant and anti-inflammatory properties that mitigate oxidative stress and inflammation induced by cadmium exposure, The extract might have metal-chelating properties that reduce cadmium-induced damage to muscle cells, thereby preventing CK-MM release (Da-Costa-Rocha, 2014; Bizzozero, 2009). Protein carbonyl is a biomarker of oxidative stress (Fernando, *et al*., 2016). Carbonylation is the most common protein modification that takes place as consequence of severe oxidative stress 50. The Group administered (cadmium only) CdCl2 show a significant increase in PC levels, this may be due to oxidative stress resulting to oxidation of proteins (Fernando, *et al*., 2016). *H. Sabdariffa* treated groups showed significant decrease in PC levels, The *H. Sabdariffa* extract might contain antioxidants that can scavenge reactive oxygen species (ROS) generated by cadmium exposure. This would prevent the oxidation of proteins and the formation of protein carbonyls (Farag, 2015; Pretto, *et al*., 2009). The extract could have metal-chelating properties that sequester cadmium ions. By reducing cadmium levels, the extract might indirectly prevent protein oxidation. Nitric oxide is a colorless gas that has many important roles in the human body. It is produced by various cells and tissues, such as the endothelial cells that line the blood vessels, the nerve cells that transmit signals, and the immune cells that fight infections. Nitric oxide helps to regulate blood pressure, blood flow, nerve transmission, and inflammation. It also has some effects on memory, learning, and sleep. Nitric oxide is a free radical, which means it has an unpaired electron that makes it very reactive. This can be beneficial or harmful, depending on the amount and location of nitric oxide in the body (Nui, *et al*., 2023; Singh, *et al*., 2019). The Group administered (cadmium only) CdCl2 show a significant increase in NO levels, by increasing the degradation of nitric oxide by enhancing the activity of reactive oxygen species (ROS), which are molecules that can cause cellular damage by reacting with lipids, proteins, and DNA. ROS can react with nitric oxide and form peroxynitrite, which is a highly toxic compound that can damage cellular components (Nui, *et al*., 2023; Singh, *et al*., 2019; Robbins and Grisham, 1997). *H. Sabdariffa* treated groups showed no significant change in NO levels compared to control, The extract contains antioxidants (Da-Costa-Rocha, 2014) that scavenge reactive oxygen species (ROS) generated by cadmium exposure. Reduced ROS levels would lead to lower peroxynitrite formation.

In conclusion, the findings of the study indicate that cadmium chloride triggers neurotoxic manifestations by disrupting balance in stress, muscle function markers and electrolyte balance. However, rats' treatment with aqueous extract of *Hibiscus Sabdariffa* may ameliorate this effect. This suggests that *H. Sabdariffa* can effectively act as a natural remedy to against neurotoxicity.

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Niger. J. Physiol. Sci. 39 (June 2024): 119 – 124
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Research Article

Abnormal Haematological Profile caused by Potassium Bromate in Wistar Rats is corrected by *Parkia Biglobosa* Seed

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Many biological tissues and organs are affected by the toxicity of potassium bromate ($KBrO_3$). The purpose of this study was to evaluate the *Parkia Biglobosa* (*P. Biglobosa*) seed's ability to treat $KBrO_3$-induced haematological parameters derangement. After becoming accustomed to the lab, 24 Wistar rats were randomly assigned to groups A, B, C, and D. Group A was given distilled water to drink. Each of the groups in B, C, and D got 100 mg/kg of $KBrO_3$. Also, for 28 days prior to sacrifice, groups C and D received 100 and 200 mg/kg of P. biglobosa, respectively. Blood was drawn, and the haemogram was examined using a haematology autoanalyzer. When $KBrO_3$ was added compared to the control, the results showed a substantial decrease in both haemoglobin concentration, packed cell volume (PCV), and red blood cell count from 17.26±2.84 g/dL, 39.73±2.58%, 5.12±0.83 x 10^{12}/L to 13.25±1.25 g/dL, 27.93±1.44%, and 3.47±0.22 x 10^{12}/L, respectively. The effect of $KBrO_3$ was dose-dependently counteracted by *P. biglobosa* treatments of 100 and 200 mg/kg body weight. However, there was no discernible difference in the MCV, MCH, and MCHC values between the control and test groups. Similar to how *P. biglobosa* reduced the effects of $KBrO_3$ in a dose-dependent manner, *P. biglobosa* also induced a substantial decrease in white blood cell count, its differentials, and platelet counts ($P \leq 0.05$). $KBrO_3$-induced deranged haematological parameters were mitigated by *Parkia biglobosa* in a dose dependent manner. Care must be taken with the consumption of this addictive due to its numerous toxic effects. However, consumption of *P. biglobosa*, a tropical homemade food is recommended for families to benefit from the barrage of its health benefits. This will also alleviate the toxic effect of $KBrO_3$ if consumed inadvertently. Human clinical trial is needed to substantiate these findings.

Keywords*: Haematological Parameters, Parkia Biglobosa, Potassium bromate*

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Manuscript received- August 2023; Accepted- November 2023

DOI: https://doi.org/10.54548/njps.v39i1.15

INTRODUCTION

When potassium bromide solution is electrolyzed or when bromine is infused into a warm potassium hydroxide solution, potassium bromate ($KBrO_3$) is produced (Airaodion et al., 2023). It strengthens the dough and makes it bigger, and it is utilised in the United States (US) to improve flours. Due to its oxidising ability, if excess is applied to bread that is not adequately supported, residual amounts may remain that are hazardous to the body (Kurokawa et al., 2010). When administered orally to humans or experimental animals, $KBrO_3$ is nephrotoxic and causes cancer in rats. According to studies, $KBrO_3$ causes thyroid follicular cell tumours, peritoneal mesotheliomas, and renal cell tumours (Airaodion et al. 2023; Kurokawa et al., 2010). Furthermore, research aiming at determining the route of carcinogenic action has shown that $KBrO_3$ is a full carcinogen, having both initiating and encouraging effects

on the development of rat kidney tumours (Kurokawa et al., 2010). Different organs and systems of the body are affected by potassium bromate. A recent report by Ugwu et al. (2022a) revealed that $KBrO_3$ perturbed lipid profile of experimental rats. Another study showed that hepatic indicators were unhinged in the blood and liver cells by $KBrO_3$ (Onyekachi et al., 2022).

Potassium bromate has been outlawed in some nations' food products: Canada, Nigeria, South Korea, Peru, the European Union, Argentina, Brazil, and a few other nations. China in 2005, India in 2016, and Sri Lanka in 2001 all enacted bans on it (Airaodion et al., 2019a). It has not, however, been outlawed in the United States. Before the Food, Drug, and Cosmetic Act's Delaney clause, which outlaws possibly carcinogenic compounds, took effect in 1958, the United States' Food and Drug Administration (FDA) approved the use of bromate. The FDA has, however, advised bakers to voluntarily discontinue using it since 1991. In California, using bromated flour necessitates the addition of a warning label (Airaodion et al., 2019b).

Parkia biglobosa is a dicotyledonous angiosperm in the Fabaceae family (Airaodion et al., 2019c; Thiombiano et al., 2012). It belongs to the group of vascular plants known as spermatophytes. It is a perennial deciduous plant that, under some conditions, can grow as high as 30 metres (Ntui et al., 2012). In West Africa, *P. biglobosa*, often known as African locust bean, is widely used. It is a tree of extreme importance in Burkina Faso as a source of food and money in some rural households (Termote et al., 2022). The species is indigenous to sub-Saharan Africa and is referred to as n'er'e in Francophone Africa (Hopkins, 2022). In Nigeria, the Igbos call it "Ogiri," the Yorubas call it "Iru," and the Hausas call it "Dorowa" (Onyeaghala et al., 2022). The use of the bean seeds as a soup seasoning and their monetary value have aided in their widespread acceptance.

In West Africa, notably Nigeria, the beans are commonly fermented to create "Dawadawa," a spice. The dark-colored flavour known as dawaddawa, is high in protein and lactose and widely used in local soups (Ezirim et al., 2022a; Achi, 2015; Wokoma and Aziagba, 2010). The roots of *P. biglobosa* and other parkia species, as well as paste and decoction, are used to treat a variety of skin conditions (Ajaiyeoba, 2010; Oladunmoye and Kehinde, 2011). After being soaked in water for an entire night, *P. Biglobosa* seeds are extracted by maceration. This is then filtered and used to treat diabetic mellitus (Erakhrumen et al., 2010). In folkloric medicine, it is used to treat leprosy, diarrhoea, bacterial infections, high blood pressure, and wound healing (Airaodion et al., 2020a; Ekperikpe et al., 2019). *P. Biglobosa* has significant metabolic advantages in Wistar rats. It was discovered to be hepatoprotective by reducing the rat's susceptibility to the liver-damaging effects of $KBrO_3$ (Onyekachi et al., 2022).

Another investigation revealed that *P. biglobosa* attenuated the $KBrO_3$-induced coagulopathy in a dose-dependent manner (Ugwu et al., 2023). According to Ugwu et al. (2022b), *P. biglobosa* significantly raised the antioxidant levels in rats, reducing the oxidative damage brought about by potassium bromate. Ezirim et al. (2022a) showed that treatment with *P. biglobosa* prevented the testicular toxicity effect caused by $KBrO_3$ in the rat.

It has been demonstrated that potassium bromate causes haemoglobin oxidation, oxidative stress, and impairment of the blood's antioxidant defence system (Ahmad and Mahmood, 2016). However, this impact was lessened by taurine (Ahmad and Mahmood, 2016). Furthermore, Achukwu et al. (2009) found that $KBrO_3$ caused thrombocytopenia in rats. However the investigation found no statistically significant differences between the test and control rats' mean cell haemoglobin concentrations, haematocrits, or total leukocyte counts. The same study suggested that consuming potassium bromate constantly and in large amounts is harmful to one's health (Achukwu et al., 2009). Despite NAFDAC's 2003 warning about the risk of employing $KBrO_3$ as a flour enhancer, several studies in Nigeria have revealed that $KBrO_3$ is still at the Bakeries' beck and call (Airaodion et al., 2019a,b). Researchers have recently proved that some food items in Nigeria could ameliorate the toxic effects of potassium bromate (El-Deeb and Abd-El-Hafez, 2015; Femi-Oloye et al., 2020). This study was therefore, aimed at assessing the sanative propensity of *P. Biglobosa* seed on $KBrO_3$-induced derangement on haematological parameters.

MATERIALS AND METHODS

Collection and Extraction of *Parkia Biglobosa*: A botanist identified *P. biglobosa* seeds after purchasing them from a neighbourhood market in Ibadan, Nigeria. They were sun-dried and mechanically blended into powder (Moulinex). The extraction was performed using a soxhlet apparatus and ethanol as the solvent in accordance with the steps indicated by Airaodion *et al*. (2019b; 2020). About 10.20 percent yield extract was obtained after the ethanol was evaporated in a rotary evaporator at 35 oC. For subsequent investigation, the extract was kept in a refrigerator (4 oC).

Animal Treatment: Twenty-four mature male Wistar rats (Rattus norvegicus), weighing between 140 and 160 g, were used for the experiment. They spent seven (7) days getting used to the testing setting before the test. The rats were housed in cages made of wire mesh, and they had unrestricted access to rat food and water from the market. The animals were kept within normal ranges of temperature and humidity with 12-hour cycles of light and darkness. The Helsinki Declaration and the guidelines set by the Committee for the Purpose of Regulation and Supervision of Experiments on Animals were both adhered to during the conduct of this study, which was approved by the ethical committee on research at Abia State University, Uturu, Nigeria. The National Research Council's policy on using animals in research was also followed (NRC, 2011).
Groups A, B, C, and D of rats were created. Group A was given distilled water. For each kilogramme of body weight, animals in groups B, C, and D received 100 mg of potassium bromate. Also, groups C and D received dosages of 100 and 200 mg/kg of *P. biglobosa* seed extract respectively. Diethyl ether was used to gently sedate the animals for the final 24 hours of their 28-day therapy period before they were euthanized. The haemogram was evaluated using a haematology autoanalyzer after blood was taken through a cardiac puncture.

Statistical Analysis: Using one-way ANOVA and the Tukey post hoc test, the results were analyzed. P values of 0.05 were taken into consideration to be statistically

significant for all analyses, which were performed using the Graph Pad Prism software (version 8).

RESULTS

Packed Cell Volume (PCV) and red blood cell count from 17.26±2.84 g/dL, 39.73±2.58%, 5.12±0.83 x 10^{12}/L to 13.25±1.25g/dL, 27.93±1.44%, 3.47±0.22 x 10^{12}/L respectively ($P<0.05$) when $KBrO_3$ was added compared to the control (table 1). The results showed a significant reduction in haemoglobin concentration, PCV, and red cell count from 17.26±2.84 g/dL, 39.73±2.58%, 5.12±0.83 x 10^{12}/L in control animals to 13.25±1.25g/dL, 27.93±1.44%, 3.47±0.22 x 10^{12}/L respectively in animals exposed to $KBrO_3$ only (Table 1). Treatment with 100 and 200 mg/kg body weight of *P. biglobosa* neutralized the effect of $KBrO_3$ in a dose-dependent manner. However, MCV, MCH and MCHC values showed no significant difference ($P>0.05$) in the control and test groups (Table 1). Similarly, $KBrO_3$ caused significant reduction in White blood cell count, its differentials and platelet count which was also mitigated in a dose-dependent manner by *P. biglobosa* as shown in Table 2.

DISCUSSION

The main role of red blood cells is to carry carbon dioxide to the lungs for expiration and oxygen across the body for metabolism. Haemoglobin is a special protein found in the red cell and a vehicle that the red cell uses to perform these functions (Airaodion and Ogbuagu, 2020). The purpose of platelets, also known as thrombocytes, a blood component, is to clump in response to bleeding caused by blood vessel damage and thereby producing a blood clot (Airaodion *et al*., 2019e). Aplastic anaemia, myelodysplastic syndromes, immune thrombocytopenic purpura, chemotherapy, human immunodeficiency virus infection, and other disorders can all cause thrombocytopenia (platelet counts below 150 x 109/L), which is a significant clinical issue. Platelets are necessary for haemostasis (Airaodion *et al*., 2019f). White blood cells, sometimes referred to as leukocytes or leucocytes, are immune system cells that assist in defending the body against pathogens and foreign invaders (Airaodion *et al*., 2019g). The importance of blood cells in the physiological activities of the body and the use of bromate as food additives in our environment makes this study critical. This study showed KBrO3 induced significant reduction ($P<0.05$) in haemoglobin concentration, platelets, total WBC count, neutrophils, lymphocytes, and monocytes compared to those in the control animals. Also, it was noted that therapy using *P. biglobosa* attenuated the effect of KBrO3. These findings agreed with a Saudi study that used Forty five (45) Swiss Webster (SW) mice which observed significant reduction in the red cells, white blood cells (WBC) and platelets in the study groups compared to the control group (Altoom *et al*., 2018). Another study by Mohamed and Saddek (2019) discovered a significant reduction in haemoglobin concentration , MCV and total WBC count but not for platelets. Achukwu *et al*. (2009) found only significant reduction in platelets count. Between the test and control rats, the mean Cell haemoglobin concentration, haematocrit, and total leukocyte counts did not differ statistically in their study.

Table 1:
Effect of *P. biglobosa* on Red Blood Cell Parameters of Potassium Bromate-induced Rats

Parameters	Control	100 mg/kg $KBrO_3$ only	100 mg/kg $KBrO_3$ + 100 mg/kg *P. biglobosa*	100 mg/kg $KBrO_3$ + 200 mg/kg *P. biglobosa*	p-value
Hb (g/dL)	17.26±2.84	13.25±1.25	15.34±1.18	16.92±1.27	0.01
PCV (%)	39.73±2.58	27.93±1.44	34.26±1.95	38.27±1.22	0.01
RBC (x10^{12}/L)	5.12±0.83	3.47±0.22	4.03±0.36	4.98±0.72	0.02
MCV (FL)	75.10±7.23	80.49±4.53	85.01±6.73	73.60±6.15	0.49
MCH (pg)	28.85±3.84	29.54±4.24	30.62±3.50	28.69±2.31	0.74
MCHC (g/dL)	3.84±0.96	3.67±0.28	3.60±1.00	3.90±0.27	0.82

Values are presented as Mean±S.D, where n = 6.
PCV = Packed Cell Volume; Hb = Haemoglobin; RBC = Red Blood Cell; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Haemoglobin; MCHC = Mean Corpuscular Haemoglobin Concentration

Table 2:
Effect of *P. biglobosa* on White Blood Cell and Platelets Parameters of Potassium Bromate-induced Rats

Parameters	Control	100 mg/kg $KBrO_3$ only	100 mg/kg $KBrO_3$ + 100 mg/kg *P. biglobosa*	100 mg/kg $KBrO_3$ + 200 mg/kg *P. biglobosa*	P Value
WBC (x10^9/L)	9.12±1.05	7.03±1.37	7.98±2.00	9.08±1.15	0.01
Platelet (x10^9/L)	241.26±11.34	218.25±17.34	223.74±14.83	230.26±22.43	0.00
Neutrophils (x10^9/L)	5.84±1.35	2.46±0.11	3.78±0.93	4.79±0.38	0.03
Lymphocytes (x10^9/L)	4.17±1.12	2.68±0.26	3.10±0.75	3.86±1.27	0.00
Monocytes (x10^9/L)	0.80±0.07	0.34±0.00	0.40±0.01	0.68±0.02	0.03

Values are presented as Mean±S.D, where n = 6. ; WBC = White Blood Cell

Increased lipid peroxidation and protein oxidation in both plasma and erythrocytes, which point to the development of oxidative stress, may be responsible for the reductive effect of KBrO3 on blood cells, particularly red cells. The succession of oxidative lipid breakdown events is known as lipid peroxidation. During this process, free radicals "steal" electrons from the lipids in cell membranes, harming the cells. This activity is driven by a free radical chain reaction mechanism (Airaodion *et al*., 2020b). Due to their role as oxygen carriers and the presence of lipids in their structure, red blood cells are vulnerable to lipid peroxidation. This study supports the oxidative action of $KBrO_3$. Previous studies have reported that $KBrO_3$ possesses oxidative propensity by showing significant increases in methemoglobin and nitric oxide as well as significant decreases in plasma, hepatic, renal, and cardiac catalase, superoxide dismutase (SOD), and glutathione peroxidase activity (GPx), reduced glutathione (GSH) concentrations, and increased malondialdehyde (MDA) levels (Ugwu *et al*., 2022c). Additionally, several toxins reduce the production of haemoglobin by preventing the activities of aminolaevulinic acid dehydratase and ferro-chelatase (Ashour *et al*., 2007). Thus, by hindering haem-biosynthesis and reducing RBC survival, $KBrO_3$ may result in anaemia at high concentrations. In a study by Femi-Oloye *et al*. (2020), rats were administered 10 mg of $KBrO_3$ per kilogramme of body weight, and there was no discernible difference in the rats' haemoglobin levels between the test and control groups which however contradicts our study. These differences may have resulted from the doses of KBrO3 used as we used 100 mg/kg against the 10mg/kg body weight used by Femi-Oloye *et al*. (2020).

The reduction of white blood cell and platelets obtained in this study corroborate with the study of Achukwu *et al*. (2009). These decreases in the leucocyte and platelet count may have been brought about by DNA strand breaks caused by the oxidative stress induced by potassium bromate in these cells. Additionally, there might have been selective megakaryocyte depression and bone marrow suppression (Hoffbrand and Petit, 2014). On the other hand, $KBrO_3$ may directly harm the platelets.

Several studies have shown attenuation or inhibition of KBrO3- induced toxicity using different nutrients. According to El-Deep *et al*. (2015), vitamin C reduced the oxidative stress caused by $KBrO_3$ on the left ventricular myocardium of adult male albino rats; the research using taurine and vanilla shown a considerable reduction in the negative effects of $KBrO_3$ toxicity on haematological parameters, renal, and testicular tissues (Mohamed and Saddek, 2019). Another investigation revealed that bilberry (Vaccinium myrtillus L.) extract has a protective effect against kidney damage brought about by KBrO3 in mice (Bao *et al*., 2018). Cloudy apple juice reduces rat kidney and liver damage brought on by $KBrO_3$ (Kujawska *et al*., 2013). In a recent study, Ugwu *et al*. (2022c) demonstrated that Corchorus olitorius leaves ameliorated oxidative stress induced by KBrO3. While it's destructive effect on the liver was attenuated by *Parkia Biglobosa* (Onyekachi *et al*., 2022) and Corchorus olitorius leaves (Aguh *et al*., 2022).

Parkia Biglobosa in addition to its mitigative effect on Potassium bromate-induced deranged haematological parameters, has shown great impact on mitigating the effect of $KBrO_3$ -induced toxicity in organs and systems of the Wistar rats: *Parkia Biglobosa* seeds have been reported to exert therapeutic effect against potassium bromate-induced testicular toxicity (Ezirim *et al*., 2022a), sex hormones perturbations (Iwuoha *et al*., 2022), hepatocellular injury (Onyekachi *et al*., 2022), renal toxicity (Abali *et al*., 2022), sperm cell abnormalities (Ezirim *et al*., 2022b), cardiotoxicity (Ugwu *et al*., 2022a) and coagulation abnormality (Ugwu *et al*., 2023) induced by KBrO3 on Wistar rat..

A very significant multipurpose plant, *Parkia Biglobosa* is used in the environment for food, medicine, and economic purposes (Airaodion *et al*., 2020a). *P. biglobosa* is rich in saponins, tannins, flavonoids, resins, carbohydrates, terpenoids, phenols, sterols, and cardiac glycosides, using various aqueous and organic extracts (Musara *et al*., 2020). Antimalarial, antihelminthic, antibacterial, antidiabetic, antihypertensive, anti-inflammatory, analgesic, anti-carcinogenic, anti-trypanosomic, and antioxidant characteristics were demonstrated by *P. biglobosa* in pharmacological studies (Airaodion *et al*., 2020a; Musara *et al*., 2020). It also contains high level of omega 3 which authenticates it cardioprotective and antioxidant functions (Wokoma and Aziagba, 2010). More studies should be done to unravel the latent potential of this multipurpose plant.

In conclusion, this study revealed that potassium bromate-induced deranged haematological parameters were mitigated by seed extract of *Parkia Biglobosa* in a dose dependent manner. Care must be taken with the consumption of KBrO3 due to its numerous toxicological effects. However, consumption of *P. biglobosa*, a tropical homemade food is recommended for families to benefit from the barrage of its health benefit. This will also alleviate the toxicity effect of $KBrO_3$ if consumed inadvertently. Human clinical trial is needed to substantiate these findings.

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Niger. J. Physiol. Sci. 39 (June 2024): 125 – 135
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

A Polyherbal Remedy, PurXcel improves Cadmium-induced Male Reproductive Impairment and Testicular Antioxidant Status in Wistar Rats

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Summary: Cadmium, despite being an environmental pollutant has a wide range of applications and causes oxidative damage to the testes and impairment of male reproductive function. PurXcel, a polyherbal remedy is said to be rich in antioxidants and improves fertility. But there are no scientific records of its effect on Cadmium-induced male reproductive impairment, hence this study. Twenty male wistar rats were randomly assigned into 4 groups of 5 rats each namely control, Cadmium-only, PurXcel-only and Cadnium+PurXcel groups. Daily treatment with PurXcel lasted for 28 days after which blood samples were collected and testes and epididymis harvested for evaluation of relevant parameters. Body weight changes (BWC) as well as weights of testes and epididymis were significantly reduced in Cadmium-only group ($P<0.05$) compared with the control. PurXcel given alone and in combination with Cadmium significantly increased ($P<0.05$) the BWC as well as testicular and epididymal weights in comparison with the Cadmium-only group. Sperm function indices (count, motility, viability and normal morphology) and reproductive hormones (GnRH, FSH, LH and testosterone) activities were significantly decreased ($P<0.05$) in the Cadmium-only group compared with the control but higher in all treated groups ($P<0.05$) compared with Cadmium-only group. Testicular concentrations of MDA and TBARS were significantly increased in the Cadmium-only group compared with control ($P<0.05$) but reduced in treated groups ($P<0.05$) when compared with Cadmium–only group. The activities of testicular SOD, GPx and Catalase as well as total antioxidant capacity were significantly reduced in the Cadmium-only group compared with control ($P<0.05$) but increased in the treated groups compared with Cadmium-only group ($P<0.05$). Testicular morphometric parameters showed decreases in Sertoli cell count, Leydig cell count, Johnson's score, seminiferous tubules diameter and germinal epithelial height ($P<0.05$) in the Cadmium-only group compared with the control but these were significantly higher in the Cadmium+PurXcel than in the Cadmium-only groups ($P<0.05$). A section of the testes also revealed mainly empty luminal cavities and scanty intervening interstitium in the Cadmium-only group compared with the control. There were loosely packed epididymis and which were mainly empty. We conclude that PurXcel improves Cadmium-induced male reproductive toxicity and given alone, it improves testicular antioxidant status in Wistar Rats.

Keywords*: PurXcel, Cadmium, reproductive parameters, male rats, oxidative stress*

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Manuscript received- November 2023; Accepted- March 2024

DOI: https://doi.org/10.54548/njps.v39i1.16

INTRODUCTION

Infertility, defined by the failure of a couple to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse is a disease of the male or female reproductive system (WHO, 2023, Araoye, 2003). Infertility affects millions of people, having economic and social implications. The WHO (2015) estimates that 1 in 6 people of reproductive age, experience infertility in their life time. Fifteen percent (15%) of all couples in the United States of America and about 180 million couples worldwide are said to have infertility issues with 40 – 50% of the cases attributed to male factor (Brugh and Lipshutz, 2004). Male infertility is regarded as the inability of a male to make a fertile female pregnant after 12 months of regular unprotected intercourse (Leslie *et al.*, 2023; Huang *et al*., 2023). About 42.4% of infertility cases is attributed to the male factor in Nigeria (Ikechebulu *et al*., 2003).

Over the past decades, a downward trend in the quality and quantity of male fertility indices has been observed. Sengupta *et al* (2017) and Levine *et al* (2023) reported a decline in sperm count among men. A downward trend in fertility as demonstrated by decreases in morphologically normal sperms, motility and count as well as a decrease in seminal fluid volume among males over the past decades was also reported by Carlsenal *et al* (1992) and Sengupta *et al* (2012).

Causes of male infertility or sub-fertility vary widely but can be related to congenital anatomical factors, testicular dysfunction, endocrinopathies as well as lifestyle and gonadotoxic exposures (Agarwal *et al*., 2020, Eisenberg *et al*., 2023). Research activities in the recent past have drawn the world's attention to the negative effects of environmental toxins or pollutants on male reproductive health. One of such environmental toxins which also has a wide range of application is Cadmium (Chandel and Gyan, 2014; Angelis *et al*, 2017, Predes *et al*., 2010).

Cadmium (Cd) is one of the most toxic industrial and agriculture-associated heavy metasl that has been widely dispersed in the range of applications (Unsal *et al*, 2020; WHO, 2019). It is used as a coating material in PVC and ship building industry. Cadmium products are also used in the production of plastics, battery, petrochemicals, cigarette, glass, ceramics, rubber, paint, fireworks etc. (WHO, 2019). Exposure to Cd is often through industrial and non-industrial links through drinking water and food affected by contamination by these products. Humans are also exposed to Cd through inhalation of dust or air that have been contaminated by industrial activities and vehicle exhaust fumes as well as exposure to cigarette smoke (Pappas, 2011; WHO, 2019; IPCS, 1992)

Exposure to Cadmium has been reported to have detrimental effects on various organs and systems including the cardiovascular, hepatic, respiratory, renal, bone and reproductive systems (Godt, *et al*, 2006, Gore *et al.*, 2015, Gu *et al*., 2022). Exposure to Cadmium has also been associated with derangement in seminal fluid parameters (Abarikwu *et al*.., 2016; Olanijan *et al.*, 2022; Yang *et al.*, 2022) as well as levels of testosterone, follicle stimulating hormone and luteinizing hormone (Almeer *et al*., 2018; Alkhedaide *et al*., 2016; Olaniyam *et al*., 2022) among others. The pathophysiology of Cadmium-induced cytotoxicity is partly blamed on oxidative stress, which is proven by the ameliorating effects of different antioxidants on Cadmium-induced cytotoxicities (El-Neweshy *et al*, 2012; Abarikwu *et al* 2016).

PurXcel is a plant-based polyherbal dietary supplement that works using the tri-active technology to improve the body's antioxidant defense system (Lobo *et al*., 2010; Lewis *et al.*, 2013) PurXcel is said to contain 18 bioactive substances which are believed to work synergistically to affect health. These bioactive substances include glutathione, superoxide dismutase, Aloe vera, vitamin C, vitamin E, Selenium, N-acetyl L-cysteine, alpha lipoic acid, *Morringa oleifera*, turmeric, broccoli, *Cordyceps simensis*, milk thistle, blue berry, schisandra, grape seed extract, pomegranate and black pepper extract (https://livepure.com) which have antioxidant and anti-inflammatory activities (Forman *et al.*, 2009; Pickspiteteller, 2007; Dean *et al*., 2011). PurXcel is said to provide nutritional support to the body's antioxidant system (Richie *et al.*, 2015; Carillon *et al*., 2014) and improves reproductive health (Lobo *et al.*, 2011). Though PurXcel is said to contain a wide range of bioactive substances individually known to have pro-health effects, there is paucity of information on the effect of this product on male reproductive parameters either given alone or in Cadmium-induced male reproductive toxicity and hence this study.

MATERIALS AND METHODS

Preparation of Stock Solution of PurXcel: The content of one capsule (435mg) of PurXcel (Live Pure, Frisco, Texas, USA) purchased from Puregen African Nigeria Limited, Lagos, Nigeria was dissolved in 200ml of distilled water.

Preparation of stock solution of Cadmium: This was made by dissolving 50mg of Cadmium Chloride, $CdCl_2$, (Sigma-Aldriech, Chemical Company, St Louis, MO, USA) in 50ml of distilled water.

Acute toxicity study: The median lethal dose LD_{50} of PurXcel was estimated using Lorke's method (Lorke, 1983) and followed up by the up and down method as described by Erhirhie *et al*, 2018).

Experimental design: Twenty male wistar rats were randomly divided into four groups (n=5) namely control, PurXcel-only, Cadmium-only and Cadmium+PurXcel. Cadmium (as Cadmium Chloride) was administered at 5mg/kg (Da-Costa et al., 2016; Nna et al., 2017). PurXcel was administered daily for twenty eight days at an oral dose of 38.4mg/kg based on the computation of effective dose described by Nair and Jacob (2016). The control group was given 0.5ml of the vehicle daily. All animals were given rat feeds and water ad libitum. Duration of experimentation was twenty eight days.

Collection of samples: At the end of the treatment period, the animals were anaesthetized, blood samples collected from them via cardiac puncture into plain bottles for determination of relevant serum parameters. The animals were then sacrificed and their testes dissected out for assay of necessary parameters.

Determination of body, testes and epididymal weights: The weights of the animals, their testes and epididymis were measuared with an electronic weighing scale (Scout Pro, Ohaus Corporation, USA) while the relative organ weight was computed as Absolute weight of organ/final body weight x 100/1.

Preparation of testicular homogenate: The left testis of each rat was homogenized separately in 50mm Tris-HCl buffer (pH 7.4) containing 1.15% KCl to prepare a 20% (1/5 w/v) tissue homogenate using Potter Elvelyem homogenizer (BEE International, Apion Company, USA). The homogenate was then centrifuged at 10,000g for 10 minutes in a cold centrifuge (4^{o}C) (YSCF0424AR, Multipurpose centrifuge, Guanghzhou, China). The supernatant was then obtained for determination of necessary testicular parameters.

Evaluation of Sperm Count: The method of Raji *et al* (2006) was used to determine sperm count. In brief, the harvested left cauda epididymis was put in 2ml of normal saline and prewarmed to 37^{o}C. Small incisions were then made in the cauda epididymis and released sperms suspended in normal saline. Two hundred microliter (200µL) of the sperm suspension was transferred into both chambers of the improved Neubauer hemocytometer (Hawksley, England) using a Pasteur pipette. The sperms

were counted in five large Thorma squares with the help of a microscope (Leica DM 750, Switzerland).

Sperm viability: The method of Wyrobek *et al.,* (1983) was used to assess sperm viability. In brief 20µl of sperm suspension was stained with 20µL of 0.05% eosin Y-nigrosine and the mixture incubated for 120 seconds at room temperature. The slides were then viewed under microscopy (Leica DM 750, Switzerland) using X400 magnification. Viable sperms were unstained while non-viable ones stained pink.

Sperm motility: This was done using the Makler's counting chamber as demonstrated by Nna *et al*., (2019). A sperm sample obtained from the vas deferens was introduced into 1ml of normal saline and the mixture stirred gently. A drop of the mixture was then dropped on the Makler's chamber (Self Medical Instruments, Israel) and examined microscopically, counting the cells and expressing it as percentage of the total number of spermatozoa.

Determination of sperm morphology: The method described by Narayana et al., (2005) was used to evaluate this parameter. A drop of epididymal sperm suspension used for sperm count was smeared on a glass slide and stained with 1% eosin Y. The slide was air-dried and examined microscopically (Leica DM 750, Switzerland) at X100 magnification.

Determination of serum concentration of FSH: Serum FSH concentration was evacuated in triplicate using rat FSH Elisa Kits cat No. E.El-R0391 (Elabscience Biotechnology, Wuhan China) and following manufacture's protocol.

Determination of serum LH: Serum LH concentration was determined with rats LH ELISA kit, Cat No. ABIN6574078 (ElabScience Biotechnology, China) and following manufacturer's protocol.

Determination of testosterone: Rat: ELISA Kit (ElabScience Biotechnology, China) was used for this assay and following manufacturer's protocol.

Determination of serum concentration GnRH: This was done with rat GnRH Kit (ElabScience Biotechnology, China) and following manufacturer's protocol.

Evaluation of testicular lipid peroxidation

Malondialdehyde (MDA): The concentration of MDA in testicular homogenate was evaluated using Ohkawa *et al* method (Ohkawa et al, 1979) as also described by Chatterjee *et al* (2000) using commercially available reagents. In brief, a 100ml aliquot of testicular homogenate was added to the reaction mixture that contained 200ml of 8.1% (wt/v) Lauryl sulphate, 1.5ml of 20% (wt/v) acetic acid, 1.5ml of 0.8% (wt/v) thiobarbituric acid and 100ml of distilled water. The mixture was then boiled and centrifuged and the absorbance of the supernatant measured spectrophotometrically.

Thiobarbituric acid reactive substances (TBARS): The level of TBARS in telsticular homogenate was determined as described by Armstrong *et al* (1991) using commercial reagents. Malomdialdehyde as one of the end products of lipid peroxidation reacts with thiobarbituric acid to form a coloured substance whose absorbance is measured spectrophotometrically at 532nm.

Determination of testicular activities of antioxidant enzymes

Superoxide dismutase (SOD): The activity of superoxide dismutase in testicular homogenate was determined according to the method of Sun *et al* (1988) and used by Al-Batran *et al*, (2013) which is based on the ability to inhibit the reduction of nitro tetrazolium-blue and using commercialy available reagents (Sigma-Aldrich, St Louis, USA). Briefly, the homogenate supernatant was recentrifuged at 12000 rpm and the SOD evaluated on the resultant supernatant. 1ml of the reactant (13nM L-methionine, 100nM EDTA, 300uL of 2uM riboflavin and 50nN phosphate buffer, pH 7.8) and the activity read spectrophotometrically at 560nm

Catalase (CAT): Calatase activity was evaluated in testicular homogenate by the method described by Chandran *et al* (2014) using commercially available reagents (Sigma-Aldrich St Louis USA).The method is based on enzyme-catalysed decomposition of H_2O_2 which forms a yellowish complex with molybdate and the absorbance read at 405nm.

Glutathione peroxidase (GPx): This was evaluated in testicular homogenate as described by Luchese *et al* (2009) using H_2O_2 as a substrate.

Total antioxidant capacity (TAC): The TAC was assayed using the method described by Koracevic *et al* (2001). The TAC assay employs a thermal radical generator which produces a steady flux of radicals in solution. The addition of antioxidants results in competitive inhibition of the substrstes.

Histological studies: The harvested right testes were cleaned of connective tissues and fixed in Bouin's fluid and then dehydrated with ethanol before being embedded in paraffin blocks. The blocks were then sectioned and stained with haematoxylin and eosin (H&E) and viewed using light microscope (Leica, DM, 750 Switzerland) at a magnification of x400. The number of Leydig cells per intertubular region and thereafter the average Leydig cell count was computered. Sertoli cells were counted in 20 seminiferous tubules. Johnsen score was assessed in 10 seminiferous tubules (Johnsen, 1970) as used by Aksu *et al* (2017). Image Analyser software (Soft Imaging System, VGA, Utilities Version 3.67c) was used to measure seminiferous tubular diameter and germinal epithelial height in 20 seminiferous tubules chosen from serial sections and their averages computed.

Statistical analysis

Results were presented as mean ± SEM and analysed using statistical package for Social Sciences (SPSS) version 20. One-way analysis of variance (ANOVA) was employed to analyse the data and Tukey Post hoc test used to compare the mean values. Values of $P<0.05$ were considered statistically significant.

RESULTS

Acute toxicity study: There were no mortality or significant behavioural changes up to a dose of 5000mg/kg. The LD_{50} of PurXcel was therefore greater than 5000mg/kg.

Body Weights in different groups: There was significant reduction (P<0.05) in body weight in the Cadmium-only (15.2±5.42) compared with control (28±9.92) groups but higher body weights in the PurXcel-only (25.4±7.23) and Cd+PurXcel (5.6±1.14) than in the Cadmium-only group (P<0.05). This is shown in table 1.

Testes and epididymal weights: The absolute/relative weights of the testes were significantly reduced in the Cadmium-only (1.98±0.19/ 0.92±0.10)) group compared with control (3.56±0.16/ 1.37±0.06) group (P<0.05) but significantly higher in the PurXcel-only (3.52±0.15/ 1.37±0.04) and Cd+PurXcel (2.36±0.17/ 1.00±0.07) than in the Cadmium-only (1.98±0.19/ 0.92±0.10) groups (P<0.05). The absolute/relative weights of the epididymis were significantly reduced (P<0.05) in the Cadmium-only (1.4±0.15/ 0.53±0.06) compared with control (1.5±0.16/ 0.58±0.05) but significantly higher in the PurXcel-only (1.5±0.17/ 0.60±0.06) than in the Cadmium-only (1.4±0.15/ 0.53±0.06) group (P<0.05). These results are shown in Table 1.

Comparison of sperm parameters in different groups

Sperm count: The mean±SEM sperm count ($\times 10^6$/L) of the control, Cadmium–only, PurXcel-only and Cadmium+PurXcel groups were 55.60± 4.57, 29.92±3.47; 62.60± 3.73 and 46.70±4.35 respectively. Sperm count was significantly decreased in the Cadmium-only and Cd+PurXcel compared with control groups (P<0.05) and significantly higher in the PurXcel-only compared with control and Cadmium-only groups (P<0.05). It was also signfiicnalty higher in the Cadmium+PurXcel than in the Cadmium-only groups (P<0.05). These result are shown in Table 2.

Sperm motility: Sperm motility (%) was significantly reduced (P<0.05) in the Cd-only (52.20±3.90) and Cd+PurXcel (67.20 ± 3.12) groups compared with control (76.20±3.11) though significantly higher (P <0.05) in the Cd+PurXcel (67.30±3.12) group compared with control (76.20 ± 3.11) and higher in the PurXcel–only (86.20± 5.12) compared with control (P<0.05). It was also significantly lower in Cd+PurXcel than PurXcel–only groups (P<0.05) as shown in Table 2.

Sperm viability: Sperm viability (%) was significantly reduced (P<0.05) in Cadmium–only (50.80 ± 6.28) compared with control (70.20±4.49) groups. It was however significantly higher (P<0.05) in the PurXcel-only (77.80± 7.36) and Cd+PurXcel (71.40±6.88) than in the Cd-only groups (P<0.05). This is shown in Table 2.

Sperm morphology: The percentage of morphologically abnormal sperms was significantly higher in the Cd–only (28.20±3.27) than in the control (9.60±2.41) and significantly reduced (P<0.05) in the PurXcel–only (7.60±3.05) compared with Cadmium-only (P<0.05) groups. It was significantly higher in the Cd+PurXcel (16.40±4.16) than in the PurXcel–only groups (P<0.05). This is shown in Table 2

Testicular oxidative stress biomarkers

Malomdialdehyde concentration: Testicular concentrations of MDA (mmol/mg protein) were 2.88±0.25, 9.66±0.59, 2.92±0.23 and 4.40±0.44 for control, Cd-only, PurXcel-only and Cd+PurXcel groups respectively. Malondialdehyde was significantly increased in Cd-only and Cd+PurXcel compared with control (P<0.05) but significantly lower in PurXcel-only and Cd+PurXcel than Cd-only groups (P<0.05). It was however higher in the Cd+PurXcel than in the PurXcel–only groups (P<0.05) as shown in Table 3.

Table 1:
Body weight, absolute and relative weights of testes and epididymis

Group	Initial body weight (g)	Final body weight (g)	body weight change (g)	Absolute Testis weight (g)	Relative testis weight (g)	Absolute epididymis weight (g)	Absolute epididymis weight (g)
Control	231.00±8.46	259.00±5.34	28.00±9.92	3.56±0.21	1.37±0.06	1.50±0.16	0.58±0.05
Cadmium	230.00±1.58	214.80±5.45*	15.20±5.45*	1.98±0.19*	0.92±0.10*	1.14±0.15*	0.53±0.06
PurXcel	230.80±6.98	256.20±5.26[a]	25.40±7.23[a]	3.52±0.15[a]	1.37±0.04[a]	1.54±0.17[a]	0.60±0.06
Cadmium + PurXcel	231.40±2.70	237.00±2.74*[ab]	5.60±1.14*[ab]	2.36±0.17*[ab]	1.00±0.07*[a]	1.32±0.13	0.56±0.05

*Values are presented as mean ±SEM, n = 5. * = p<0.05 vs control; a = p<0.05 vs Cadmium ; b = p<0.05 vs PurXcel*

Table 2:
Sperm count, motility, viability and morphology

Group	Sperm count	Motility	RPFM	SPFM	RM	Sperm viability	Abnormal Morphology
Control	55.60±4.57	76.20±3.11	32.40±1.67	21.20±3.77	22.60±2.30	70.20±4.49	9.60±2.41
Cadmium	29.92±3.47	52.20±3.90*	22.40±2.88*	17.00±2.12	12.80±1.92*	50.80±6.38*	28.20±3.27*
PurXcel	62.603.73[a]	86.205.12*[a]	44.605.50*[a]	23.601.67[a]	18.001.58*[a]	77.807.36[a]	7.603.05[a]
Cadmium+ PurXcel	46.704.35*[ab]	67.205.63*[ab]	36.402.41[ab]	15.603.44[b]	15.202.39*	71.406.88[a]	16.4016*[ab]

*Values are presented as mean ±SEM, n = 5. * = p<0.05 vs control; a = p<0.05 vs Cadmium; b = p<0.05 vs PurXcel*

Testicular TBARS: Activities of TBARS (nmol/mg protein) for control, Cd–only, PurXcel–only and Cd+PurXcel groups were 1.76±0.32, 11.02±0.64, 1.98±0.65 and 5.52±0.59 respectively. The activities of TBARS was significantly increased in Cd–only and Cd–PurXcel compared with control (P<0.05) but lower in PurXcel–only and Cd+PurXcel than Cd- only groups (P<0.05). It was however significantly higher in the Cd+PurXcel than in the PurXcel-only groups as shown in Table 3.

Superoxide dismutase (SOD) acivity: The activities (IU/mg protein) of SOD in the control, Cd–only, PurXcel–only and Cd+PurXcel groups were 7.62±0.67, 3.04±0.38, 11.1±1.16 and 8.14±0.84 respectively. Superoxide dismutase was significantly reduced (P<0.05) in Cd–only but higher (P<0.05) in the PurXcel groups compared with control. It was significantly higher in PurXcel–only and Cd+PurXcel than Cd–only group though significantly lower in Cd+PurXcel than PurXcel-only groups as shown in Table 3.

Glutathione peroxidase activity (GPx): The activities of GPx (IU/mg protein) were 4.08±0.39, 1.02±0.16, 6.74±0.34 and 3.24±0.46 for control, Cd–only, PurXcel–only and Cd+PurXcel respectively. The activity of GPx was significantly reduced (P<0.05) in the Cd–only, and Cd+PurXcel (P< 0.05), but increased in PurXcel-only (P < 0.05) groups compared with control. It was significantly higher in PurXcel–only and Cd+PurXcel (P < 0.05) than Cd–only groups though lower in the Cd+PurXcel compared with Cd–only groups as shown in Table 3.

Catalase (CAT) activity: The activities of CAT were 77.7±2.20, 49.82±1.19, 82.03±2.48 and 70.98±2.20 for control, Cd–only, PurXcel–only and Cd+PurXcel groups respectively. The activity was significantly reduced in the Cd–only, and Cd+PurXcel (P< 0.05), but higher in PurXcel-only (P < 0.05) groups than the control. It was higher in PurXcel–only and Cd+PurXcel (P < 0.05) than Cd–only groups (P<0.05) but lower in Cd+PurXcel than in the PurXcel–only (P<0.05) groups as shown in Table 3.

Total antioxidant capacity (TAC): The TAC (nmol uric acid Eq/mg protein) in control, Cd–only, PurXcel-only and Cd-PurXcel were 171.82±5.46,95.82±4.60, 194.46±4.16 and 165.54±4.59 respectively. It was significantly reduced (P<0.05) in the Cd–only but increased (P< 0.05) in PurXcel-only groups compared with the control. TAC was significantly higher in PurXcel–only and Cd+PurXcel groups (P< 0.05) than Cd–only groups though significantly lower (P< 0.05) in the Cd+PurXcel than PurXcel-only groups as shown in Table 3

Histology of testes and epididymis

Johnsen scores: The Johnsen score for control, Cd–only, PurXcel-only and Cd+PurXCel groups were 8.72 ± 0.49, 3.67 ± 0.70, 9.22 ± 0.33 and 6.68 ± 1.16 respectively. It was significantly decreased (P<0.05) in Cd–only and Cd+PurXcel groups compared with control but higher in the PurXcel–only and Cd+PurXcel groups than in the Cd-only group. It was lower in the Cd+PurXcel than in the PurXcel-only group as in Table 4.

Leydig cell count: Leydig cell count (cells/ITR) in the control, Cd–only, PurXcel–only and Cd+PurXcel groups was 4.40 ± 0.41, 2.76 ± 0.30, 4.24 ± 0.24 and 3.66 ± 0.38 respectively. There was significant decrease in Leydig cells in Cd-only group compared with control (P < 0.05) but significantly increased in the PurXcel–only and PurXcel+Cd groups (P< 0.05) compared with the control as shown in Table 4.

Sertoli cell count: Sertoli cell counts (cells/STR) in the control, Cd-only, PurXcel–only and Cd+PurXcel were 9.32 ± 0.38, 2.76 ± 0.30, 9.14 ± 0.78 and 6.28 ± 0.55 respectively. Sertoli cell count was significantly reduced in the Cd–only and Cd+PurXcel groups compared with the control (P< 0.05) but significantly higher (P< 0.05) in the PurXcel–only and Cd+PurXcel than Cd-only groups. It was also significantly lower in Cd+PurXcel than in the PurXcel–only (P< 0.05) groups as shown in Table 4.

Seminiferous tubule diameter: The tubular diameter (µm) in the control, Cd–only, PurXcel–only and Cd+PurXcel were (30.30 ± 3.36, 97.79 ± 3.98, 136.87 ± 3.00 and 120.45 ± 3.34 respectively. It was significantly decreased in Cd–only and Cd+PurXcel (P < 0.05) compared with the control but higher in PurXcel–only and Cd+PurXcel than in the Cd–only groups (P< 0.05). It was also significantly lower in Cd+PurXcel than in the PurXcel–only (P< 0.05) groups as shown in Table 4.

Germinal epithelial thickness: Germinal epithelial thickness (µm) in control, Cd–only, PurXcel–only and Cd+PurXcel groups were 36.63 ± 2.78, 17.46 ± 2.74, 32.62 ± 2.69 and 25.98 ± 3.48 respectively. It was significantly decreased in Cd–only and Cd+PurXcel groups compared with control (P< 0.05) though significantly higher in PurXcel–only and Cd+PurXcel than PurXcel–only groups. It was also significantly lower in Cd+PurXcel than in the PurXcel–only groups as shown in Table 4.

Table 3:
Antioxidant activity of the different experimental groups

	MDA	TBARS	SOD	GPx	CAT	TAC
Control	2.88±0.25	1.76±0.32	7.62±0.67	4.08±0.39	77.79±2.20	171.82±5.46
Cadmium	9.66±0.59*	11.02±0.64*	3.04±0.38*	1.02±0.16*	49.82±1.19*	95.82±4.60*
PurXcel	2.92±0.23[a]	1.98±0.47[a]	11.10±1.16*[a]	6.74±0.34*[a]	82.03±2.48*[a]	194.46±4.16*[a]
Cadmium + PurXcel	4.40±0.44*[ab]	5.52±0.59*[ab]	8.14±0.82[ab]	3.24±0.46*[ab]	70.98±1.75[ab]	165.54±4.59*[ab]

Values are presented as mean ±SEM, n = 5.
** = p<0.05 vs control; a = p<0.05 vs Cadmium; b = p<0.05 vs PurXcel*

Table 4:
Testicular morphometric indices of the different experimental groups

	Johnsen's Score	Leydig cell count	Sertoli cell count	Tubular diameter (Microns)	Germinal Epithelial Height
Control	8.72±0.49	4.40±0.41	9.32±0.38	130.30±3.16	36.63±2.78
Cadmium	3.67±0.70*	1.88±0.35*	2.76±0.30*	97.79±3.98*	17.46±2.74*
PurXcel	9.22±0.33[a]	4.24±0.24[a]	9.14±0.78[a]	136.87±3.00[a]	32.62±2.69[a]
Cadmium + PurXcel	6.68±1.16*[ab]	3.66±0.38[a]	6.280. ±55*[ab]	120.45±3.34*[ab]	25.98±3.48*[ab]

Values are presented as mean ±SEM, n = 5.
** = p<0.05 vs control; a = p<0.05 vs Cadmium ; b = p<0.05 vs PurXcel*

Male reproductive hormones

Serum gonadotropin-releasing hormone (GnRH): The mean ± SEM serum concentrations of GnRH (Pg/ml) were 2.32±0.32, 1.18±0.15, 3.80±0.84 and 2.34±0.38 for control, Cadmium-only, PurXcel–only and Cd+PurXcel respectively. The result shows a significant decrease (P<0.05) of GnRH in the Cd-only compared with the control groups and increased in the PurXcel–only group compared with control. GnRH was significantly higher (P<0.05) in both PurXcel–only and Cd+PurXcel groups compared with Cd-only group, though lower in the Cd+PurXcel than PurXcel–only groups (P<0.05). This is shown in Fig 1.

Serum testosterone: The concentrations of testosterone (ng/ml) were 3.08 ± 0.40, 6.14 ± 0.14, 1.98 ± 0.28, 6.06± 0.21 and 4.24 ± 0.43 for control, Cd-only, PurXcel-only and Cd+PurXcel respectively. Testosterone was significantly reduced in the Cd-only compaà lolred with the control and higher (P<0.05) in the PurXcel–only compared with the control. It was also higher in the PurXcel-only and Cd+PurXcel (P< 0.05) than in the Cd-only groups but lower in the Cd+PurXcel (P<0.05) than in the PurXcel–only groups as shown in Fig. 2

Serum LH: The mean ± SEM of LH (IU/ml) were 5.20 ± 0.45, 2.76 ± 0.11, 6.74 ± 0.29 and 4.12 ± 0.31 for control, Cd-only, PurXcel-only and Cd+PurXcel respectively. Serum LH was significantly reduced in Cd-only and Cd+PurXcel (P<0.05) compared with control but higher (P<0.05) in PurXcel–only than in the control. It was significantly higher in Cd+PurXcel and PurXcel–only (P<0.05) groups than Cadmium-only groups though it was lower in the Cd+PurXcel than PurXcel-only groups as shown in Fig. 3.

Serum follicle stimulating hormone: Serum levels of FSH (ng/ml) were 6.20±0.53, 2.68±0.30, 7.96±0.18 and 5.13±0.36 for control, Cadmium-only, PurXcel-only and Cadmium+PurXcel groups respectively. Serum FSH levels were significantly reduced in the Cadmium-only and Cadmium+PurXcel (P<0.05) compared with the control but higher in the PurXcel-only than in the control groups. It was significantly higher in the PurXcel-only and Cadmium+PurXcel than in the Cadmium-only groups as shown in Fig. 4.

Photomicrographs of sections of the testes and epididymis in different experiment groups: Plate 1a shows a section of the testis in the control group exhibiting numerous seminiferous tubules of various sizes with intact basement membranes, most of the tubules containing numerous spermatozoa. There are 10 – 12 Sertoli cells per tubule and 3 – 5 Leydig cells per interstitium.

Plate 1b is a section of the testis in Cd–only group showing uniform seminiferous tubules with an intact basement membrane containing spermatogonia at various stages of maturation. The tubules are mostly 3 to 5 cell layers thick with the luminal cavities mainly empty. The intervening interstitium are scanty with 3 – 5 Leydig cells. The Sertoli cells are up to 9 to 10 per tubule.

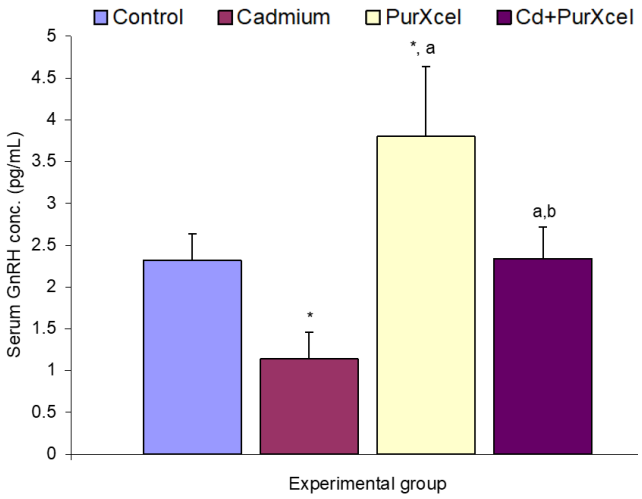

Figure 1
Serum gonadotropin-releasing hormone concentration in the different experimental group.
Values are presented as mean ±SEM, n = 5.
** = p<0.05 vs control; a = p<0.05 vs Cadmium ; b = p<0.05 vs PurXcel*

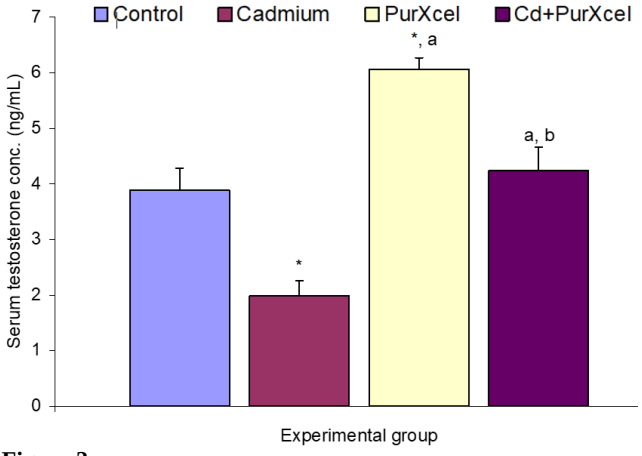

Figure 2
Serum testosterone concentration in the different experimental group.
Values are presented as mean ±SEM, n = 5.
** = p<0.05 vs control; a = p<0.05 vs Cadmium;*
b = p<0.05 vs PurXcel

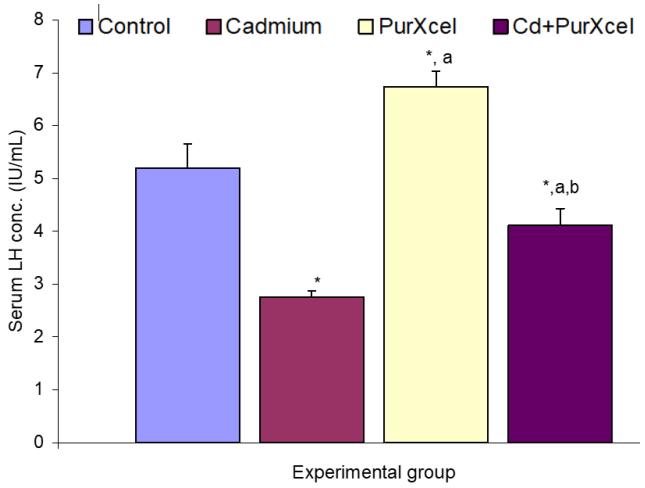

Figure 3
Serum LH concentration in the different experimental group.
Values are presented as mean ±SEM, n = 5.
** = p<0.05 vs control; a = p<0.05 vs Cadmium;*
b = p<0.05 vs PurXcel

Plate 1c is a section of testis in PurXcel–only group showing numerous widely spaced seminiferous tubules with intact basement membrane. The spermatogonic cells are in various stages of maturation. The luminal cavities are filled with spermatozoa.

Plate 1d shows a section of testis in Cd+PurXcel group which shows closely packed seminiferous tubules with intact basement membrane. The tubules contain proliferating spermatogomia at various stages of development. The cells are 3 - 5 cell layers thick consisting of spermatogonia, spermatocytes, and moderate amounts of spermatids and spermatozoa.

Plate 2a is a section of the epididymis in the control group showing prominent tubules separated by a loose stroma with an intact basement membrane. The lumens are filled with spermatozoa.

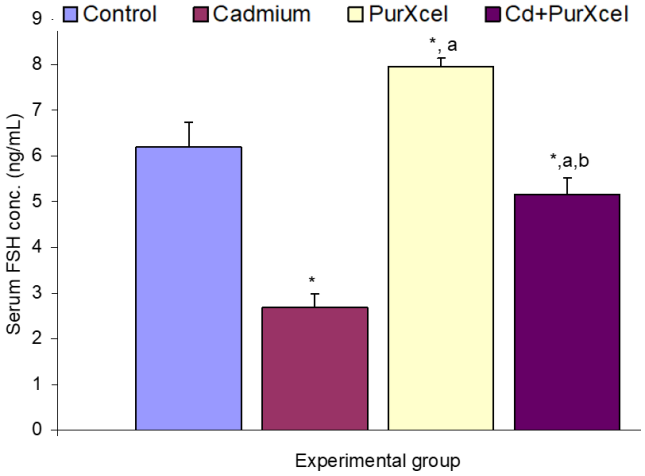

Figure 4
Serum FSH concentration in the different experimental group.
Values are presented as mean ±SEM, n = 5.
** = p<0.05 vs control; a = p<0.05 vs Cadmium;*
b = p<0.05 vs PurXcel

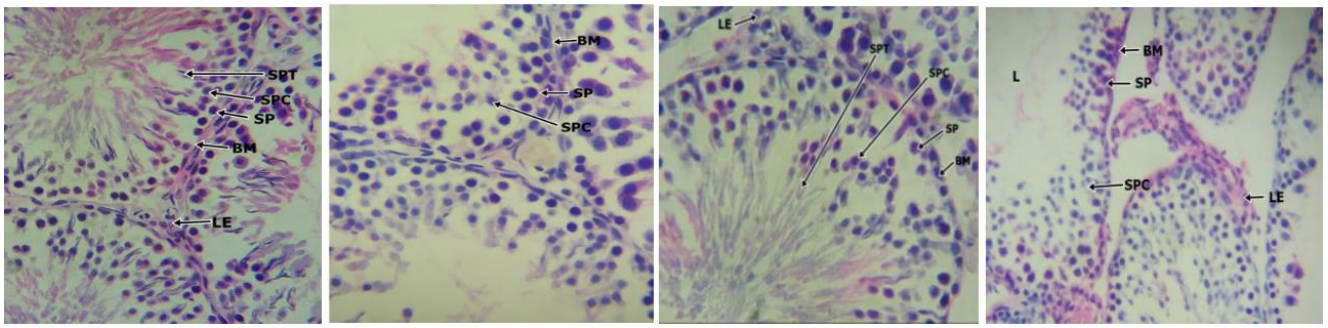

Plate 1:
Section of testis showing seminiferous tubules in a) control group, b) cadmium group, c). PurXcel group and d). Cd + PurXcel group, x400 magnification.
BM-basement membrane, SP-spermatogonia, SPC-spermatocytes, SPT- spermatid, L – Lumen; LE – Luminal endothelium

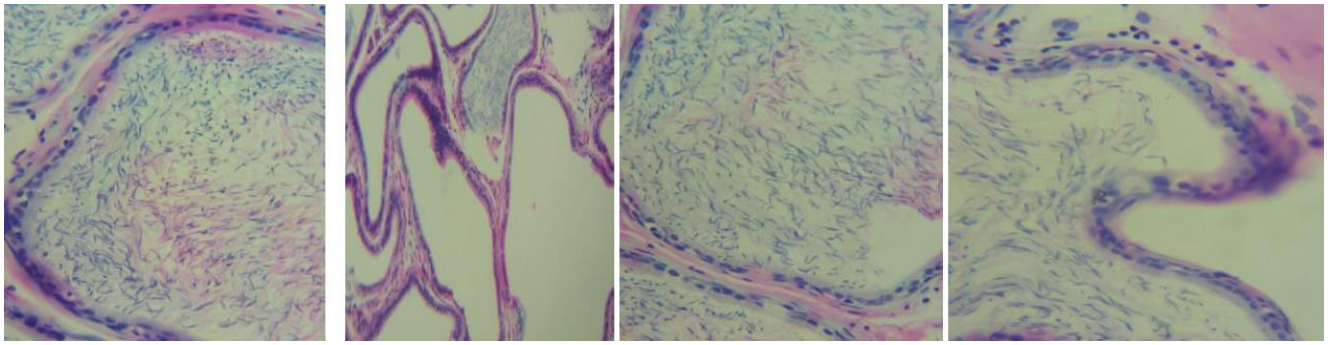

Plate 2:
Section of epididymis in a) control group; b) cadmium group; c) PurXcel group and d) Cd + PurXcel group, x400 magnification
STRO = loose stroma, EPI = lining epithelium, SPZ = spermatozoa

Plate 2b shows a section of epididymis in the Cd–only group and shows loosely packed, fibrotic epididymal tubules with an intact basement membrane. The tubules contain scanty spermatozoa with majority of the tubules being empty.

Plate 2c is a photomicrograph of the section of epididymis in the PurXcel–only group and shows prominent tubules separated by stroma. The tubules are dilated with intact basement membrane. The lumens contain dense spermatozoa.

Plate 2d is a section of epididymis of the Cd+PurXcel groups and shows widely spaced tubules with intact basement membrane and abundant intervening stroma. The tubules contain scanty spermatozoa with majority of the tubules being empty while few contain degenerated spermatozoa.

DISCUSSION

The study investigated the effect of PurXcel which is said to contain antioxidants and improves reproductive health on Cd–induced male reproductive toxicity associated with oxidative stress.

The non-significant differences in the initial body weights of the rats means they were weight-matched. The observed progressive weight loss in the Cadmium-only group is similar with the finding of Nna *et al* (2017) and Olaniyan *et al,* (2021).The decrease in body weight might have been due to the toxic nature of the metal (Godt *et al*., 2006, Aitken and Curry, 2010). The rather increase in body weight that was noted in the PurXcel–only group and Cd+PurXcel compared with Cd–only groups suggests the ability of PurXcel to counter the mechanisms responsible for the weight loss.

Our results show decreased testicular and epididymial weights in the Cd–only group, a finding consistent with that made by El-neweshy *et al* (2012) and Nna *et al.,* (2017) which they attributed to necrosis and degeneration of the testis. PurXcel given alone or in combination with Cd improved these weights likely because of their possible antioxidant effects occasioned by the numerous antioxidant factors it contains. Necrosis and degeneration of tissues are associated with oxidative stress (Choi *et al.*, 2009).

Semen analysis is the cornerstone for male fertility evaluation (WHO, 2003). Therefore, alterations in any of the constituents will impair sperm function leading to infertility. Our study shows derangement in sperm function indices (count motility, viability and morphology) in the Cadmium-only group compared with the control which is in line with the findings made by Asadi *et al* (2014), Oliveira *et al.,* (2009) and Abarikwu *et al.,* (2016). The improvement in these indices following administration of PurXcel alone or in combination with Cadnium shows the ability of PurXcel to improve sperm function indices and to combat the negative effects of Cadnium on sperms. This might have been made possible due to supplemented antioxidants in PurXcel (Richie *et al.,* 2015) snce a major mechanism by which these indices are disrupted is oxidative stress (Abarikwu *et al.*, 2016; El-Neweshy *et al.*, 2012).

Administration of Cadmium resulted in significant decreases in serum GnRH, luteinizing hormone follicle stimulating hormone and testosterone compared with the control producing a hypogonadotropic hypogonadism. This effect on FSH, LH and testosterone secretion is similar to the report by Olaniyan *et al* (2021) and Almeer et al (2018). Cadmium is a known endocrine disruptor (Takiguchi and Yoshihara, 2006). The decrease in serum GnRH could have been due to a primary toxic effect on the GnRH–secreting cells in the hypothalamus.

The reduced levels of FSH and LH might have been due to insufficient stimulation of the gonadotropes in the anterior pituitary by the low GnRH levels (Guyton and Hall, 2011). The low levels of testosterone might have been due to testicular toxicity or the low LH and FSH which are necessary for proper functioning of the testis and secretion of testosterone (Guyton and Hall, 2011). The concentrations of these hormones significantly increased when Cd was co-administered with PurXcel demonstrating the ability of PurXcel to ameliorate the effect of Cd. PurXcel administered alone significantly improved the serum concentration of these hormones compared with the control and the Cadmium+PurXcel groups indicating that PurXcel improves male reproductive hormones function. These effects could be attributed to its rich phytonutrients (Livepure.com).

Oxidative stress is a major mechanism implicatied in Cadmium-induced cytotoxicity (Abarikwu *et al.,* 2016, Takeshima *et al.,* 2021). Our observed increases in MDA and TBARS and decreased levels of GPx, SOD, CAT and TAC in the Cd-only group compared with control supports other studies that Cd induces oxidative stress in tissues (Turner and Lysiak, 2008; Abarikwu *et al.,* 2016). Oxidative stress is evaluated indirectly by measuring the final products of lipid peroxidation (example, MDA and TBARS) and concentration of antioxidants (GPx, SOD and CAT) as well as TAC (Aprioku, 2013, Zim and Schlegel, 1996). Lipid peroxidation (MDA and TBARS) though lower, in the PurXcel-only and the Cd+PurXcel than in the Cd–only groups, it was significantly reduced in the Cd+PurXcel compared with the Cadmium-only group indicating that the ability of PurXcel to relief lipid peroxidation could be limited. Increases in the levels of SOD, GPx CAT and TAC in same groups were observed and supports the fact that PurXcel alone or even if administered with Cd could relief or improve oxidative processes in testicular tissue.

Johnsen score (graded 1 – 10) provides a connection between the results of seminal analysis and those of testicular biopsy/histology and considers the thickness of the germinal epithelium, number and type of sperm cells seen/spermatogenesis as well as presence or absence of Sertoli cells etc (Johnsen, 1970; Gune *et al.,* 2019). The reduced Johnsen score noted in the Cd-only rats compared with the control is similar to the observation by Mohammad *et al.*, (2019) which positively relates with impaired spermatogenesis by Cd as earlier reported. The tubular diameter, Sertoli cells, germinal epithelial height and Leydig cells were also decreased in Cd-only group compared with the control. However, there were improvements in Johnsen score following co-administration of Cd with Purxcel though significantly lower in this group than in the control. This indicates that Purxcel improves Johnsen score indices in Cd-induced testicular toxicity but does not have significant effect on these indices in normal/healthy testes.

A section of the epididymis in the control group (Plate 5) shows prominent tubules separated by loose stroma and an intact basement line. The lining epithelium is columnar with thin lamina propria and muscular layer. The lumens are filled with spermatozoa. In the epididymal section of the Cadmium-only group (Plate 6), the tubules have an intact basement membrane and are loosely packed. The tubules are largely empty containing scanty spermatozoa. Plate 7 (PurXcel-only) is a section of epididymis showing prominent dilated tubules with an intact basement membrane and lumens filled with spermatozoa. The epididymal section of the Cd+Purxcell group (plate 8) shows widely spaced tubules with an intact basement membrane and abundant intervening stroma. The tubules contain scanty spermatozoa and most of them are empty.

Findings from this study indicate that given alone to normal rats, Purxcel does not significantly affect lipid peroxidation (MDA, TBARS) but improves the antioxidative status (SOD, CAT, GPx, TAC) of the testis. In Cadmium-administered rats, PurXcel ameliorates oxidative stress in the rats testes. Purxcel given to normal rats does not affect sperm parameters except motility which it increases. Administered alone, Purxcel increases the serum levels of male reproduction hormones and when given to Cd-administered rats, it improves the levels of these hormones and Johnsen score.

In conclusion, PurXcel ameliorates Cadmium-induced male reproductive toxicity and and oxidative stress and improves sperm motility and testicular redox status when administered alone

Ethical Approval: *The ethical approval for this study was obtained from the Animal Research Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria (Approval number 256PHY2103).*

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Niger. J. Physiol. Sci. 39 (June 2024): 137 – 147
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Effect of *Moringa oleifera* Feed Inclusion on Nω-nitro-L-arginine methyl ester (L-NAME)-induced Hypertension in a Rat Model

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Summary: *Moringa oleifera* (MO) has been recognized for its numerous beneficial properties. This study aimed to evaluate the potential antihypertensive effects of MO seeds in rats subjected to Nω-nitro-L-arginine methyl ester (L-NAME) exposure. Fifty male Wistar rats were randomly divided into five groups of 10 rats each for the experiment. Group A served as the control, received normal saline only, Group B received L-NAME (40 mg/kg) only, Group C received L-NAME (40 mg/kg) + 10% MO feed, Group D received L-NAME (40 mg/kg) + 20% MO feed, and Group E received L-NAME (40 mg/kg) + Lisinopril (10 mg/kg). Treatment was daily and covered a period of 5 weeks. Blood pressure and electrocardiographic measurements were obtained using a non-invasive tail cuff blood pressure device and a 6/7 lead computer ECG equipment, respectively. Heart and kidney tissues were analyzed for oxidative stress parameters, and immunohistochemistry and histopathology of the heart and kidney were conducted using standard methods. L-NAME treatment led to a significant increase in diastolic and systolic values compared to the control group. Serum nitric oxide concentration significantly decreased in rats that received L-NAME alone, while co-treatment with MO and Lisinopril showed a significant increase in nitric oxide levels. Co-treatment with MO and Lisinopril significantly reduced malondialdehyde (MDA) concentrations in the cardiac and renal tissues, whereas L-NAME alone caused a significant increase in MDA concentration. The expressions of cardiac and renal caspase-3 significantly increased in L-NAME alone treated rats, while co-treatments with MO and Lisinopril significantly reduced the expressions of caspase-3. In conclusion, co-treatment with MO effectively reduced arterial pressure and indices of hypertension in rats, mitigated the oxidative stress and apoptosis induced by L-NAME. Therefore, the inclusion of MO seeds in hypertension management may serve as an effective remedy.

Keywords*: Moringa oliefera, L-NAME, Hypertension, Oxidative Stress, Nitric oxide*

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Manuscript received- December 2023; Accepted: March 2024

DOI: https://doi.org/10.54548/njps.v39i1.17

INTRODUCTION

Hypertension poses significant health burden and has profound implications for critical organs such as the kidney and the heart (Aydogdu *et al*., 2019; Panthiya *et al*., 2022). Epidemiological studies have indicated an increase trend in the occurrence of hypertension globally (Li *et al*., 2020). Effective control and prevention of hypertension will largely reduce the occurrence of cardiovascular and kidney diseases (Aydogdu *et al*., 2019). A common underlying factor in the pathophysiology of hypertension is inhibition of endothelial nitric oxide synthase (NOS) activity, which leads to reduction in nitric oxide (NO) production and consequently hypertension (Boe *et al*., 2013; Panthiya *et al*., 2022).

Nω-nitro-L-arginine methyl ester (L-NAME) is a nitric oxide synthase inhibitor which has been used widely to induce hypertension in rat models. Previous research has elucidated that the reduction of NO due to the actions of L-NAME can potentially lead to the senescence of endothelial cells (Silva *et al*., 2017). Production of NO has shown to decrease with increase in age (Leo *et al*., 2015). Long term inhibition of NOS in rats by L-NAME has been reported to result in increased vascular expression of plasminogen activator inhibitor-1 (Boe *et al*., 2013). However, the effects of long term use of L-NAME does not only mediate

inhibition of endothelial NOS, but has also been reported to increased oxidative stress (Chia *et al*., 2021). Chronic L-NAME treatment in rats has been deployed as a chemical method for the induction of nitric oxide deficiency - inducing endothelial malfunction (Leo *et al*., 2015). L-NAME administration has been found to increase arterial blood pressure, cause vascular dysfunction, and alterations of vascular tissues after many weeks of continuous administration (Panthiya *et al*., 2022). Liver injury has also been reported after chronic exposure of rats to L-NAME, with increased serum concentrations of cholesterol, triglyceride, alanine transaminase and aspartate transaminase activity (Li *et al*., 2020). Nitric oxide acts in parts by stimulating soluble guanylyl cyclase (sGC). The sGC coverts guanosine triphosphate to cyclic guanosine monophosphate which in turn activates cyclic guanosine monophosphate dependent protein kinase, leading to relaxation of vascular smooth muscle (Leo *et al*., 2015). A number of NO signaling pathways in the smooth muscle contribute to increase development of vascular diseases (Ma *et al*., 2023). Increase in oxidative stress, reduction of NO concentration, and down-regulation of endothelial NOS in vascular tissues are associated with the toxic effects of L-NAME in Wistar rats (Panthiya *et al*., 2022). Oxidative stress has been associated with the pathophysiology of hypertension (Aremu *et al*., 2019).

Moringa oleifera (MO) is a plant widely distributed in tropical and subtropical countries (Ghasi *et al*., 2000). It has been used as a very rich source of protein in animals feed (Tutubalang *et al*., 2022). MO has been studied for its high quality of antioxidants, including, polyphenols, polysaccharides, alkaloids and nutritional benefits (Abdel-Raheem and Hassan, 2021). It has been reported to be abundant in secondary metabolites, vitamins, and carotenoids that are of good benefits and could cause improvement in animals' performance (Lu *et al*., 2016; Emam *et al*., 2022). It has hypocholesterolemic effects in obese individuals (Ghasi *et al*., 2000). The use of MO in animals' diet has attracted immense research works due to its numerous biological and pharmacological actions (Sultana *et al*., 2021; El-Kassas *et al*., 2022). MO has been demonstrated to significantly increase the concentration and activities of antioxidant enzymes and pro-inflammatory cytokines (Leo *et al*., 2015; El-Kassas *et al*., 2022). The carotenoids and vitamin E present in MO have been demonstrated to support diverse physiological functions of epithelial tissues, visceral organs, mucosal epithelial secretions, and cellular immunity by shielding cells from harmful free radicals (Zaneb *et al*., 2017; Mansour *et al*., 2018; Tutubalang *et al*., 2022). In addition, MO has been sobserved for its antimicrobial and immunomodulatory attributes in poultry (Zaneb *et al*., 2017; Moreno-Mondoza *et al*., 2021). Considering the reported escalating cases of hypertension globally and the proven health benefits of MO, this study was designed to investigate the impact of MO seed-inclusion in feed on L-NAME-induced hypertension in male Wistar rats.

MATERIALS AND METHODS

Preparation of MO feed inclusion: Dried seeds of MO were obtained and ground into powder. Thereafter, they were formulated into 10% and 20% feed content in a commercial feed mill and pelleted.

Experimental Animals: Fifty male Wistar rats, weighing approximately 90-150 g each were obtained from the animal house of Faculty of Veterinary Medicine of University of Ibadan. They were kept in plastic cages and allowed to acclimatize for 4 weeks after which their weights were >170-250 g in the Faculty of Veterinary Medicine animal house before the experiment. The animals were maintained on pelletized growers' feed and tap water ad libitum. The animals were divided into five groups of ten each and kept under natural photoperiod of about 12 h light and 12 h darkness daily.

Experimental Design: The animals were randomly divided into five (5) groups of ten (10) animals per group and assigned as follows: Group A: Normal saline only (orally); Group B: L-NAME only (orally); Group C: L-NAME+ 10% *Moringa oleifera* inclusion in feed; Group D: L-NAME + 20% *Moringa oleifera* inclusion in feed; Group E: L-NAME + 10 mg/kg bw Lisinopril (orally).

Induction of Hypertension and Blood Pressure Measurement: Hypertension was induced through oral administration of L-NAME (40 mg/kg/day) for 5 weeks. A non-invasive tail cuff blood pressure system (CODA™ tail-cuff blood pressure system, Connecticut, USA) was used to measure rats' blood pressure. The blood pressure of rats was measured on day 36 after the commencement of L-NAME administration. The systolic and diastolic blood pressures were measured after drug administration. Blood pressure was measured, three readings were recorded per group and the mean value was used as the blood pressure measurement.

Electrocardiography: The electrocardiographic evaluation of the rats was done using a 6/7 lead computer ECG machine, EDAN VE-1010 as earlier described (Omóbòwálé *et al*., 2018).

Blood collection: Blood was collected on day 36 of experiment through the retro-orbital sinus using capillary tubes into heparinised tubes (Parasuraman *et al*., 2010). Blood samples were allowed to stand for 20 mins to allow coagulation and thereafter centrifuged at 10,000rpm for 10 mins. Sera were stored at -20^{o}C until analysed.

Animal Sacrifice: All rats were sacrificed on day 36 of the experiment by quick cervical dislocation (Aguwa *et al*., 2020). The hearts and kidneys were harvested, rinsed briefly in normal saline and blotted with filter paper before being kept at -20^{o}C until biochemical analysis. The samples for histology were preserved in 10% neutral buffered formalin for 7 days (until tissues were well fixed) and thereafter subjected to histological processing.

Evaluation of Biochemical Assays: Tissue levels of nitric oxide were quantified indirectly by measuring the total nitrite, as described by Olaleye *et al*. (2007). Malondialdehyde concentration was determined by measuring the thiobarbituric acid reactive substances (TBAR) produced during lipid peroxidation. This procedure was carried out using the method of Varshney and Kale

(1990). The values were expressed as µmol/mg/protein. Hydrogen peroxide levels in the cardiac and renal tissues were determined by the method of Wolff (1994). The values were expressed as µ/mg protein.

The cardiac and renal reduced glutathione (GSH) were estimated by the method of Jollow *et al*. (1974).

Glutathione S-transferase (GST) was estimated by the method of Habig *et al*. (1974). Glutathione peroxidase (GPx) activity in tissues was measured according to the method of Buetler *et al*. (1963).

Histology and immunohistochemistry Methods

Histology: Histological studies on the cardiac and renal tissues stained with Haematoxylin and Eosin (H&E) stain were carried out as described by Avwioro (2002).

Immunohistochemistry: Immunohistochemistry procedure was done as described by Oyagbemi *et al*. (2021) with slight modification using 2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution (Catalog number: E-IR-R217 from Elabscience Biotechnology®, China). The cardiac and renal samples were fixed with 10% paraformaldehyde, embedded in paraffin and sectioned at a thickness of 5µm. The slides were subsequently dewaxed in xylene (100%) solution for 2minutes and afterward, hydration was carried out in different concentrations of ethanol (100%, 90%, 80% and 70%) for 2 minutes each. The hydrated tissue sections were rinsed and put in a phosphate buffered saline (PBS) tank for 5 minutes. The antigen retrieval was performed with citrate buffer (pH 6.0), in a microwave oven. Endogenous peroxide (H2O2) block was carried out following manufacturer's instructions on the kit (E-AB-15447). Drops of H2O2 were added to cover the sections and incubated in a humidifying chamber at room temperature for 10 min. The slides were rinsed afterwards and put back in the PBS tank for 5 minutes. Goat serum (E-1R-R217A) was added onto the slides to prevent non-specific binding and incubated in a humidifying chamber at room temperature for 30 minutes. Thereafter, the tissues were probed with Synthetic peptide of human HTR1A (E-AB15447:1:100) as primary antibodies for the cardiac and renal tissues. They were then incubated for 2 hours at room temperature. Following incubation, the tissue slides were rinsed with PBS and a secondary antibody labelled (E-1R-R217B) was added and the slides were incubated in a humidifying chamber at room temperature for 20 min. The slides were subsequently rinsed and immersed in PBS tank for 5 minutes. Finally, a few drops of the substrate diaminobenzidine (DAB) (50 µL of DAB concentrate (E-1R-R217D) + 1mL DAB solution (E-1R-R217E)) were added at room temperature for 10 seconds in the dark. The reaction was terminated with deionised water and slides were immersed in haematoxylin for 3 seconds before rinsing with PBS. The slides were placed in 70%, 80%, 90% and 100% of ethanol and then xylene (100%) for 2 minutes each. Slides were removed, allowed to dry and a DPX mountant was applied. Sections were observed with a light microscope (Leica LAS-EZ®) using Leica software application suite version 3.4 equipped with a digital camera.

Statistics Analyses: The results were expressed as mean ± standard deviation. One-way ANOVA was used to analyse the differences among them. Comparisons between the groups were done using the Student's t-test. Data were analysed using GraphPad Prism for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com.). Values of $P < 0.05$ were considered significant.

RESULTS

Blood pressure and Nitric oxide (NO): Figure 1 shows the result of treatments on blood pressure of the rats. A significant ($P < 0.05$) increase was observed in systolic and diastolic in L-NAME treated group compared to the control group. The concentration of NO rose significantly ($P < 0.05$) in rats co-treated with 20% MO group compared with the NO concentration obtained in rats treated with L-NAME alone.

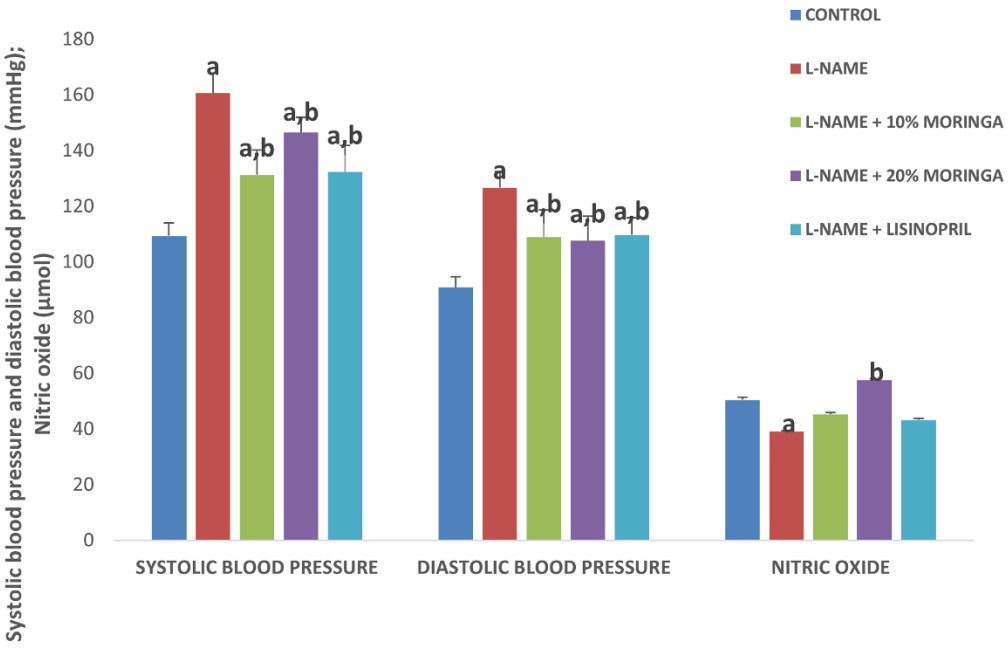

Figure 1: Effect of *Moringa oleifera* on systolic blood pressure, diastolic blood pressure and serum nitric oxide (NO) level in the experimental rats treated with L-NAME. Values are presented as mean ±standard deviation. *Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B.*

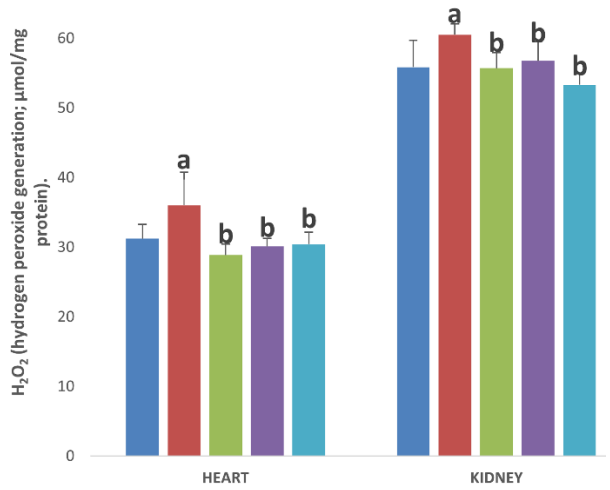

Figure 2:
The effect of *Moringa oleifera* on hydrogen peroxide generation (H_2O_2) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B.

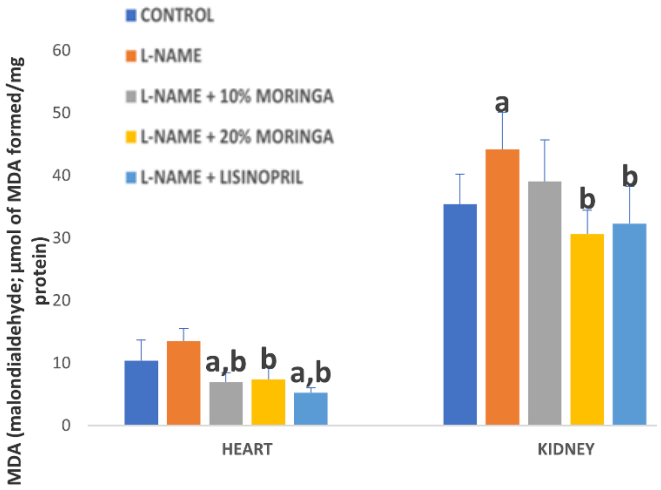

Figure 3:
The effect of *Moringa oleifera* on malondialdehyde (MDA) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B.

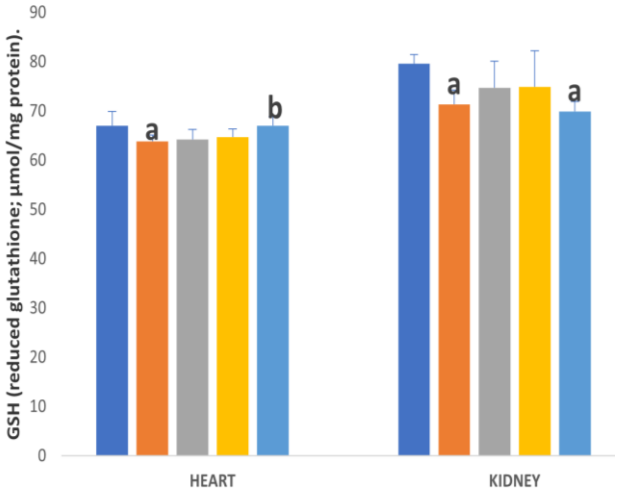

Figure 4:
The effect of *Moringa oleifera* on reduced glutathione (GSH) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B.

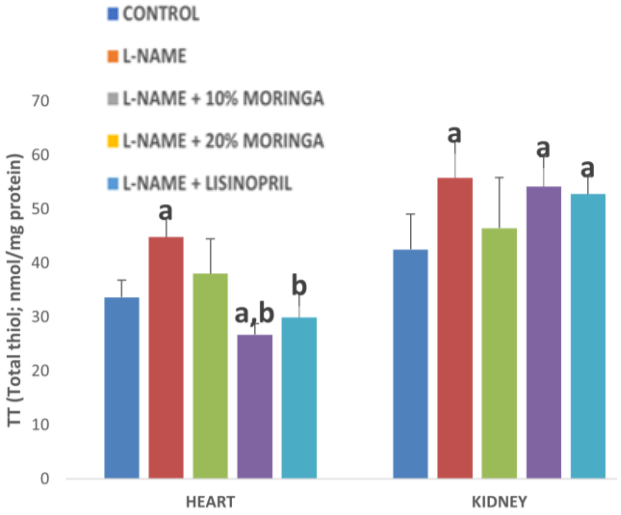

Figure 5:
The effect of *Moringa oleifera* on total protein thiol (TPT) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B

Hydrogen peroxide (H_2O_2): The concentration of H_2O_2 deceased significantly (P < 0.05) in the L-NAME-treated group when compared with the MO co-treated groups (Figure 3).

Malondialdehyde (MDA): Figure 3 showed the concentration of MDA in the heart and kidney tissues. There was significant (P < 0.05) increase in the concentration of MDA in rats treated with L-NAME compared to the level obtained in the groups co-treated with MO seeds.

Antioxidant parameters: The level of GSH in cardiac and renal tissues is shown in Figure 4. The level of GSH significantly (P < 0.05) reduced in the rats treated with L-NAME alone when compared with the co-treatment groups. The value of TPT was significantly higher (P < 0.05) when compared with the co-treatment groups showed in Figure 5. The concentration of non-protein thiol (NPT) increased significantly (P < 0.05) in L-NAME compared with the value obtained in co-treatment groups (Figure 6). The glutathione S-transferase (GST) activity in the co-treatment groups increased significantly (P < 0.05) when compared with L-NAME group (Figure 7). Figure 8 showed the activity of glutathione peroxidase (GPx) in the heart and kidney tissues. The activity of GPx obtained in MO seed feed inclusion treatment groups was higher (P < 0.05) than

that obtained in L-NAME alone group. Vitamin C concentration was found to reduce significantly ($P < 0.05$) in L-NAME alone treated group compared with co-treatment groups (Figure 9). Protein carbonyl level increased significantly in L-NAME alone treated rats when compared with the co-treatment groups (Figure 10).

Histology: The photomicrograph of the kidney of the rats (Plates 1) showed mild haemorragic lesion and thrombosis in hypertensive rats, while the hypertensive rats treated with MO had mild haemorrhagic lesion, mild tubular inflammation, glomerular infilteration. The group treated with lisinophil had slight perivascular inflammation, mild thrombosis and glonerulonephrosis. In the heart tissue (plate 2), photomicrograph revealed moderate thrombosis and perivascular inflammation in hypertensive rats. These abnormalities were not found in the photomicrograph of the groups treated with MO exract and lisinophl.

Immunohistochemistry Immunohistochemistry showed a higher expression of immune positive C – Reactive Proteins (CRP) on the heart of the hypertensive rats when compared to that of the control. However, there was lower expression of the CRP in heart tissue of rats treated with MO and lisinopril when compared to the heart tissue of the hypertentive rats that were not treated. There was higher expression of extracellular regulated kinase (ERK) in the kidney tissues of the hypertensive rats when compared to the control. A high expression of ERK was also observed the group treated with 10% MO. Whereas a lower expression of MO was observed by the groups treated with 20% MO and lisinopril.

Electrocardiogram: When compared with the controls, no statistically significant ($p<0.05$) differences were observed between the L-NAME-only treated groups and the others.

DISCUSSION

The objective of the current investigation was to assess the antihypertensive efficacy of Moringa oleifera. Hypertension was generated in rats with the oral administration of L-NAME at a dose of 40mg/kg. This phenomenon can be attributed to its capacity to inhibit NOS activity, resulting in a subsequent decrease in NO levels within the circulatory system (Gardina *et al*., 2010; Krol and Kepinska, 2020). The L-NAME treated group exhibited a notable reduction in serum NO levels compared to the control group. This decrease can be attributed to the down-regulation of endothelial nitric oxide synthase expression in hypertensive rats, as previously reported by Zhou and Frohlich (2007) and Panthiya *et al*. (2022). Rats which were fed MO seed inclusion in their diet, in conjunction with L-NAME, exhibited a statistically significant ($p<0.05$) elevation in NO levels as compared to the groups that received L-NAME alone. The observed phenomenon can likely be attributed to the antioxidant properties of the MO, which consequently mitigated the oxidative damage generated by L-NAME.

In this study, oxidative stress markers were significantly increased in L-NAME group compared with groups treated with MO and Lisinopril. The MDA is a product of lipid peroxidation and the contents of tissue MDA is reported to be a reliable marker of lipid peroxidation (Ayala *et al*., 2014). The administration of L-NAME alone exhibited a significant increase in the MDA contents, which is an indication of oxidative stress-induced cardiac and renal damage. However, co-treatment with MO caused a significant reduction in this MDA contents implying that MO impact an antioxidant activity, which involves chelating of ions and scavenging of ROS, thus protecting the cardiac and renal tissues from lipid peroxidation. These present results are in agreement with those of several authors who reported the association of oxidative stress and lipid peroxidation (Bergin *et al*., 2021; Sabanna and Ratan, 2021; Cordiano *et al*., 2023).

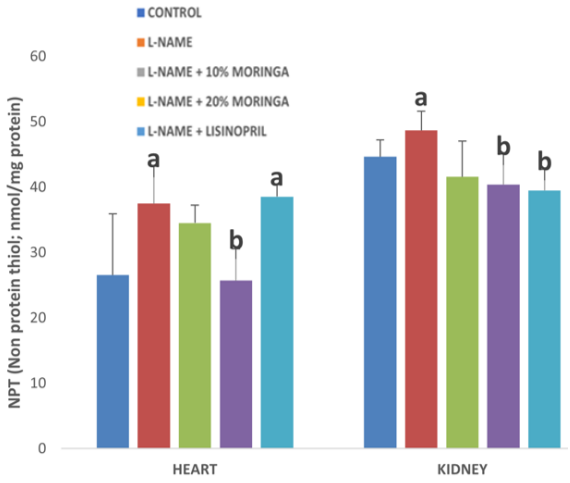

Figure 6:
The effect of *Moringa oleifera* on non-protein thiol (NPT) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at $P < 0.05$ compared with control (Group A) while superscript (b) Indicates significant difference at $P < 0.05$ compared with Group B.

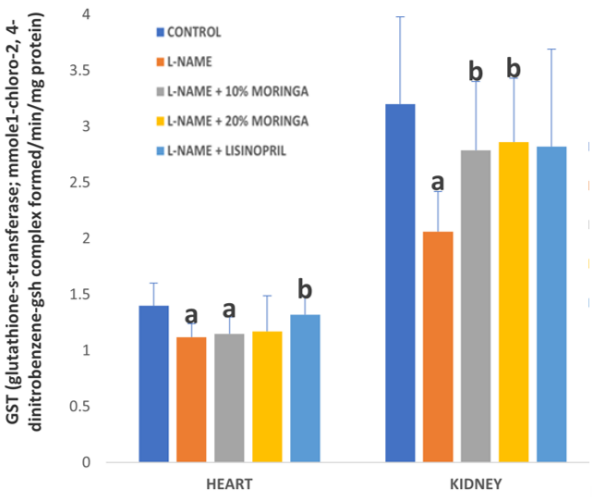

Figure 7:
The effect of *Moringa oleifera* on glutathione S-transferase (GST) activity in the cardiac and renal tissues of L-NAME treated Wister rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at $P < 0.05$ compared with control (Group A) while superscript (b) Indicates significant difference at $P < 0.05$ compared with Group B.

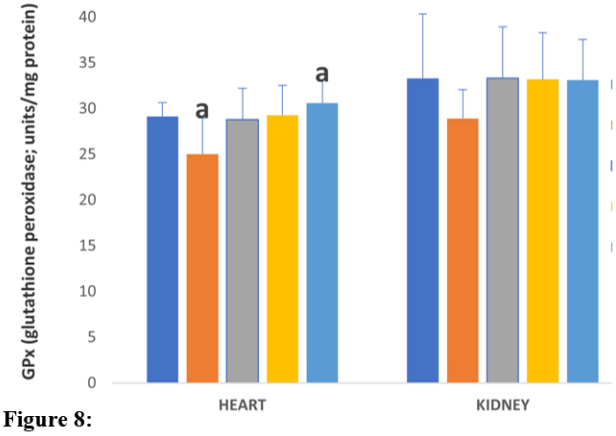

Figure 8:
The effect of *Moringa oleifera* on glutathione peroxidase (GPx) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B

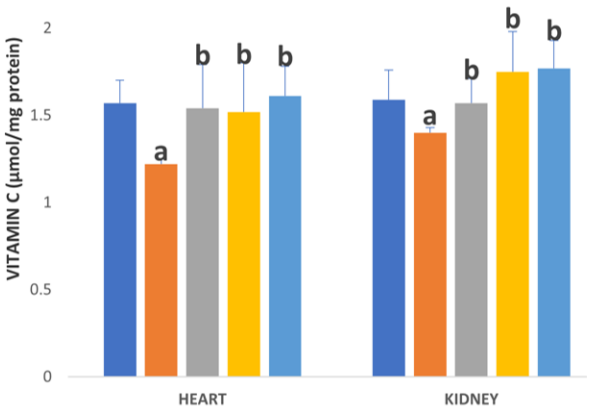

Figure 9:
The effect of *Moringa oleifera* on Vitamin C levels in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B

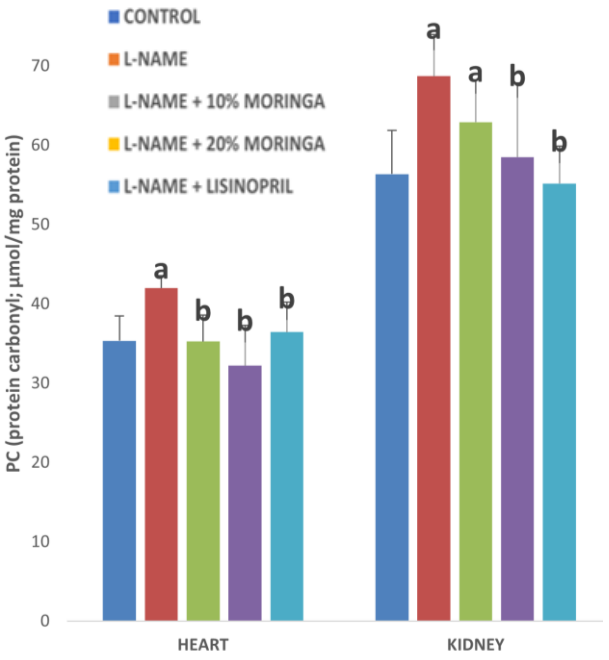

Figure 10:
The effect of *Moringa oleifera* on protein carbonyl (PC) activity in the cardiac and renal tissues of L-NAME treated Wistar rats. Values are presented as mean ± standard deviation.
Superscript (a) indicates significant difference at P < 0.05 compared with control (Group A) while superscript (b) Indicates significant difference at P < 0.05 compared with Group B.

Treatment with L-NAME produced a significant increase in the H2O2 level in both cardiac and renal tissues, which might be due to its ability to raise levels of oxidative stress markers as reported by Pal *et al*., 2023. Co-treatment with *Moringa oleifera* and Lisinopril however, caused a significant reduction in the H2O2 levels, indicating the ability of MO to counteract the deleterious effects of free radical generation by the L-NAME.

Our results showed that GPx was significantly decreased following L-NAME treatment. This however corresponds to previous work by Efosa *et al* (2023) who reported a decrease in GPx activity following administration of L-NAME in Wistar rats; and in agreement with the findings that oxidative stress led to decreased activities of antioxidative enzymes. The finding in this study could be as a result of an adaptive response of the GPx antioxidant system against a sudden and aggressive attack by free radicals produced following administration of L-NAME. Co-treatment with MO caused a significant increase in the enzyme activity compared to the L-NAME alone and this could probably be due to the antioxidant protective ability of the MO that involves quenching of the free radicals generated by L-NAME treatment, thereby ameliorating L-NAME-induced oxidative damage in cardiac and renal tissues.

In this present study, GST activity was significantly inhibited in the cardiac and renal tissues in L-NAME treated rats. This is in contrast with the results obtained by (Gogebakan *et al*., 2012) which showed a significant increase in GST in L-NAME treated rats. However, co-treatment with MO and Lisinopril counteracts the inhibition of GST against free radical generation and oxidative stress. The significant increase in the activity of GST in MO and Lisinopril treatment groups implied that MO ameliorates L-NAME induced free radical generation due to its antioxidant and free radical scavenging properties.

The thiols are organic compounds that contain sulphydryl group which constitute the major portion of the total body antioxidants which play an important role in the defense against reactive oxygen species. Both intracellular and extracellular thiols are components of total thiol. In this present study, the levels of both protein and non-protein thiol increased significantly in the L-NAME treatment group when compared with the control group. This is not in consonance with previous studies that decreased levels of thiols which reported in some diseases of the kidney, cardiovascular system and several other organs in the body (Prakash *et al*., 2009). Co-treatment with MO and Lisinopril caused a significant decrease in the total thiol level and this could be as a result of the antioxidant property of MO.

Blood pressure measurement in this study showed a significant increase in the systolic and diastolic pressure in L-NAME treatment group when compared with the control group. Also, the systolic, diastolic and mean arterial

pressure decreased significantly in the MO and Lisinopril treated groups when compared with L-NAME treated group indicating that MO has the ability to reverse the inhibition of NOS by L-NAME, thereby, increasing the level of nitric oxide in the circulation.

The findings showed that ascorbic acid activity is reduced in the L-NAME treated group which inhibits the reduction and neutralisation of ROS such as hydrogen peroxide whereas MO and Lisinopril treated groups caused an increase compared to the L-NAME group and thus promotes free radical formation and however, will also reduce metal ions that generate free radicals through the Fenton reaction (Stohs and Bagchi, 1995). There is also an increased PC of L-NAME treated group which in turn will aid generation of free radicals and increase metal ions whereas MO and Lisinopril treated group has reduced PC activity compared to the L-NAME treated group only.

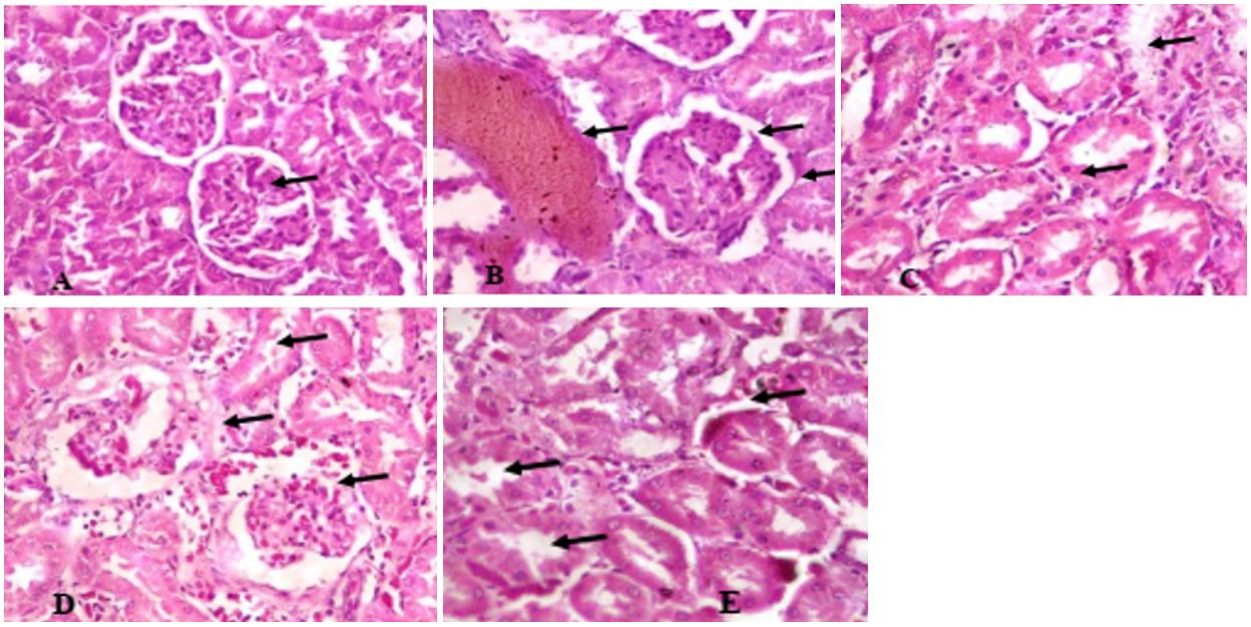

Plate 1:
Photomicrograph showing kidney of rats (Group A) - Control: Shows mild haemorrhagic lesion; (Group B) administered with L-NAME 40 mg/kg) shows mild haemorrhagic lesion and thrombosis (black arrow); (Group C: 10% *Moringa oleifera* + L-NAME 40 mg/kg) shows mild glomerular congestion and mild haemorrhagic lesion, (Group D: 20% *Moringa oleifera* + L-NAME 40 mg/kg shows mild haemorrhagic lesion, glomerular inflammation, and mild peritubular inflammation; Group E (10 mg/kg lisinopril + L-NAME 40 mg/kg) shows slight peritubular inflammation, mild thrombosis and glomerulosclerosis. Plates are stained with H and E stains and viewed with X 100 objectives.

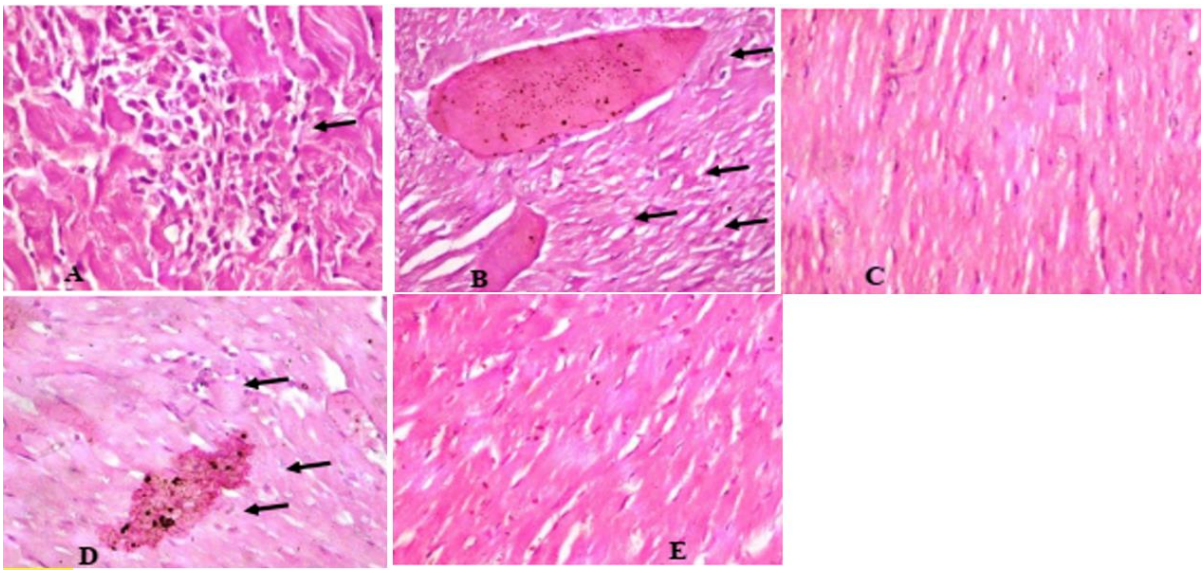

Plate 2:
Photomicrograph showing heart of rats (Group A)-Control: moderate infiltration of inflammatory cells (thin arrow) to the myocardium; (Group B) administered with (L-NAME)-40 mg/kg) shows moderate thrombosis (black arrow) and mild perivascular inflammation (black arrow); (Group C: 10% *Moringa oleifera* + L-NAME 40 mg/kg) shows normal architecture and cellularity of myocytes. No visible lesion seen. (Group D: 20% *Moringa oleifera* + L-NAME 40 mg/kg shows moderate thrombosis; Group E (10 mg/kg lisinopril + L-NAME 40 mg/kg) shows normal architecture and cellularity of myocytes. No visible lesion seen. Plates are stained with H and E stains and viewed with X 100 objectives

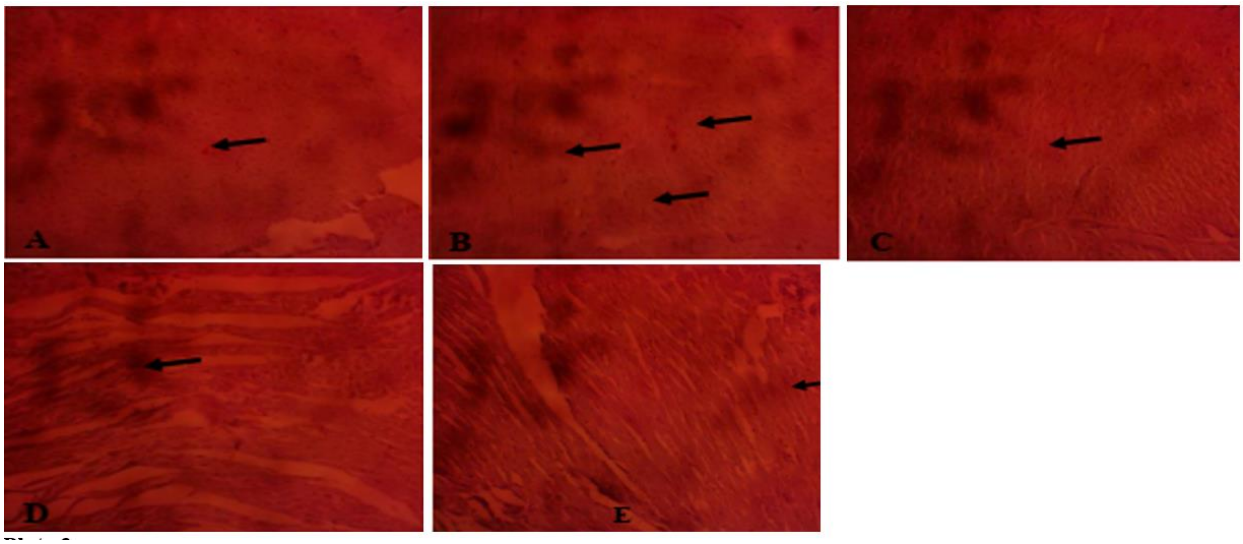

Plate 3:
Immunohistochemistry of C-reactive proteins (CRP) in the heart of rats. A – Control: There were lower immune-positive expressions of CRP. Group B L-NAME 40 mg/kg shows higher immune-positive expression of CRP when compared to the control; Group C: 10% *Moringa oleifera* + L-NAME 40 mg/kg) shows lower expressions of CRP when compared with the L-NAME only treated group (black arrow) and (Group D) administered 20% *Moringa oleifera* + L-NAME 40 mg/kg also shows lower expressions of CRP when compared with the L-NAME only treated group (black arrow) and Group E (10 mg/kg lisinopril + L-NAME 40 mg/kg shows lower expressions of CRP compared with L-NAME alone. The slides were counterstained with high-definition hematoxylin and viewed x 100 objectives.

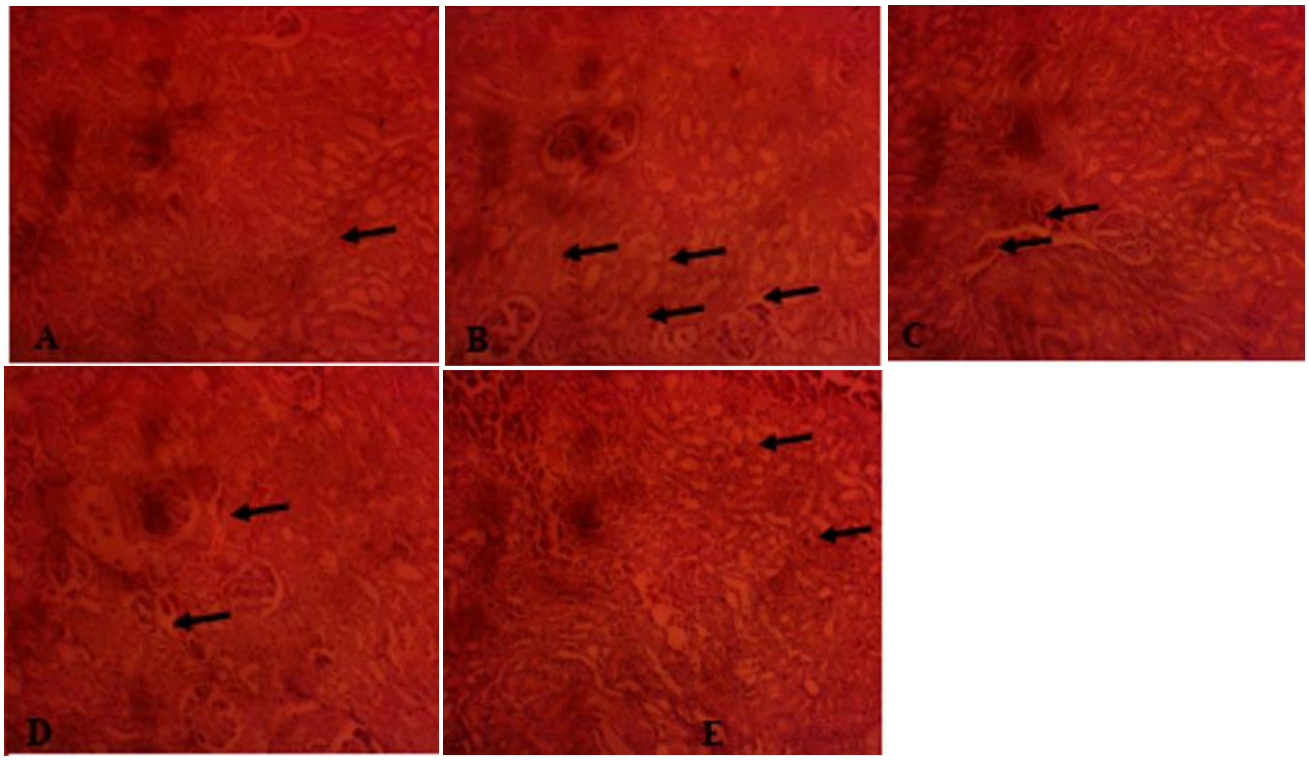

Plate 4:
Immunohistochemistry of extracellular regulated kinase (ERK) in the kidney of rats. A – Control: There was lower immune-positive expression of ERK. Group B L-NAME 40 mg/kg) shows higher immune-positive higher expression of ERK when compared to the control; Group C: 10% *Moringa oleifera* + L-NAME 40 mg/kg) shows higher expressions of ERK when compared with the L-NAME only treated group (black arrow) and (Group D) administered 20% *Moringa oleifera* + L-NAME 40 mg/kg also shows higher expressions of ERK when compared with the L-NAME only treated group (black arrow) and Group E (10 mg/kg lisinopril + L-NAME 40 mg/kg shows higher expressions of ERK compared with L-NAME alone. The slides were counterstained with high-definition hematoxylin and viewed x 100 objectives.

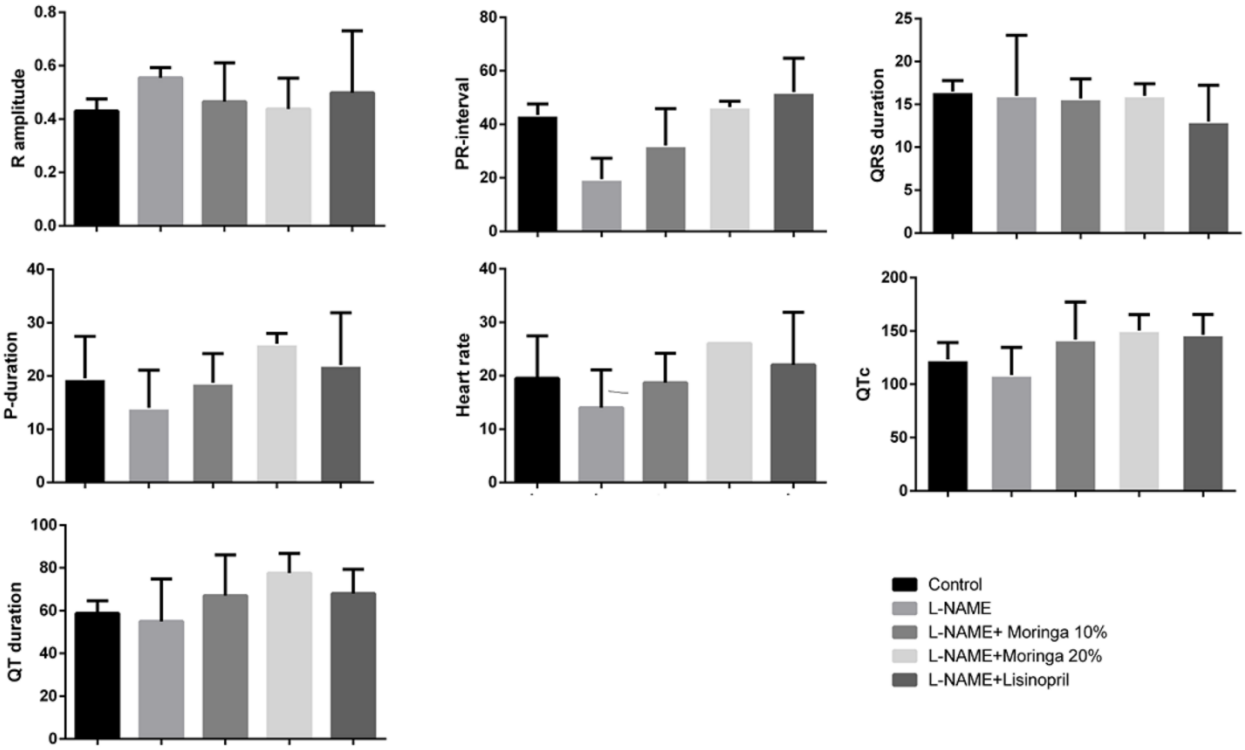

Figure. 11
Effect of *Moringa oleifera* seed inclusion and lisinopril on the electrocardiogram in L-Name-induced hypertension

The immunohistochemistry results revealed a higher expression of cardiac and renal Caspase-3 in L-NAME treated rats, thus indicative of apoptosis. Caspase-3 is an enzyme that plays a central role in the execution-phase of apoptosis following its activation by Caspase 8, 9 and 10. Caspases are actually inactive pro-enzymes that undergo proteolytic cleavage to produce two subunits, a large and small, that dimerizes to form the active enzyme (Redza-Dutordoir and Averill-Bates, 2016). Caspase-3 is activated in apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (Rodriquez-Gonzalez and Gutierrez-Kobeh, 2024).

As an executioner caspase, the caspase-3 zymogen has virtually no activity until it is cleaved by an initiator caspase after apoptotic signaling events have occurred (Waters *et al*., 2009). One of such apoptotic signaling event is the introduction of granzyme B (an enzyme shown to be involved in inducing inflammation), which can activate initiator caspases into cells targeted for apoptosis by killer T cells (Metkar *et al*., 2003; Dalken *et al*., 2006). This extrinsic activation triggers the hallmark caspase cascade that characterizes the apoptotic pathway, in which caspase-3 plays a dominant role (Rodriquez-Gonzalez and Gutierrez-Kobeh, 2024). In intrinsic activation, cytochrome c released from the mitochondria, works in combination with caspase-9, apoptosis-activating factor 1 (Apaf-1), and ATP to process procaspase-3 (Garrido *et al*., 2006; Brentnall *et al*., 2013). These molecules along with some other regulatory proteins activate caspase-3 in vivo (Brentnall *et al*., 2013). This current study shows that L-NAME administration elicited an increased expression of Caspase-3, indicating an apoptotic process associated with the L-NAME-induced hypertension; whereas co-treatment with MO and Lisinopril caused downregulation in the L-NAME induced apoptosis, characterized by a marked decrease in the Caspase-3 expression, suggesting anti-apoptotic property of MO against the L-NAME induced apoptosis and hypertension.

In conclusion, the results of this study indicate that *Moringa oleifera* has the potential for antihypertensive therapy as our study revealed. Co-treatment with Moringa oleifera, alongside of L-NAME, effectively maintained the systolic and diastolic pressure to that of normotensive animals. The findings confirmed the anecdote that *Moringa oleifera* seed possess antihypertensive properties, hence can be used for the management of hypertension with less side effects.

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Niger. J. Physiol. Sci. 39 (June 2024): 149 – 155
www.njps.physiologicalsociety.com

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Full length Research Article

Inhibition of α-Amylase and α-Glucosidase Activities by 2-Hydroxy-1,4-Naphthoquinone

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Summary: The purpose of the present study was to investigate the α-amylase and α-glucosidase inhibitory potentials of 2-hydroxy-1,4-naphthoquinone (2HNQ). The inhibition of these two carbohydrates metabolizing enzyme was done by varying the concentrations of 2HNQ in the presence of α-amylase, α-glucosidase with starch and p-nitrophenylglucopyranoside (pNPG) respectively as their substrates. The mode of inhibitions of the two enzymes by 2HNQ was determined using double-reciprocal transformation. The result obtained indicated that 2HNQ inhibited α-glucosidase activity with an IC50 0.260 mg/mL lowered than acarbose (1.530 mg/mL). Meanwhile, a moderate inhibitory potential of 2HNQ against α-amylase was observed with an IC_{50} of 1.757 mg/mL compared with acarbose (IC_{50} of 3.600 mg/mL). Furthermore, 2HNQ amazingly mops up reactive oxygen species. The observed inhibitions of α-amylase and α-glucosidase activity as well as radical scavenging potentials of 2HNQ suggest that it may be a potential target for the management of diabetes mellitus.

Keywords*: α-amylase; α-glucosidase; inhibition; 2-hydroxy-1,4-naphthoquinone; antioxidants*

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Manuscript received- March 2023; Accepted: October 2023

DOI: https://doi.org/10.54548/njps.v39i1.17

INTRODUCTION

Diabetes is a life-threatening ailment caused by the failure of pancrease to produce insulin or the ineffectiveness of the insulin produced by the pancrease (Salehi et al., 2019). This will in turn lead to postprandial hyperglycemia and other various secondary complications like blindness, kidney damage, cardiovascular disease, and lower-limb amputations (Chukwuma et al., 2019). According to Sun et al. (2022), just about 537 million of people between the ages of 20 – 79 yrs have diabetes and this figure has been anticipated to increase to 643 and 783 million by 2030 and 2045 respectively (Ogurtsova et al., 2022). The disease is recognized as a global health challenge and it has translated to a modern-day plague which poses a threat and socioeconomic problems (Mercer et al., 2019). Diabetes mellitus was responsible for the death of about 1.5 million individuals in 2019 according to World Health Organization (WHO). An effective way of managing diabetes mellitus is by the reduction in the postprandial blood glucose and this has been made possible by targeting α-amylase and α-glucosidase inhibitors (Masood et al., 2021). These two enzymes are vital to the regulation of postprandial blood glucose concentration and the inhibitions of these two enzymes can be used to lower the postprandial hyperglycemia thereby preventing or treating type 2 diabetes mellitus (Tacias-Pascacio et al., 2020). To this end, a lot of drugs have been employed in the management of diabetes mellitus and they have shown tremendous and encouraging results. However, despite the wide arrays of positive results shown by those drugs, they are not without shortcomings. Some effects such as nausea, diarrhea, heart failure, drug resistance, cancer, weight gain, hypoglycemia and soon have been reported (ADA, 2003). Diabetes Mellitus been a multifactor life-threatening disease requires a multidimensional therapeutic approach. Therefore, a molecule that is capable to act on more than one mechanism and with a little or no side effect will be an ideal candidate for an antidiabetic drug development. 2-Hydroxy-1,4-naphthoquinone (2HNQ) which is the principal natural compound found in the plant Lawsonia inermis has widely been used as paint and hair dye. Lawsonia inermis Linn. is a traditional plant which has an English name Henna (Kumar et al., 2017). The plant has been reported to have medicinal benefits (Salih et al., 2017). Widyawati et al. (2015) conducted a survey among the diabetes patients in Medan, North Sumatera, Indonesia, and reported that the leaves of Lawsonia inermis control the blood glucose level.

Therefore, this study investigates the α-amylase and α-glucosidase inhibitory potentials of 2-hydroxy-1,4-naphthoquinone (2HNQ).

MATERIALS AND METHODS

Chemical and Reagents: 2-hydroxy-1,4-naphthoquinone (2HNQ), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric chloride ($FeCl_3$), potassium ferricyanide, porcine pancreatic α-amylase, rat intestinal α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (pNPG), Potassium persulfate, methanol, DMSO were products of Sigma Chemical Co., St. Louis, Missouri, USA. Starch, dinitrosalicylic acid (DNS), maltose and sucrose were products of J.T. Baker Inc., Phillipsburg, USA, while acarbose, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Trichloroacetic acid (TCA) were procured from Santa Cruz Biotechnology, Heidelberg, Germany. Distilled water was obtained from Medical Biochemistry and Pharmacology Department, Kwara State University, Malete, Ilorin, Nigeria. All other chemicals and reagents used were of analytical grade.

In vitro Antidiabetic Assays

Alpha-Amylase Inhibitory Assay: The α-amylase inhibitory assay was done by following a method described by McCue and Shetty (2004) with slight modification. In brief, 250μL of 2HNQ (1.25–10mg/mL) was mixed with 250 μL of α-amylase solution (0.5mg/mL) prepared in 0.02M sodium phosphate buffer (pH 6.9) in test tube. The mixture was pre incubated at 25 ºC for 10 min and after the addition of 250 μL of 1% starch solution, it was incubated again at 25 ºC for 10min. The reaction was quenched by adding 500 μL of quenching (dinitrosalicylic acid (DNS)) reagent. The mixture was boiled for for 5min and cooled to room temperature and 5mL of distilled water was used to dilute the mixtures after which the absorbance was monitored at 540 nm using spectrophotometer. DMSO was used as control replacing 2HNQ and the α-amylase inhibitory potential was expressed as percentage inhibition:

$$\%\text{Inhibition} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extracts}}}{\text{Abs}_{\text{control}}}\right] \times 100.$$

Concentrations of 2HNQ resulting in 50% inhibition of α-amylase (IC_{50}) were determined graphically.

Mode of α-Amylase Inhibition: The mode of inhibition of α-amylase by 2-hydroxy-1,4-naphthoquinone (2HNQ) was carried out using the concentration of 2HNQ with the lowest IC_{50} according to the modified method described by Ali et al. (2006). Briefly, 250 μL of 2HNQ (5mg/mL) was mixed with 250 μL of α-amylase solution and the mixture was first incubated for 10min at 25°C. Varying concentrations of starch solution (5.0 - 0.3125 mg/mL) were added to the mixture and incubated for another 10min at 25°C. The reaction was stopped by adding 500 μL of DNS solution. The mixture were placed in boiling water for 5min, cooled to room temperature and 5mL of distilled water was added after which the absorbance was monitored at 540 nm using spectrophotometer. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/(S)) where V is reaction velocity and (S) is substrate concentration was plotted. The type (mode) of inhibition as well as Km and Vmax was determined (Nelson and Cox, 2008).

Alpha-Glucosidase Inhibitory Assay: The procedure described by Kim et al. (2005) with slight modification was adopted for the In vitro α-glucosidase inhibitory potential of 2HNQ. Briefly, 100 μL of α-glucosidase (1.0 U/mL) was mixed in a test tube with 50 μL of the varying concentrations of 2HNQ (1-0.0625mg/ml). the reaction was left to stand for 10min. Then 50 μL of 3.0mM (pNPG) prepared in 20 mM phosphate buffer (pH 6.9) was then added to kick start the reaction. The reaction mixture was incubated at 37°C for 20min and quenched by adding 2mL of 0.1M Na_2CO_3. The yellow-colored paranitrophenol released from pNPG was measured at 405 nm. The results were expressed as percentage of the blank control. Control was set up by replacing 2HNQ with phosphate buffer and acarbose was also used as a positive control. The inhibition percentage (%) was calculated using the formula:

$$\%\text{Inhibition} = \left[\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}}\right] \times 100.$$

Concentrations of 2HNQ resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

Mode of α-glucosidase Inhibition: The mode of inhibition of α-glucosidase by 2-hydroxy-1, 4-naphthoquinone was determined using the concentration with the lowest IC_{50} according to the modified method described by Ali et al. (2006). Briefly, 50 μL of the (1mg/mL) 2HNQ was added to 100 μL of α- glucosidase solution in a test tube and incubated for 10 min at 25°C. Then, varying concentrations of 50 μL of pNPG (5–0.3125mg/mL) was added to the mixture to kick start the reaction. The mixture was then incubated for 10min at 25°C. The reaction was terminated by adding 500 μL of Na_2CO_3. The amount of reducing sugars released was determined spectrophotometrically using a paranitrophenol standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was plotted. A double reciprocal plot (1/V versus 1/(S)) where V is reaction velocity and (S) is substrate concentration was plotted. The type (mode) of inhibition as well as Km and Vmax was determined.

In vitro Antioxidant Assays:

Reducing Power Assay: The reducing power of 2HNQ was evaluated by adopting the method of Belkacem et al. (2017) with slight modification. Varying concentrations of 2HNQ (1000 – 5000 µg/mL) were added to 2.5 mL of 0.2M phosphate buffer (pH6.6) and 2.5mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50°C for 20 min prior to addition of 2.5mL of trichloroacetic acid (TCA). The solution was centrifuged at 3000 rpm for 10min and 2.5mL of the supernatant was mixed with an equal amount of distilled water and 0.5mL of 0.1% $FeCl_3$. The absorbance of the resulting solution was then read at 700nm.

DPPH Radical Scavenging Assay: The ability of 2HNQ to bleach the purple-coloured ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was done by following the

procedure described by Turkoglu et al. (2007). Briefly, 1mL of various concentrations (1000 – 5000 µg/mL) of 2HNQ were mixed with 1mL of a 0.2 mmol/L sample of DPPH in methanol and were left to stand for 30 minutes at room temperature. The absorbance was then read at against blank at 516 nm. Inhibition rate (I%) on the DPPH radical was calculated using the expression:

$$\text{Radical scavenging } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the control, A_{sample} is the absorbance of the compound. Using standard calibration curve, the concentration of 2HNQ causing 50% inhibition (IC_{50}) of DPPH radical was estimated

ABTS Radical Scavenging Assay: The ABTS radical cation method described by Nicoletta (1999) and was modified to evaluate the free radical-scavenging effect of 2HNQ. The ABTS reagent was prepared by mixing 5mL of 7mM ABTS with 88 μL of 140mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted with water (1: 44, v/v). To determine the scavenging activity, 100 μL ABTS reagent was mixed with 100 μL of sample in a test tube and was incubated at room temperature for 6 min. After incubation, spectrophotometer was used to measure the absorbance at 734 nm. The control used was 100 % methanol. The ABTS scavenging effect was measured using the following formula:

$$\text{Radical scavenging } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Statistical Analysis of Data: Free radical and reactive oxygen species (ROS) scavenging activities were expressed in percentage while other data were expressed as the mean ± standard error of mean (SEM) of triplicate determinations. One way analysis of variance with Dunnett's post hoc test using GraphPad prism version 5.02. Values were considered statistically significant at the 95% confidence level.

RESULTS

In vitro Inhibitory Effect of 2HNQ on α –amylase and α-glucosidase: The potential of 2HNQ to inhibit α -amylase was determined (Figure 1). There was no significant difference in the inhibition of α- amylase by 2HNQ at concentrations between 0.63– 1.25 mg/mL but at higher concentrations between 2.5–5mg/mL, a significant increase in the percentage inhibition was observed ($P < 0.05$). The effectiveness of α-amylase was extrapolated from a dose response curve and this revealed that 2HNQ has a lower IC_{50} (1.757 mg/mL) when compared with acarbose (3.6 mg/mL) (Table 1). The mode of inhibition of 2HNQ showed that it displayed an uncompetitive mode of inhibition (Figure 2).

The ability of 2HNQ to inhibit α-glucosidase was displayed in Figure 3. 2HNQ inhibited α-glucosidase strongly in that it has an IC50 of 0.26 mg/mL compared to acarbose (1.53 mg/mL). The double-reciprocal transformation plot used to determine the mode of inhibition of the2HNQ revealed that 2HNQ displayed an uncompetitive mode of inhibition (Figure 4).

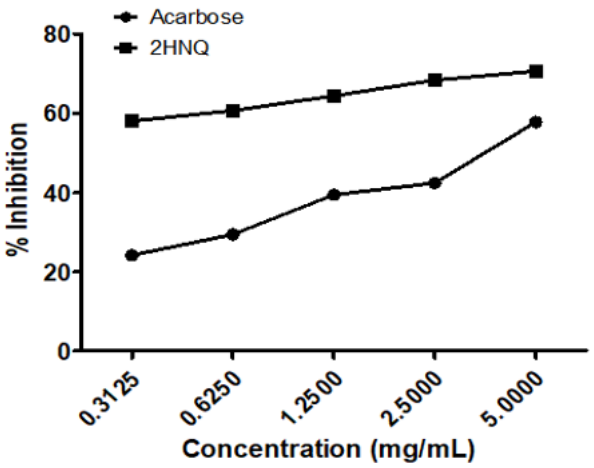

Figure 1:
Inhibitory potential of 2HNQ against α-amylase activity. The values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Table 1:
IC_{50} values for α-amylase and α-glucosidase inhibitory potential of 2HNQ.

Compound	IC_{50} (mg/mL)	
	α-amylase	**α-glucosidase**
2HNQ	1.757	0.260
Acarbose	3.600	1.530

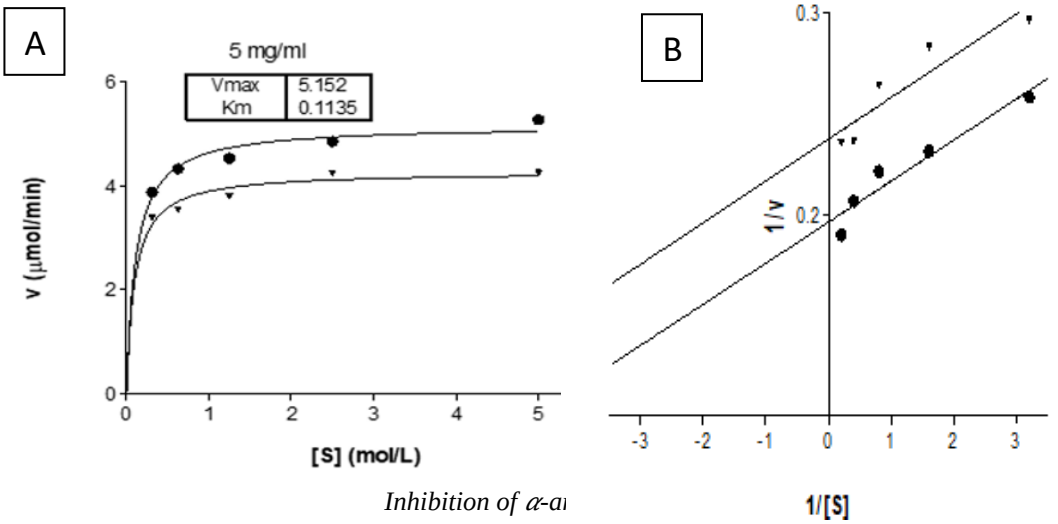

Figure 2:
Mode of inhibition of α-amylase by 2HNQ. (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot

Inhibition of α-a

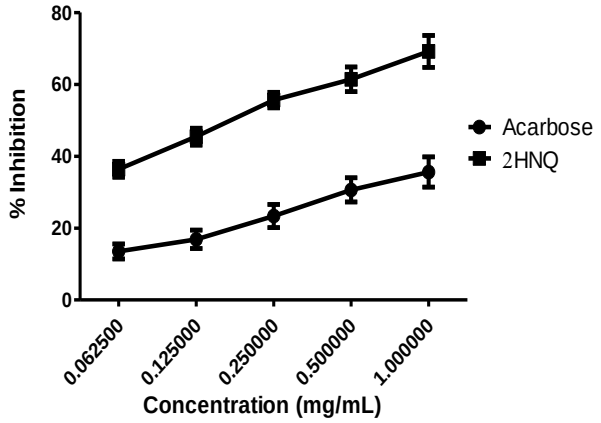

Figure 3:
Inhibitory potential of 2HNQ against α- glucosidase activity. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Antioxidant Activity: The reducing power of potential of 2HNQ was shown in figure 5. The results showed a dose dependent increased in the reducing power ability of 2HNQ and these were significantly higher than that of silymarin (Figure 5). There was a dose dependent increase in the DPPH radical scavenging potential of 2HNQ in a somewhat similar manner to that of the silymarin (Figure 6). The IC50 of 4.52µg/mL was obtained for 2HNQ as compared to silymarin (4.33µg/mL) (Table 2). The potential of 2HNQ to scavenge ABTS was shown in figure 7. The compound (2HNQ) scavenged ABTS in a dose dependent manner in a similar way to the reference silymarin (Figure 7). The IC50 of 2HNQ was 1.34µg/mL as compared to silymarin which has an IC50 of 3.67µg/mL (Table 2).

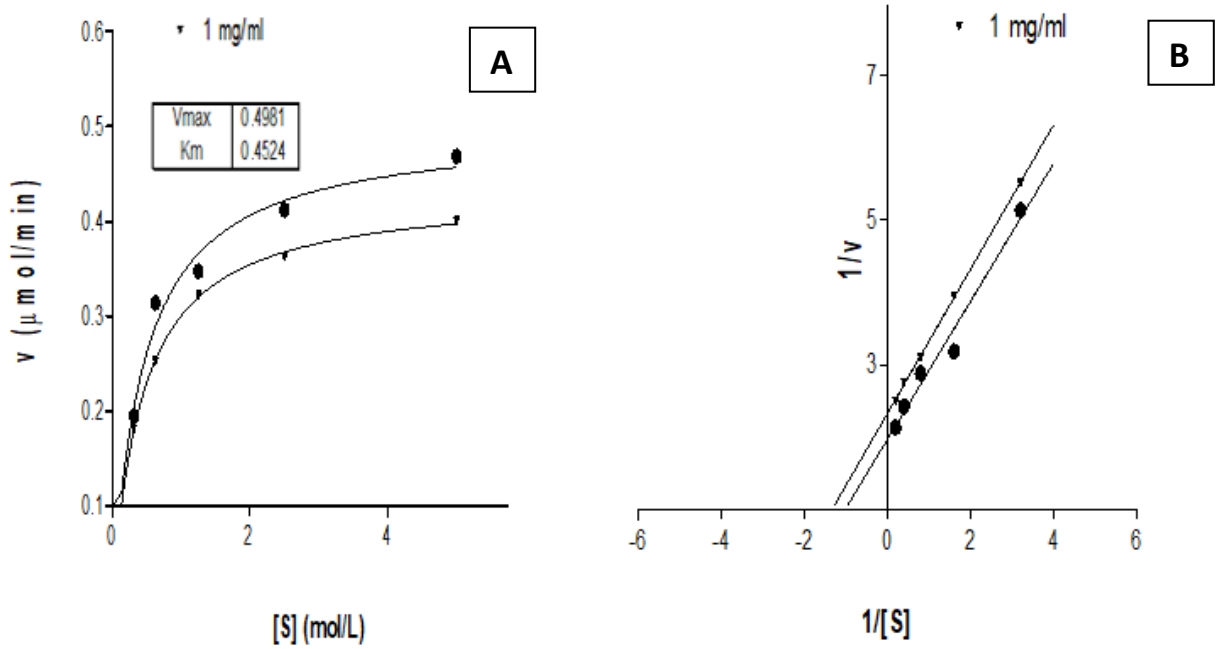

Figure 4:
Mode of inhibition of α-glucosidase by 2HNQ. (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot.

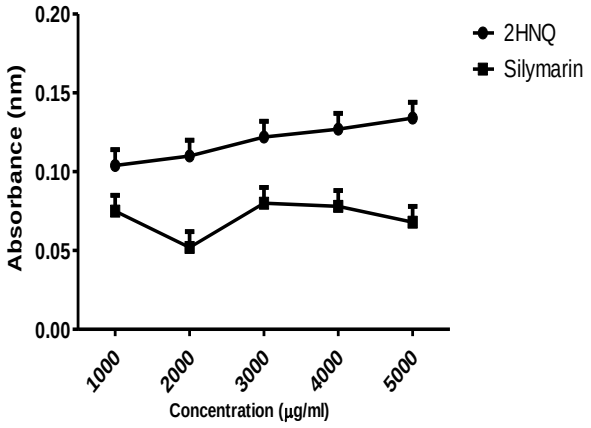

Figure 5:
Reducing power potential of 2HNQ. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

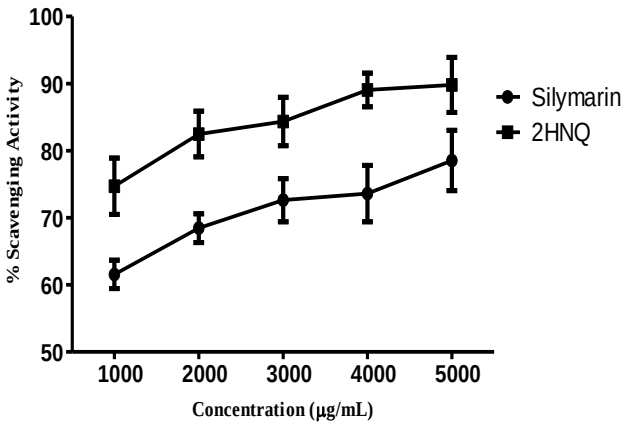

Figure 6:
DPPH scavenging effect of 2HNQ. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Inhibition of α-amylase and α-glucosidase activities

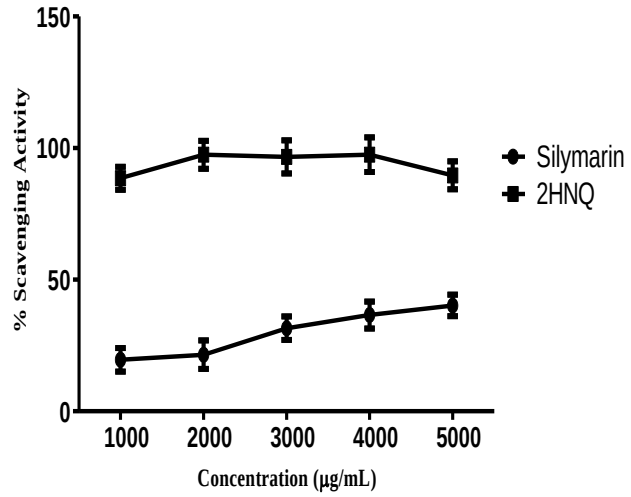

Figure 7:
ABTS scavenging effect of 2HNQ. Values are expressed as means ± SEM of triplicate determinations. Means not sharing a common letter at the same concentration were significantly different ($p < 0.05$).

Table 2:
IC_{50} of ABTS and DPPH scavenging capabilities of 2HNQ

ABTS	**Silymarin**	**2HNQ**
IC_{50}	3.67	1.34
R^2	0.9602	0.0062
Regression equation	y = 0.0056x + 12.96	y = 0.0002x + 93.302
DPPH		
IC_{50}	4.33	4.52
R^2	0.9448	0.9136
Regression equation	y = 0.0039x + 59.23	y = 0.0037x + 73.039

DISCUSSION

Generally, the reduction in the plasma blood glucose towards normal range can be achieved through the applications of oral hypoglycaemic agents and insulin. Nevertheless, these oral hypoglycaemic agents have some significant metabolic side effects (Campbell et al., 1996), and have encouraged scientists to work on the alternatives therapy with less toxicity for the management of this ailment. The upsurge in the blood glucose observed in diabetic patient is a consequence of an uncontrolled conversion of polysaccharides to an oligosaccharide by pancreatic α- amylase and the subsequent utilization of monosaccharides usually glucose by by intestinal α-glucosidases (Sabiu et al., 2016). An efficient way to handle this ailment is to sturdily prevent α-glucosidases from acting on the oligosaccharides as well as stylishly inhibit pancreatic α-amylase activity, which in turn regulate the availability of glucose in the blood (Kerru et al., 2018). A tactic to be employed in bringing down the blood glucose is to thwart carbohydrates absorption. Complex polymers need to be broken down respective monomeric form before they can be mobilized for enzyme production. This hydrolysis can be achieved by enzymes like α-amylase and α-glucosidases. Therefore, inhibition of these two enzymes prevents or stops the hydrolysis of disaccharides to glucose. Natural compound from plant origin with outstanding antioxidant ability present pretty substitute in this regard (Kumavat et al., 2012).

The compound (2HNQ) displayed a very strong inhibition of α-amylase activity. This finding agreed with Kwon et al. (2007) who reported that a mild inhibition of α-amylase activity is desirable because undue inhibition of pancreatic α- amylase could lead to an abnormal bacterial fermentation of undigested carbohydrates in the colon. The double reciprocal transformation of the data obtained revealed that 2HNQ inhibited α-amylase in an uncompetitive manner. This implies that this compound (2HNQ) binds to the complex formed between the α-amylase and substrate thereby decreasing both the Kcat and Km. It has been reported that this mode of inhibition is desirable for drug designs as the inhibitor binds to the enzyme target only when the target is active and in the presence of substrate.

The 2HNQ showed potent inhibition against α-glucosidase activity and this agreed with the finding of Kwon et al. (2007) who reported that natural compounds from plant have been reported to inhibit α-glucosidase and therefore can effectively be used as therapy for postprandial hyperglycemia with no side effect. The uncompetitive inhibition types obtained from double reciprocal transformation of data revealed that 2HNQ did not compete with enzyme for substrate binding but rather bind to a complex formed between the enzyme and the substrate which led to a decreased in both the Km and Vmax of the enzyme. The binding of 2HNQ to the complex formed between enzyme and substrate will inhibit the conversion of disaccharides to monosaccharides (Mogale et al., 2011).

Oxidative stress has been broadly reported as a contributor in the progress and evolution of diabetes (Rolo et al., 2006). Strangely high levels of reactive oxygen species and spontaneous reduction in the antioxidant defense mechanisms could result to dent of cellular organelles and enzymes, upsurge in lipid peroxidation, and progression of insulin resistance (Weyer et al., 2001). Ajiboye et al. (2013) reported that the antiradical potentials of any compound or extract can be used to test for the DPPH and ABTS scavenging effect as wells as reducing power potential. Since oxidative stress has been implicated in the pathogenesis of diabetes mellitus, it is thought that the disorders in this disease may be because of an increase in the oxidative stress (Modak et al., 2007).

The results obtained from this study revealed that 2HNQ scavenged free radical comparable to the silymarin used as a reference drug. The reduction in the IC50 values of 2HNQ is a pointer to the radical scavenging potential of the compound. This is supported by the closeness of the R2 values to 1.0.

Overall, the present study elucidates the mechanisms of α- amylase and α-glucosidase inhibitory potential of 2HNQ and apart from the fact that the compound inhibited α-amylase and α-glucosidase, it also scavenged free radicals resulted from increased oxidative stresses.

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Niger. J. Physiol. Sci. 39 (June 2024): 157 – 161
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Haematological and Serum Biochemical Reference Intervals for Nigerian White Fulani Neonatal Calves

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Summary: Reference ranges for blood and serum parameters aid in diagnosing diseases, monitoring health, and distinguishing between normal and abnormal values. There is a lack of available information and research data establishing reference ranges for haematological and serum biochemical parameters in newborn White Fulani calves. We aim to establish reference intervals for haematology and serum biochemistry analytes in apparently healthy White Fulani neonatal calves. A cross-sectional study was conducted on 30 White Fulani neonate calves under 28 days old from different farms in Ibadan, Nigeria. Blood samples were collected for haematology and serum chemistry. The haematological analysis involved packed cell volume, haemoglobin, red and white blood cell counts, differential leukocyte counts, and platelet counts using standard methods. Serum was analysed for proteins, enzymes, metabolites, electrolytes and lipid profiles using spectrophotometric techniques. Normally distributed data was analysed using 2.5th-97.5th percentiles as 95% reference intervals, with 90% confidence intervals per IFCC recommendations, using SPSS software. Haematological intervals included packed cell volume (30.11-32.29%), haemoglobin (9.26-10.04 g/dL), and white blood cell count (4.61-5.18 x 109/L) among others. Key serum biochemistry intervals were total protein (5.61-6.50 g/dL), glucose (67.12-76.78 mg/dL), cholesterol (49.98-60.52 mg/dL), creatinine (0.52-0.61 mg/dL), and electrolytes like sodium (122.25-143.95 mmol/L). The study establishes haematological and serum biochemical reference intervals for White Fulani neonate calves, suggesting their use for future research and comparisons.

Keywords*: Newborn calves, haematology, serum biochemistry, White Fulani, reference interval*

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Manuscript received- February 2024; Accepted: May 2024

DOI: https://doi.org/10.54548/njps.v39i1.19

INTRODUCTION

Establishing reference intervals for haematological and serum biochemical parameters in healthy newborn calves is important for clinicians to accurately diagnose and monitor disease conditions, and to determine whether any alterations are due to physiological changes or pathological processes and in the determination of animal welfare. Laboratory diagnoses are often made by comparing an animal's values to reference intervals from clinically healthy animals (Mohri *et al*., 2007; Marcato *et al*., 2020).

If performed properly, laboratory testing and interpretation of laboratory data have the potential to offer valuable insights into diseases and their treatment (Thrall, 2004). Specific reference intervals are required for haematological and serum biochemical test results to be accurately interpreted for each animal species. In some cases, a distinct reference value may be needed for an analyte from a particular age or breed within a species. Many analyte values fluctuate with the age of the animal, with significant changes often occurring before puberty. Therefore, some analytes necessitate separate reference intervals for different age groups (Meyer and Harvey, 2004) to account for developmental variations. Establishing species-specific and age-adjusted reference ranges allows for meaningful comparison of individual animal results in the context of normal physiological ranges for that parameter.

Alterations in haematological and serum biochemical parameters have been associated with various neonatal diseases in calves. Common disorders presenting with haematological changes include neonatal isoerythrolysis, diarrhoea, pneumonia and sepsis. Elevations in liver enzymes indicate hepatic disorders while abnormal kidney function biomarkers point to renal issues. Metabolic abnormalities are reflected by perturbations in serum electrolyte, protein and enzyme levels. Serial monitoring aids in disease diagnosis, progression and response to treatment.

These parameters are important for clinical evaluation and in identifying animal diseases. Diseases of the newborn and neonatal mortality are major causes of economic loss in livestock production, as newborn calves are highly susceptible to infectious diseases and metabolic disorders in their first few weeks of life (Windeyer *et al*., 2014) due to an immature immune system. Thus, the knowledge of

specific haematological and serum biochemical reference ranges can underscore the need to improve and promote the ability of clinicians to accurately interpret clinical pathology data and diagnose neonatal diseases.

Factors such as species, breed, age, rearing systems, feeding, and number of parturitions influence serum biochemical values; thus, the identification of these factors and their interactions is crucial for the correct interpretation of the blood parameters (Klinkon and Jezek, 2012). This study focuses on the White Fulani breeds of cattle in their first three weeks of life. White Fulani is the most numerous and widely accepted cattle breed in Nigeria (Mbap and Bawa, 2001). It is an indigenous breed derived from Bos indicus, sourced through the derived Guinea savannah, Sahel and subarid climatic zones of northern Nigeria and border countries such as Niger, Chad and Mali (Jeremiah and Banwo, 2019). The breed is highly prized for its adaptability to tropical environments, endurance, and milk production, making it a crucial component of the livestock sector and agriculture in the West African region. There is scarce breed-specific data on White Fulani calves from the first three weeks of life, a period greatly associated with birth-related changes and colostrum intake (Pérez-Santos *et al*., 2015) in normal birth with access to colostrum in the first week of life. This helped control for potential confounding effects during the critical neonatal stage when reliable reference values were being established.

There is a dearth of data on reference intervals for haematology and serum biochemistry analytes in White Fulani neonatal calves. This study aims to establish reference intervals for haematology and serum biochemistry analytes in apparently healthy White Fulani neonatal calves. Understanding reference values is crucial for veterinarians to utilize clinical pathology effectively as a diagnostic and monitoring tool.

MATERIALS AND METHODS

Study design: A cross-sectional study, which considered data at a single point in time, was carried out.
Study animals: A total of 30 White Fulani neonate calves (6 males and 24 females) under 28 days old were sampled in the present study. The neonate calves were owned by different individuals and farms in Ibadan, Oyo State, Nigeria. Only healthy neonate calves were included in the present study while calves with known health issues or under any medical treatment were excluded.

Sample collection: A 6 ml jugular venous blood sample was obtained early in the morning before feeding using a 10 ml syringe and 21G needle by slow suction. The sample was divided into EDTA bottles (3 ml) for haematological analysis and plain bottles (3 ml) for serum chemistry analysis. Within 30 minutes, the samples were transported in an ice chest to the Clinical Pathology Laboratory, Department of Veterinary Pathology, University of Ibadan.

Laboratory analysis: The haematological indices of the samples were analysed using standard methods. The packed cell volume (PCV) was determined by the microhaematocrit method (Thrall and Weiser, 2002). The haemoglobin concentration (Hb) was determined by the cyanomethaemoglobin method (Higgins *et al*., 2008). The red blood cell (RBC) and white blood cell counts were determined manually using a Neubaeur haematocytometer. Differentiation of white cells into segmented neutrophils, lymphocytes, eosinophils, and monocytes was carried out by microscopic examination of Giesma-stained thin blood smear. The platelet count (PC) was done following the Rees and Ecker direct counting method as adopted by Ihedioha and Agina (2014).

Blood samples were subjected to centrifugation at 1008 g (3,000 revolutions per minute) for 10 minutes using a table centrifuge (TDL4®, B. Bran Scientific and Instruments Co., England) to isolate the serum. The blood serum was then analysed for various parameters, including total protein and its fractions (albumin and globulin), urea, creatinine, glucose, sodium, potassium, chloride, phosphorus, calcium, and the activity of creatine kinase, blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH), as well as triglycerides, total cholesterol, and high-density lipoproteins (HDL). These analyses were conducted through spectrophotometry using RANDOX® laboratory reagent kits sourced from RANDOX Laboratories Ltd., Ardmore, United Kingdom, following the manufacturer's instructions. The content of low-density lipoproteins (LDL) in the blood serum was calculated based on the Friedewald equation (Friedewald *et al*., 1972).

Data analysis: Exploratory analysis revealed a normal distribution of the parameters. Mean, standard deviation (SD), and 95% reference intervals were calculated by excluding the upper and lower 2.5% of the range for each haematological and serum biochemical parameter, resulting in the 2.5th and 97.5th percentiles. Additionally, 90% confidence intervals (CI) were calculated for each reference limit to assess their precision for clinical use, following the International Federation of Clinical Chemistry's (IFCC) Approved Recommendations on the Theory of Reference Values (Solberg, 1987). The Statistical Package for Social Sciences (SPSS®, version 26) was used for the analysis.

RESULTS

Haematological reference intervals established for White Fulani neonate calves: The haematological reference interval for White Fuani neonate calves in the present study include the following: packed cell volume (30.11-32.29 %), haemoglobin (9.26-10.04 g/dL), red blood cell count (4.58-4.95 x 106/µL), mean corpuscular volume (63.80-66.72 fL), mean corpuscular haemoglobin concentration (32.32-33.03 g/dL), mean corpuscular haemoglobin (21.18-22.05 pg), white blood cell count (4.61-5.18 x 109/L), platelets (105.38-113.91 x 109/µL), lymphocyte count (2.77-3.20 x 109/L), neutrophil (1.32-1.56 x 109/L), monocyte (0.12-0.15 x 109/L), and eosinophil (0.18-0.22 x 109/L) (Table 1).

Serum biochemistry reference intervals established for White Fulani neonate calves: The serum biochemical reference interval for White Fuani neonate calves in the present study include the following: total protein (5.61-6.50 g/dL), albumin (2.51-2.94 g/dL), globulin (3.11-3.62 g/dL), aspartate transferase (168.72-186.28 IU/L), alanine

transferase (10.79-13.01 IU/L), alkaline phosphatase (170.66-199.04 IU/L), blood urea nitrogen (11.30-12.48 mg/dL), creatinine (0.52-0.61 mg/dL), total bilirubin (0.1-0.14 mg/dL), glucose (67.12-76.78 mg/dL), cholesterol (49.98-60.52 mg/dL), triglyceride (24.29-29.29 mg/dL), high-density lipoprotein (23.95-30.14 mg/dL), low-density lipoprotein (144.18-188.62 U/L), creatine kinase (151.64-195.26 U/L), sodium (122.25-143.95 mmol/L), potassium (2.44-2.79 mmol/L), chloride (93.10-96.90 mmol/L), phosphorus (2.19-2.68 mmol/L), and calcium (9.07-11.09 mmol/L) (Table 2).

DISCUSSION

The key finding of the results is the establishment of haematological and serum biochemical reference intervals for White Fulani neonate calves. The results suggest a comprehensive set of reference intervals for various haematological and serum biochemical parameters in White Fulani neonate calves.

The haemoglobin (9.26-10.04 g/dL) and red blood cell count (4.58-4.95 x 106/µL) of the neonatal calves in the present study are lower than those of the White Fulani adults reported in several studies (Ihedioha *et al*., 2017; Ewuola *et al*., 2014; Unigwe *et al*., 2022). This could be because neonatal calves are born with a lower number of red blood cells, a condition known as physiological anaemia of the newborn. This is a normal, transient phenomenon that occurs due to the rapid breakdown of foetal red blood cells after birth and the slower rate of red blood cell production by the calf's bone marrow initially (Mohri *et al*., 2007).

Table 1:
Haematological reference intervals established for White Fulani neonate calves

Parameter	Mean reference value	SD	Reference interval	90% CI for lower reference limit	90% CI for upper reference limit
PCV (%)	31.20	2.33	30.11-32.29	29.23-30.99	31.41-33.17
HB (g/dL)	9.26	0.84	9.26-10.04	8.94-9.58	9.73-10.36
RBC (x 10^6/µL)	4.76	0.39	4.58-4.95	4.43-4.73	4.80-5.09
MCV (fL)	65.80	3.12	63.80-66.72	62.62-64.98	65.54-67.91
MCHC (g/dL)	32.67	0.77	32.32-33.03	32.03-32.61	32.74-33.32
MCH (pg)	21.62	0.93	21.18-22.05	20.83-21.53	21.70-22.40
WBC (x 10^9/L)	4.90	0.62	4.61-5.18	4.37-4.84	4.95-5.42
Platelets (x 10^9/ µL)	109.65	91.20	105.38-113.91	101.92-108.83	110.46-113.91
Lymph (x 10^9/L)	2.99	0.45	2.77-3.20	2.60-2.94	3.03-3.37
Neut (x 10^9/L)	1.44	0.26	1.32-1.56	1.22-1.42	1.46-1.66
Mono (x 10^9/L)	0.13	0.04	0.12-0.15	0.10-0.13	0.14-0.16
Eos (x 10^9/L)	0.20	0.04	0.18-0.22	0.16-0.19	0.20-0.24

Reference interval= 2.5 and 97.5 percentiles, SD= standard deviation, CI= confidence interval, PCV= packed cell volume, HB= haemoglobin, RBC= red blood cell, MCV= mean corpuscular volume, MCHC= mean corpuscular haemoglobin concentration, MCH= Mean corpuscular haemoglobin, WBC= white blood cell, lymph= lymphocyte, neut= neutrophil, mono= monocyte, and eos= eosinophil

Table 2:
Serum biochemical reference intervals established for White Fulani neonate calves

Parameter	Mean reference value	SD	Reference interval	90% CI for lower reference limit	90% CI for upper reference limit
Total Protein (g/dL)	6.05	0.95	5.61-6.50	5.24-5.97	6.20-6.80
Albumin (g/dL)	2.73	0.46	2.51-2.94	2.34-2.68	2.77-3.22
Globulin (g/dL)	3.37	0.55	3.11-3.62	2.90-3.32	3.41-3.83
AST (IU/L)	177.50	18.77	168.72-186.28	161.60-175.83	179.17-193.40
ALT (IU/L)	11.90	2.38	10.79-13.01	9.88-11.69	12.11-13.92
ALP (IU/L)	184.85	30.31	170.66-199.04	159.17-182.15	187.44-210.53
BUN (mg/dL)	11.89	1.25	11.30-12.48	10.83-11.78	12.00-12.95
Creatinine (mg/dL)	0.57	0.09	0.52-0.61	0.49-0.56	0.57-0.64
Total bilirubin (mg/dL)	0.12	0.06	0.1-0.14	0.0746-0.1166	0.13-0.17
Glucose (mg/dL)	71.95	10.32	67.12-76.78	63.21-71.03	72.87-80.69
Cholesterol (mg/dL)	55.25	11.27	49.98-60.52	45.71-54.25	56.25-64.79
Triglyceride (mg/dL)	26.70	5.14	24.29-29.29	22.34-26.24	27.16-31.05
HDL (mg/dL)	27.05	6.61	23.95-30.14	21.45-26.16	27.64-32.65
LDH (u/L)	166.40	47.48	144.18-188.62	126.18-162.17	170.63-206.62
LDL (mg/dL)	45.07	12.49	39.22-50.92	34.49-35.95	46.19-55.65
Creatine kinase (u/L)	173.45	46.60	151.64-195.26	133.98-169.30	177.60-204.92
Sodium (mmol/L)	133.10	23.19	122.25-143.95	113.46-131.04	135.16-152.74
Potassium (mmol/L)	2.61	0.37	2.44-2.79	2.29-2.58	2.64-2.93
Chloride (mmol/L)	95.00	4.05	93.10-96.90	91.10-94.64	95.36-98.44
Phosphorus (mmol/L)	2.44	0.53	2.19-2.68	2.14-2.23	2.64-2.73
Calcium (mmol/L)	10.08	2.16	9.07-11.09	8.25-9.89	10.27-11.91

Reference interval= 2.5 and 97.5 percentiles, SD= standard deviation, CI= confidence interval, AST= aspartate transaminase, ALT= alanine transaminase, ALP= alkaline phosphatase, BUN= blood urea nitrogen, HDL= high density lipoprotein, LDH= lactate dehydrogenase, LDL= low density lipoprotein

The mean corpuscular volume (MCV) (63.80-66.72 fL) of the neonatal calves in the present study is higher than the MCV of White Fulani adults reported in several studies (Ihedioha *et al*., 2017; Ewuola *et al*., 2014; Unigwe *et al*., 2022). Since neonatal calves are born with a lower number of red blood cells compared to adult cattle (Mohri *et al*., 2007), the body compensates for this physiological anaemia by producing larger red blood cells, leading to an increased MCV (Mohri *et al*., 2007). However, the mean corpuscular haemoglobin concentration (MCHC) (32.32-33.03 g/dL) of the neonatal calves is lower than the MCHC of White Fulani adults reported in several studies (Ihedioha *et al*., 2017; Ewuola *et al*., 2014; Unigwe *et al*., 2022). This can be attributed to the fact that neonatal calves are born with lower haemoglobin levels due to physiological anaemia, which can lead to a lower MCHC value (Brun-Hansen *et al*., 2006). The white blood cell count (4.61-5.18 x 109/L), lymphocyte count (2.77-3.20 x 109/L), and neutrophil count (1.32-1.56 x 109/L) in the present study are lower than those of adult White Fulani cattle reported by Ihedioha *et al*., (2017). This could be because neonatal calves are born with an immature immune system, which results in lower production of white blood cells, including lymphocytes and neutrophils. The immune system develops gradually during the first few weeks and months of life (Barrington and Parish, 2001; Chase *et al*., 2008).

Our study shows that the total protein level in the White Fulani neonate calves is in the same range as adult White Fulani cattle in studies carried out by Ihedioha *et al*., (2017) and Ewuola *et al*., (2014). However, studies (Knowles *et al*., 2000; Brun-Hansen *et al*., 2006; Mohri *et al*., 2007) have shown that neonatal calves have lower total protein than adult cattle. Our result, being different from other studies, might be due to the absorption of colostral immunoglobulins, which contribute significantly to the total protein content, and can result in neonatal calves having similar or even higher total protein levels compared to adult cattle. This mechanism of immunoglobulin transfer from the mother to the calf through colostrum can help explain why some cattle breeds like White Fulani calves may exhibit comparable total protein levels in both neonatal calves and adult cattle, despite their different physiological stages and protein requirements (Quigley and Drewry, 1998).

The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the White Fulani neonates in this study are higher than AST and ALT levels in adult White Fulani cattle reported by Ewuola *et al*., (2014). This is in agreement with several studies where they compared the levels of these liver enzymes between neonatal calves and adult cattle. The general consensus is that neonatal calves tend to have higher levels of AST and ALT compared to adult cattle (Knowles *et al*., 2000; Mohri *et al*., 2007).

The blood urea nitrogen and creatinine levels in the White Fulani neonates in this study are lower than the blood urea nitrogen and creatinine values in adult White Fulani cattle reported by Ewuola *et al*. (2014). The lower blood urea nitrogen and creatinine levels in neonatal calves are attributed to several factors: (1) immature renal function and lower glomerular filtration rate in neonatal calves; (2) lower muscle mass and lower protein turnover in neonatal calves, resulting in lower creatinine production; and (3) lower dietary protein intake and lower urea production from protein metabolism in neonatal calves. Also, several studies (Knowles *et al*., 2000; Brun-Hansen *et al*., 2006; Mohri *et al*., 2007) have shown that blood urea nitrogen and creatinine levels are lower in neonatal calves compared to adult cattle.

The cholesterol level in the neonate calves in the present study is lower when compared with a study on adult White Fulani cattle. In agreement with this, several other studies have shown lower cholesterol levels in neonate calves when compared to adult cattle (Knowles *et al*., 2000; Brun-Hansen *et al*., 2006; Mohri *et al*., 2007). The lower cholesterol levels in neonatal calves are thought to be due to several factors, including immature lipid metabolism and cholesterol synthesis pathways in newborn calves, lower dietary intake and absorption of cholesterol in neonatal calves as they are primarily consuming milk which is low in cholesterol, and lower cholesterol requirements for growth and development in neonatal calves compared to adult cattle (Rauprich *et al*., 2000).

Abnormal values outside these reference intervals in White Fulani neonate calves may signal potential health issues, and continuous monitoring of these parameters can aid in the early detection of diseases or nutritional imbalances. Veterinarians can utilize these reference intervals as a diagnostic tool to assess the health status of individual calves and the overall herd, while researchers can use the data as a foundational basis for further studies on the health and physiology of White Fulani neonate calves.

A potential limitation of this study is the modest sample size of 30 White Fulani neonate calves included in the cross-sectional study. This limited sample size may not fully represent the diversity within the population, and the findings might not be entirely reflective of the broader range of health conditions or variations in this specific calf population. The sample size could potentially limit the external validity and applicability of the established reference intervals to a larger population of White Fulani neonate calves. Further research with an expanded sample size could help validate and refine the reference intervals established here, strengthening their application and relevance to the broader population.

In conclusion, the study has successfully established comprehensive haematological and serum biochemical reference intervals for White Fulani neonate calves. We recommend that researchers use these reference intervals as a foundation for further studies, comparisons with other breeds, and investigations into factors influencing haematological and serum biochemical parameters in neonate calves.

Acknowledgments

The authors appreciate the technical assistance of the Staff of the Clinical Pathology Laboratory, Department of Veterinary Pathology, University of Ibadan, Nigeria

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Niger. J. Physiol. Sci. 38 (June 2023): 163 – 169
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

Anti-Diabetic Activities of the Hydromethanolic Leaf Extract of *Rauvolfia vomitoria*.

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Summary: *Rauvolfia vomitoria* (African snake root) is an herbal shrub which has been reported to possessing strong anti-diabetic action; however, the method(s) by which this plant effects its reported anti-diabetic action is poorly understood. Hence, this research through in vitro and in vivo studies investigated the methods (mechanisms of action) through which this plant effects its reported anti-diabetic action. In the in vitro study, the effect of the hydromethanolic leaf extract of the plant on alpha amylase and alpha glucosidase enzymes were investigated in comparison with acarbose; while in the in vivo study, the effect of the extract on blood sugar and plasma insulin levels of normal and streptozotocin-induced diabetic albino rats were investigated in comparison with glyburide. Results from the in vitro study showed the percentage inhibition of alpha amylase by the extract (100 mg/ml) to be 62.28 (4.73) with an IC_{50} values of 74.35 mg/ml, while acarbose had a percentage inhibition and IC_{50} values of 72.81 (2.52) and 66.05µg/ml, respectively. The percentage inhibition of alpha glucosidase by the extract (100 mg/ml) was 79.63 (4.09) and an IC50 values of 58.85 mg/ml, while acarbose had a percentage inhibition and IC_{50} values of 82.11 (1.84) and 56.79µg/ml, respectively. From the in vivo study, the result showed that the extract caused a dose and treatment-duration dependent significant increases ($P<0.05$) in the plasma insulin levels of streptozotocin-induced diabetic rats in a manner comparable to glyburide. These results showed that *Rauvolfia vomitoria* leaf effects its anti-diabetic actions via two separate mechanisms; the plasma insulin increasing mechanism and the alpha amylase and alpha glucosidase inhibitory mechanism.

Keywords*: Rauvolfia vomitoria leaf extract, alpha amylase, alpha glucosidase, plasma insulin, acarbose, glyburide*

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Manuscript received- January 2023; Accepted: November, 2023

DOI: https://doi.org/10.54548/njps.v39i1.20

INTRODUCTION

Diabetes mellitus is a health condition distinguished by severely elevated plasma sugar level ensuing from either a dysfunction of the beta cells of the islets of Langerhans (pancreatic islets) which often results to insufficient insulin secretion (type-1) or cells of the body not responding to insulin (type-2) (N'doua *et al*., 2016; Akpojotor and Ebomoyi, 2021a).

The prevalence of diabetes is constantly increasing globally. In 1980, it was 108 million people that was estimated to have diabetes worldwide (World Health Organization, 2016). By 2013, the figure had increased to 382 million (Shi and Hu, 2014) and as at 2019, the figure has further increased to 463 million (Saeedi *et al*., 2019). More increases have been forecasted (World Health Organization, 2008; Piero *et al*., 2014; Saeedi *et al*., 2019). This health condition has become a great source of concern due to its increasing prevalence and the inability of orthodox drugs in offering absolute cure to it (Okpuzor *et al*., 2009; Kumari *et al*., 2013; Akpojotor and Ebomoyi, 2021a).

The use of medicinal plants (herbs) as therapy for management of diabetes mellitus and other ailments is an age long practice (Mbaka *et al*., 2010; Akpojotor and Kagbo, 2016; Udia *et al*., 2016). The ailment-specific pharmacological basis (such as active phytoconstituents, mechanisms of action and possible side effects) of some of these medicinal plants and their formulations have been elucidated by scientific investigations (Udia *et al*., 2016), while many others including *Rauvolfia vomitoria* are yet to be elucidated.

Rauvolfia vomitoria commonly known as African snake root is a plant species in genus *Rauvolfia* under the apocynaceae family (Ajayi, 2021). It mainly grows wildly in different forests across the globe especially in tropical Africa, South America and Asia (Amole, 2003; Ogbe *et al*., 2009; Akpojotor and Ebomoyi, 2021a). Various studies have attributed a range of medicinal actions to it including treatment of cancer (Yu *et al*., 2013), hypertension (Amole, 2003; Ezejindu *et al*., 2013), convulsion (Olatokunboh *et al*., 2009), mental derangement (Bisong *et al*., 2010), diabetes (Campbell-Tofte *et al*., 2011), etc. On its diabetic medicinal action, available literatures mainly report its plasma sugar lowering (hypoglycemic) ability (Compbell-Tofte *et al*., 2011; N'doua *et al*., 2015; N'doua *et al*., 2016), while vital information such as the biochemical constituents through which the plant effect this action as well as the method (mechanism of action) are still lacking. This

therefore, necessitated this investigation which was designed to examine the mechanisms through which *Rauvolfia vomitoria* leaf effects its anti-diabetic action by examining its effects on plasma insulin, alpha amylase and alpha glucosidase levels in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

This study was carried out in two phases (an *in vitro* and an *in vivo* phase). In the *in vitro* phase, the effect of the hydromethanolic leaf extract of the plant on alpha amylase and alpha glucosidase enzymes were investigated in comparison with acarbose, a standard alpha amylase and alpha glucosidase inhibitory drug. In the *in vivo* phase, the action of the plant on plasma insulin was investigated in comparison with glyburide, a standard anti-diabetic drug that effects its anti-diabetic action by enhancing insulin secretion. Streptozotocin-induced diabetic male adult albino rats was used as models.

Research Protocol Approval: Approval for research and animal handling for this research investigation was granted by the Research Ethics Committee in the College of Medical Sciences of the University of Benin, Benin City, Nigeria. App. No.: CMS/REC/2021/159.

Chemicals and Drugs: Insulin and Glucagon immunoassay kits (Calbiotech, Inc. 1935 Cordell Ct., CA, USA); Streptozotocin, *Saccharomyces cerevisiae* and paranitrophenyl-glucopyranoside (Sigma-Adrich Co., St Louis, USA.); Purified porcine pancreatic *α*-amylase (ICN Biochemicals, Ohio, USA) were acquired from BioRapid Diagnostic Nig. Ltd., Abuja. Accu‑Chek Active blood glucose meter and Accu‑Chek Active blood sugar testing strips; glyburide and acarbose were obtained from Green House Pharmacy, at the University of Port Harcourt Teaching Hospital, Rivers state, Nigeria.

Plant Sample (Collection and Identification): *Rauvolfia vomitoria* leaves were collected from open farmlands in Orhuwhorun village in the South-South region of Nigeria. The plant sample was authenticated by E. Nwosu at the Ecoland Herbarium (EH) Unit, University of Port Harcourt Choba, Rivers State, with EH. ID. number EH – P – 051.

Extraction of Active Components from Plant sample: Collected leaves of *Rauvolfia vomitoria* were rinsed in clean water to rid all dirt and then air-dried in a well-ventilated room for two weeks. The dried leaves were then milled to fine powder and thereafter subjected to maceration extraction using hydromethanol (ratio of water to methanol is 1:4) as extraction solvent following the method of Akpojotor and Kagbo, 2016. In brief, the milled sample was immersed in sufficient extraction solvent. The mixture was stirred from time to time within forty-eight hours at room temperature after which Whatman No. 1 filter paper was used to separate it into two portions (a filtrate and a residue). Thereafter, the filtrate was concentrated in a vacuum at 40°C using a rotary evaporator (Searl Instruments Ltd. England) into the extract used in the experiments.

Preliminary Phytochemical Profiling of Extract: Preliminary phytochemical profiling of the extract was done following the methods of Sofowora, 2008; Ibironke and Olusola, 2013; and Amita and Shalini, 2014.

***In Vitro* Experiments**

Alpha-Amylase Enzyme inhibition test: The effect of the hydromethanolic leaf extract of the plant on alpha amylase enzyme was investigated following the method used by Chelladurai and Chinnachamy (2018), with slight modification.

In preparing the starch (substrate) solution used in this experiment, 1 gram of starch from potato was dissolved in 10mls of distilled water, the mixture was boiled, cooled, and the volume increased to 100mls by the addition of more distilled water. 1 milligram of porcine pancreatic alpha amylase dissolved in 100mls of 20 mM phosphate (pH 6.9) was used as the enzyme solution. Different masses of the extract (20, 40, 60, 80 and 100 milligrams) were dissolved in dimethyl sulfoxide to prepare the different concentrations (20 to 100mg/ml) of sample solutions used. The colouring reagent was Dinitrosalicylic (DNS) solution.

Three sets of tests (test, blank and control) were conducted each in triplicate. In brief, 1ml each of the sample and enzyme solutions were mixed in a test tube and incubated under room temperature (32-33^{o}C) for 20 minutes, thereafter, 1ml of the incubated solution was mixed with 1ml of the substrate solution and incubated for 3 min at room temperature. After the 3 minutes' incubation, 1 ml of the coloring reagent was added and the mixture heated in water bath at 85^{o}C for 15 minutes after which it was diluted with distilled water (9ml) and the absorbance was recorded at 540 nm. In the blank experiment, the coloring reagent was added before the substrate solution, while the rest of the method was the same as in the test experiment. For control, all procedures were as in the test experiment except that the sample solution was replaced with 1ml of dimethyl sulfoxide. Acarbose (20 to 100µg/ml) was used as a positive (comparative) control.

The percentage inhibition of the sample was determined by mathematical calculation using the formula:

$$\% \text{ Inhibition} = \frac{[(Ac - As)/Ac] \times 100}{}$$

Ac-absorbance of control;

Where As=absorbance of sample

Alpha-Glucosidase inhibitory test: The alpha glucosidase inhibitory action of the extract was investigated using Kim *et al*. (2005) method as described by Kazeem *et al*. (2013) with slight modifications. 1.01M/ml alpha glucosidase from brewer's yeast, p-nitrophenyl glucopyranoside prepared in 20mM phosphate buffer (pH 6.9) and 0.1M sodium carbonate (Na_2CO_3) were used as enzyme, substrate and reaction-stopping solutions respectively.

In brief, 100μl of the enzyme solution was added to 50μl of the different concentrations of the extract (20-100mg/ml) and incubated for 10 minutes under room temperature. Thereafter, 50μl of the substrate solution was added and incubated for 20 minutes. After the 20 minutes' incubation, 200μl of the reaction-stopping solution was added and the alpha glucosidase activity was immediately determined by measuring the yellow-colored para-nitrophenol released from p-nitrophenyl glucopyranoside at

405 nm. For control, dimethyl sulfoxide was used in place of test solutions, while for positive controls, acarbose (20, 40, 60, 80 and 100µg/ml) was used in place of the sample extract. The alpha glucosidase inhibitory activity of the extract was calculated using the mathematical formula:

% Inhibition = [1 – Ae/Ac] × 100

Where Ae=Absorbance of Extract, and Ac=Absorbance of Control

All samples were assayed in triplicate.

***In Vivo* Studies on Rats**

Induction of Diabetes: Diabetes was induced in experimental animals (male albino rats) following the method of Rossini (1977) as used by Akpojotor and Ebomoyi (2021b). In brief, experimental animals were fasted overnight and diabetes induced via intraperitoneal administration of 55mg/kg bwt streptozotocin solution (streptozotocin dissolved in citrate buffer, pH 4.5). Following the streptozotocin administration, animals having blood sugar equal to or more than 280 mg/dl were recruited as diabetic animals for this study.

Grouping of Experimental Animals: Fifty (50) albino rats (male) weighing between 180-220g were recruited for this study. Experimental animals were grouped into five groups of ten animals each as follows; **1**-Normal control group (Non-diabetic animals)**; 2**-Diabetic control group**; 3**-250mg/kg bwt extract treated group**; 4**-500mg/kg bwt extract treated group**; 5-** 5mk/kg bwt glyburide treated group. The three treated groups (group 3-5) were treated with 250mg/kg) bwt dose of the extract, 500mg/kg bwt dose of the extract and 5mg/kg bwt dose of glyburide respectively following the method of Diehl *et al*., 2001. Administrations were done daily. In brief, a flexible cannula is attached to the syringe containing the extract or drug to be administered. The delivery end of the cannula is inserted into the mouth of the animal and gently advanced into the esophagus and towards the stomach, then the extract or drug is emptied gently into the animal.

Blood Sample Collection: After 14 and 28 days of treatment, five rats from each group were sacrificed and blood collected from each sacrificed animal for hormonal assay of insulin via cardiac puncture.

Measurement of Blood Glucose Levels: The Accu-Chek Active blood glucose meter and Accu-Chek Active test strips were used for the measurement of blood glucose levels of animals. In brief, the tip of the tail of the animal was pierced with a lancet and a drop from the blood that oozes out was applied to test strip inserted into the Accu-Chek blood glucose meter. The glucose concentration in the blood was displayed on the screen of the Accu-Chek blood glucose meter. This procedure was performed on all the animals. Blood glucose levels of all groups were measured just before commencement of treatment and thereafter, after every four days of treatment.

Biochemical Analysis: Collected blood samples from sacrificed animals were subjected to hormonal assays following the methods of Tietz (1995) as used by Akpojotor and Ebomoyi (2021b). In brief, 50µL of the different concentration of standard working solution were each pipetted into the first two test pots, while blood serum from experimental animals were pipetted into the other test pots. Then 50µL of biotinylated solution was pipetted into all the test pots and they were incubated for 45 minutes. After incubation, the test pots were drained and washed using wash buffer. After washing, 100µL of Horseradish Peroxidase Conjugate working solution was pipetted into each test pot and they were incubated for 30 minutes. After incubation, the test pots were again drained and washed. Thereafter, 90µL of substrate reagent was pipetted into each test pot and incubated for 15 minutes. After incubation, stop solution (50µL) was added to each test pot and a micro-plate reader set at 450nm was used to determined the optical density (OD) value of each test pot.

Note: The Standard Working Solution of different concentrations and the test pots (wells) were provided in the ELISA immunoassay kit. All incubations were done at room temperature.

Data Analysis: The data obtained from this study were recorded as mean ± standard error of mean (SEM) and were analyzed using ANOVA. Statistical differences between means were analyzed by applying Duncan Multiple Range Test (DMRT) for comparison with control groups at 95% ($p<0.05$) confidence levelThis study was carried out in two phases (an *in vitro* and an *in vivo* phase). In the *in vitro* phase, the effect of the hydromethanolic leaf extract of the plant on alpha amylase and alpha glucosidase enzymes were investigated in comparison with acarbose, a standard alpha amylase and alpha glucosidase inhibitory drug. In the *in vivo* phase, the action of the plant on plasma insulin was investigated in comparison with glyburide, a standard anti-diabetic drug that effects its anti-diabetic action by enhancing insulin secretion. Streptozotocin-induced diabetic male adult albino rats was used as models.

RESULTS

Preliminary Phytochemical Screening of Hydromethanolic Extract of *Rauvolfia vomitoria* Leaf: Investigation on the phytochemical constituents of Hydromethanolic extract of *Rauvolfia vomitoria* Leaf revealed that it contains some bio active phytochemical compounds as tabulated in Table I below.

Table I:
Preliminary phytochemical screening of hydromethanolic *Rauvolfia vomitoria* leaf extract

Constituents	Inference
Alkaloids	+
Flavonoids	+
Saponins	+
Tannins	+
Terpenes	+
Cardiac glycosides	+
Cyanogenic glycosides	-
Anthraquinones	-

\+ *indicates presence*
\- *Indicates absence*

Table 2:
Effect of treatment with hydromethanolic (HM) leaf extract of *Rauvolfia vomitoria (Rv)* on blood glucose level (mg/dl) of Streptozotocin-induced diabetic male wistar rats

Group	Description	Just b/4 induct.	72 hrs after induct.	2 days of treat.	6 days of treat.	10 days of treat.	14 days of treat.	18 days of treat.	22 days of treat.	26 days of treat.	28 days of treat.
1	Normal control	82.80±3.34	81.60±3.61	85.60±2.71	80.40±2.84	82.80±3.95	82.00±3.08	85.00±3.16	85.00±3.63	89.00±2.10	88.20±1.62
2	Diabetic control	89.80±2.92	339.00±6.38	326.60±7.81	315.00±7.56	302.80±4.19	299.60±7.88	303.40±1.94	299.80±2.48	304.00±2.19	300.00±0.71
3	Diabetes plus 250mg/kg of extract	91.00±3.81	328.40±8.10	326.80±9.55	304.00±7.21	268.40±14.08	223.40±2.56	201.20±3.44*	146.00±10.32*	128.40±6.95*	115.00±8.76*
4	Diabetes plus 500mg/kg of extract	86.60±2.23	324.80±10.72	280.40±11.10	241.60±4.24	185.40±6.45*	127.20±4.25*	106.20±5.45*	94.20±2.22*	89.40±1.54*	87.80±3.31*
5	Diabetes plus glyburide (5mg/kg)	86.60±2.23	318.00±6.74	279.00±6.72	244.20±3.07	179.40±5.97*	130.80±3.77*	110.20±4.80*	100.60±2.25*	96.20±1.83*	81.00±4.23*

*Values are expressed as Mean±SEM; n=5; *=Significant at p<0.05*

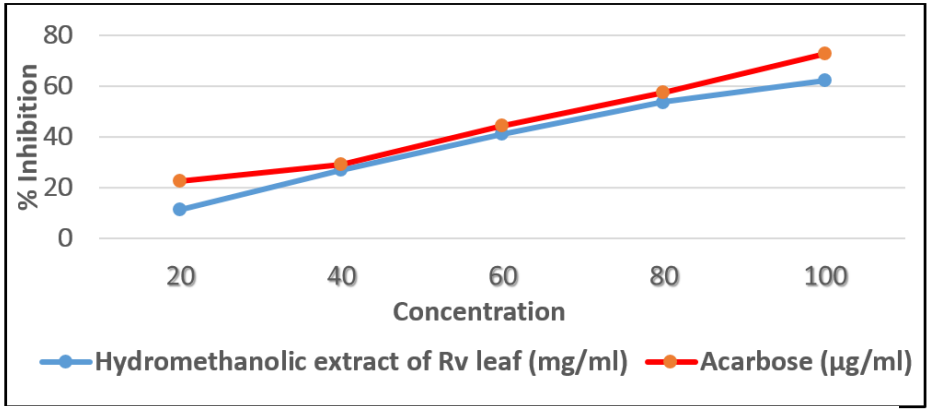

Figure I:
Effect of hydromethanolic *Rauvolfia vomitoria* leaf extract on α-amylase in comparison with acarbose

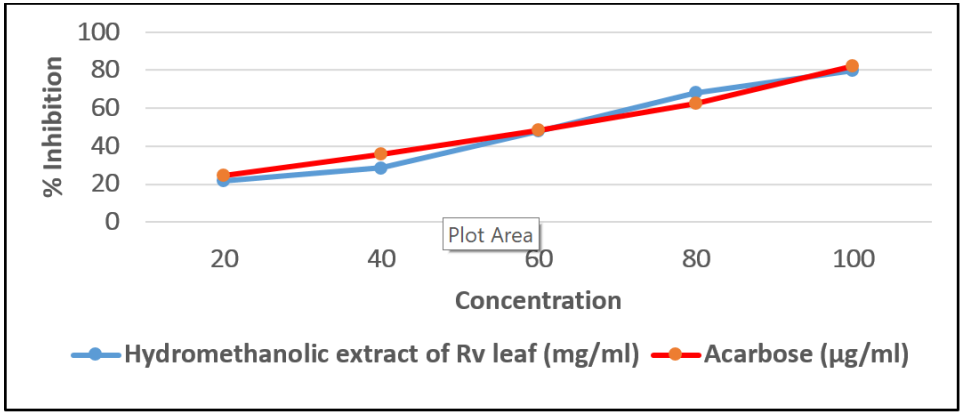

Figure 2:
Effect of hydromethanolic *Rauvolfia vomitoria* leaf extract on α-glucosidase in comparison with acarbose

***In Vitro* Study:** The in vitro study revealed that *Rauvolfia vomitoria* leaf extract inhibited alpha amylase and alpha glucosidase enzymes. As shown in figure I, different concentrations (20 - 100 mg/ml) of the extract exhibited alpha amylase inhibition in a dose dependent manner comparable to acarbose (a standard alpha amylase inhibitory drug). The IC_{50} values for the extract and acarbose were calculated as 74.35 mg/ml and 66.05µg/ml, respectively.
The result of the alpha glucosidase inhibitory action of extract (figure II) showed that the extract also has a dose dependent inhibitory effect on alpha glucosidase enzyme. The IC_{50} values for the extract and acarbose were calculated as 58.85 mg/ml and 56.79µg/ml, respectively.

The maximum percentage of inhibition for both alpha amylase and alpha glucosidase enzymes by *Rauvolfia vomitoria* leaf were obtained at a concentration of 100mg/ml and were 62.28 (4.73) % and 79.63 (4.09) %, respectively.

***In Vivo* Study:** The in vivo study revealed that treatment with *Rauvolfia vomitoria* leaf caused a dose and treatment-duration dependent effect on the insulin levels of streptozotocin-induced diabetic rats.
Table II, the tabulated result of the in vivo study indicated significant increase ($P<0.05$) in the insulin levels of streptozotocin-induced diabetic rats following treatment with 250 and 500 mg/kg bwt doses of the extract. These increases were comparable to that of the standard anti-diabetic drug (glyburide) treated group. The result also showed that the significant increase in the insulin levels of the extract treated groups were dose- and duration-dependent.

Table 2:
Effect of hydromethanolic extract of *Rauvolfia vomitoria* leaf on plasma insulin levels of Streptozotocin-induced diabetic albino rats

S/N	Description	Insulin (µU/ml) after two weeks treatment	Insulin (µU/ml) after four weeks treatment
1	Normal control group (Non-diabetic animals)	13.180 (1.550)	13.380 (0.610)
2	Diabetic control group	6.990 (0.780)	7.290 (0.720)
3	250mg/kg bwt extract treated group	9.490 (0.920)	11.190 (0.870)*
4	500mg/kg bwt extract treated group	11.800 (1.100)*	13.250 (0.720)*
5	5mk/kg bwt glyburide treated group	10.640 (0.980)*	13.980 (0.450)*

Values are expressed as Mean (SEM); n=5; *=Significant at $p<0.05$

DISCUSSION

The result of the preliminary phytochemical screening of the extract in this study is similar to those of Ojo *et al*. (2012), Okereke *et al*. (2015) and Ajayi (2021) which reported Alkaloids, flavonoids, saponins, cardiac glycosides, terpenoids, and tannins, as the bio active phytochemical compounds of *Rauvolfia vomitoria* leaf. Some of these phytochemical compounds have been shown by previous studies to possess anti-diabetic actions and can be potential natural sources for medications against diabetes. For example, Osadebe *et al.* (2010) reported that the presence of flavonoids, terpenoids and tannins in plant confer blood glucose lowering ability on it. Similarly, Etxeberria *et al*. (2012) implicated flavonoid as an inhibitor of carbohydrate digestive enzyme (α-glucosidase); Khan *et al*. (2014) listed tannins and terpenoids as the main phytoconstituents responsible for the anti-diabetic action of Chinaberry tree (*Melia azedarach*), while Poongunran *et al*. (2015) reported that flavonoids and tannins have potential inhibitory effects on alpha amylase and alpha glucosidase enzymes.
The use of medicinal plants (herbs) as therapy for management of diabetes mellitus and other ailments is an age long practice (Mbaka *et al*., 2010; Akpojotor and Kagbo, 2016; Udia *et al*., 2016). Most medicinal plants are believed to be effective in managing different ailments. But the ailment-specific pharmacological profile and actions (such as active phytoconstituents, mechanisms of action and possible side effects) of most of these medicinal plants and their formulations have not been elucidated by scientific investigations, hence our recent studies on *Rauvolfia vomitoria*.

Diabetes mellitus is a health condition distinguished by severely elevated plasma sugar level resulting from either a dysfunction of the beta cells of the pancreas which often results to insufficient insulin secretion (type-1) or cells of the body not responding to insulin (type-2). Hence, the primary focus of its treatment is to return the severely elevated blood sugar level to the normal physiological range or as close as possible. This explains why the ability to cause a decrease in the blood sugar level of an elevated blood sugar system is the first criterion a substance, drug or plant, must meet for it to be considered an anti-diabetic agent. The result of this study (table 2), showed that *Rauvolfia vomitoria* leaf has anti-diabetic potentials. Our result from this study is in line with the reports of Campbell-Tofte *et al*. (2011) and N'doua *et al*. (2016) which associated *Rauvolfia vomitoria* leaf with blood glucose lowering (hypoglycemic) activity.

The treatment of diabetes is anchored on three strategies or mechanisms which include; (1) prevention of postprandial glucose spike, (2) increasing the plasma insulin and (3) increasing body cells' sensitivity to insulin.

Alpha amylase and alpha glucosidase are the enzymes responsible for the digestion of starch (carbohydrates) in ingested food, converting them from poly-saccharides and di-saccharides into absorbable mono-saccharides (glucose and fructose) which are absorbed through the walls of the small intestine into circulation. By inhibition these enzymes, the formation of absorbable carbohydrates is inhibited and postprandial glucose spike is prevented (De Sales *et al*., 2012; Chellandurai and Chinnachamy, 2018; Alqahtani *et al*., 2020). The result from this study showed that the extract possesses strong inhibitory effects on these enzymes and therefore suggest that, one of the mechanisms through which *Rauvolfia vomitoria* leaf effects its anti-diabetic action is by alpha amylase and alpha glucosidase inhibition. This result is in line with reports of Kwon *et al*. (2006),

Kazeem *et al*. (2013) Chellandurai and Chinnachamy (2018), and Alqahtani *et al*. (2020), which associated various plants with natural alpha amylase and alpha glucosidase inhibitors.

Insulin in the body is secreted by the beta cells of the Islet of Langerhans of the pancreas. Hence, streptozotocin induces diabetes by destroying the beta cells of the pancreas (Szkudelski, 2001; Akpojotor and Ebomoyi, 2021b). Streptozotocin produces free radicals such as hydrogen peroxide and peroxynitrite which are selectively permeable to the beta cells of the pancreas (Elsner *et al*., 2000; Lenzen, 2008). Accumulation of these free radicals in the beta cell causes oxidative stress/destruction of the cell and consequently deficiency of plasma insulin (Raza *et al*., 2011; Akpojotor and Ebomoyi, 2021b). Increasing plasma insulin is one of the common approaches in the treatment of type 2 diabetes, and results obtained from this study showed that *Rauvolfia vomitoria* leaf restored physiologic insulin levels in streptozotocin-induced diabetic albino rats.

From our findings, it was concluded that *Rauvolfia vomitoria* leaf contains some bio active phytochemical compounds responsible for its anti-diabetic actions. *Rauvolfia vomitoria* leaf effects its anti-diabetic actions by minimizing the amount of glucose that is absorbed into circulation after a carbohydrate meal via its α-amylase and α-glucosidase inhibitory action; and by increasing plasma insulin levels. The inhibitory effect is more towards alpha glucosidase than alpha amylase.

This research study is the first to investigate the anti-diabetic mechanisms of action of *Rauvolfia vomitoria* leaf, it will therefore contribute significantly to the available knowledge on the anti-diabetic properties of *Rauvolfia vomitoria* and the potential for its formulation as medicine for the management of diabetes.

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Niger. J. Physiol. Sci. 39 (June 2024): 87 – 90
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

The Effect of Long-term Consumption of Calabash Chalk on Peptic Ulcer Scores in Wistar Rats

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Summary: This study was aimed at investigating the effects of long-term consumption of calabash chalk on ulcer scores in Wistar rats. Fifty (50) male adult Albino Wistar rats weighing between 160g - 180g were used. The rats were randomly divided into ten (10) groups of 5 rats each consisting of five (5) control and five (5) test rats. Group 1 was the control group and was administered 1ml of distilled water daily while group 2 was the test group and was administered 1ml of calabash chalk suspension orally daily. They were allowed food and water ad libitum. The experiment lasted for 28 days, thereafter, basal gastric acid secretion, gastric pepsin secretion, gastric pH, gastric mucous output, and gastric ulcer scores were measured. The mean basal gastric acid secretion for the control and the test group was 0.58± 0.22 and 0.61 ± 0.37uMol/10min respectively. Basal gastric acid secretion was not significantly higher in the test group when compared with the control group. The mean gastric pepsin secretion for the control and the test group was 0.46 ± 0.02 and 0.57 ± 0.02mg/100ml respectively. The mean gastric pepsin secretion in the test group was significantly higher ($P<0.01$) when compared with the control. The mean gastric pH for the control and the test group was 4.10 ± 0.37 and 2.94 ± 0.14 respectively. The pH of the test group was significantly higher ($p<0.05$) when compared with that of the control group. The mean gastric mucous output in the control and the test group was 0.14 ± 0.014g and 0.08 ± 0.01g respectively. The results showed that the gastric mucous output in the test groups was significantly reduced ($p<0.01$) when compared with the control group. The mean gastric ulcer score in the control and the test group was 3.70 ± 0.30 and 7.50 ± 1.25 respectively. The results showed that the ulcer score in the test group was significantly higher ($p<0.05$) when compared to the control group. In conclusion, the long-term consumption of calabash chalk predisposed to peptic ulceration in Albino Wistar rats.

Keywords*: calabash chalk, ulcer scores, gastric secretions, mucous, Pepsin, pH*

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Manuscript received- March 2024; Accepted: May 2024

DOI: https://doi.org/10.54548/njps.v38i2.11

INTRODUCTION

Calabash chalk is a naturally occurring substance that is common in West African countries. It is referred to as kaolin, edible clay, and marble chalk in English. It is also known by different names in different parts of the world; "Shile" in Ghana, "La-Craie" in France, "Umcako" in Zulu, "Nzu in Ibo Nigeria, and "Mabele" in Congo (Reilly & Henry 2000). Chalk is not a food substance but it is readily consumed by numerous people in different parts of the world (Halstead, 1986). This is a form of geophagy and a form of pica.

Calabash chalk is consumed by both males and females, but more by females especially during pregnancy and in the postpartum phase. It is said to prevent morning sickness, excessive salivation, postpartum depression, anxiety, and feelings of alienation (Reilly & Henry, 2000). Tests on calabash chalk done by the Foods and Standards Agency of the United Kingdom have shown high levels of lead, aluminium, arsenic, and alpha lindane which are known to be toxic to the body (Campbell & Weir, 2002).

Calabash chalk is known to cause low birth weight, iron deficiency anemia, reduced red blood cell count and platelet count, and reduced zinc and iron absorption in babies in utero and in breastfeeding infants. It has also been demonstrated to cause histomorphological changes to the architecture of the liver, stomach, and esophagus including, ulceration of the gastrointestinal mucosa and dilatation of the liver sinusoids (Ekong *et al*., 2009). Since there was no attempt to score the gastric ulceration and elucidate the causative factors that could provoke the ulcer if any; this study was therefore carried out to investigate the effect of chronic consumption of calash chalk on gastric ulcer scores, and to elucidate some factors that could provoke the ulcers notably gastric acid secretion, gastric pepsin secretion and gastric mucous output.

MATERIALS AND METHODS

Preparation of calabash chalk suspension: Blocks of non-salted calabash chalk were purchased from Watt market in Calabar, south-south of Nigeria, and ground into a powder using a manual grinder. Forty grams of the powder was dissolved in1liter of water to get a concentration of 40mg/ml. This concentrate was filtered with Whatman filter paper to remove all impurities. The suspension was stored in a plastic jug in a cool dry place.

Experimental animals: Fifty (50) adult male Albino Wistaria rats weighing between 160-180g were used for these experiments. They were housed in a well-ventilated room in the animal facility of the Department of Physiology, University of Calabar. They were kept at a temperature of 28 ± 3° C and humidity of 85 ± 5%. They werekept in a 12/12, dark/ light cycle and were allowed to acclimatize for 2 weeks before the onset of the experiments. The rats were fed with standard feed pellets and were allowed access to water and food *adlibitum*.

Experimental design: The animals were randomly divided into 10 groups of 5 rats each. Group 1 served as the control group and was administered 1ml of distilled water while group 2 served as the test group and was administered1ml of the calabash chalk suspension. This administration was done orally, every morning for a period of 28 days. An initial body weight was taken at the start of the experiments and a final body weight wastaken at the end of the experiments. During the experiments, the animals were weighed daily using an animal weighing balance. The difference between the final and initial body weight of each animal was taken as the body weight change of the respective animal. Approval for the use of the animals was obtained from the ethical committee of the faculty of basic medical sciences, University of Calabar. This is in tandem with internationally accepted standards for laboratory animal care and their use.

Table 1:
Grading of ulcer points

GRADE	INTERPRETATION
0.0	No les
0.5	Pin size ulcer
1.0	Two or more small linear or hemorrhagic ulcer
2.0	Ulcer spots greater than 3mm

Ulcer index for each group = $\frac{\text{number of rats x number of grade}}{\text{Total number of rats in a group}}$

Ulcer incidence (%) = $\frac{\text{number of rats with ulcers x 100}}{\text{Total number of rats}}$

Measurement of various gastrointestinal parameters: Measurement of mean basal gastric acid was done using the continuous perfusion method (Ghosh and Schild, 1958; Osim *et al.*, 1991). Gastric acid output in the effluent sample was measured by titrimetricanalysis. The calculation of acid in millimoles per hour (mMol/Hr) was by the principle that states that a gram equivalent of acid balances a gram equivalent of the base at the neutralisation point. The determination of the proteolytic activity of gastric acid secretion (which is the basis for the measurementof pepsin) was done using Casein as a substrate (Tarique *et al.*, 2016). The weight of adherent gastric mucous was determined using the method of (Tan *et al.*, 2006). Ulcers were induced by gastric instillation of acid-ethanol. The stomach of each animal was spread and pinned to a dissecting board tohave a good view of the mucosa of the stomach (Joshii *et al.*, 2004). A careful macroscopic examinationfor the presence of any ulcerative lesion was carried out and scoring of ulcer spots was done (Obembe *et al.*, 2011). Ulcer scoring was done according to the following grading system (Table 1).

RESULTS

Comparison of mean basal gastric acid output between the control and the test group

The mean basal gastric output (BGAO) in the control group and the test group was 0.58 ± 0.22 uMOL /10min and 0.61 ± 0.37uMol/10min respectively. The mean histamine-mediated mean gastric acid output(Hist-GAO) in the control and the test group was 1.43 ± 0.144uMol/10min and 1.33 ± 0.08uMol/10min respectively. The mean Histamine + Cimetidine-mediated gastric acid output (Hist+Cim-GAO) in the control group and the test group was 0.66 ± 0.08uMol/10min and 0.65 ± 0.04uMol/10min respectively. There was no significant difference in the various mean gastric acid outputs between the control and thetest group (Figure 1).

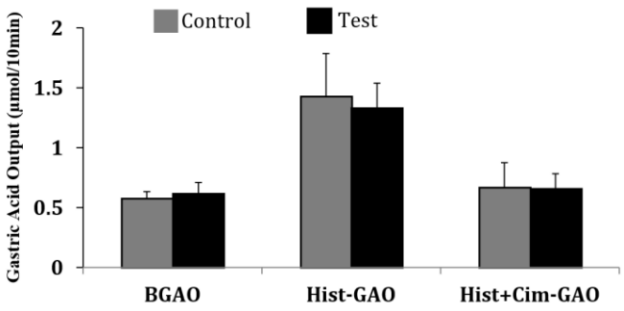

Figure 1
Gastric acid output between control and test groups.
Values are expressed as mean ± SD, n = 5.
No significant difference between groups

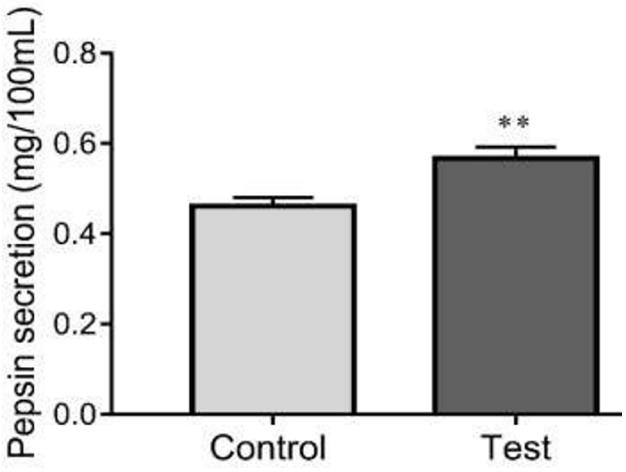

Figure 2:
Comparison of gastric pepsin level between the control and test groups.
*Values are mean ± SEM, n = 5. **p< 0.01 vs control.*

Gastric pepsin secretion in the control and the test group The mean concentration of gastric pepsin in the control and the test group was 0.46 ± 0.02mg/100ml and 0.57 ± 0.02mg/100ml respectively. The concentration of gastric pepsin in the test group was significantly higher ($p<0.01$). when compared with the control group (Figure 2).0.57 ± 0.02mg/100ml respectively. The concentration of gastric pepsin in the test group was significantly higher ($p<0.01$) when compared with the control group (Figure 2).

Gastric mucous output in the control and the test group The mean gastric mucous output in the control and the test group was 0.14 ± 0.014g and 0.08 ± 0.01g respectively. The gastric mucous output in the test group was significantly reduced ($p<0.01$) when compared to the control group (Figure 3).

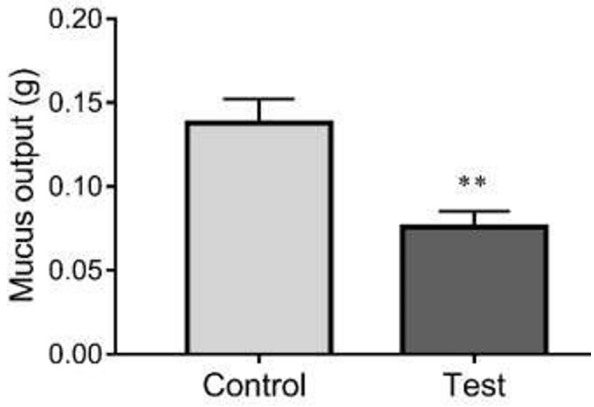

Figure 3:
Comparison of gastric mucus output between the control and test groups.
Values are mean ± SEM, n = 5. ** *p*< 0.01 vs control

Gastric ulcer scores in the control and the test group: The mean gastric ulcer score in the control and the test group was 3.70 ± 0.30 and 7.50 ± 1.25 respectively. The ulcer score in the test group was significantly higher ($p<0.05$) when compared with the control group (Figure 4).

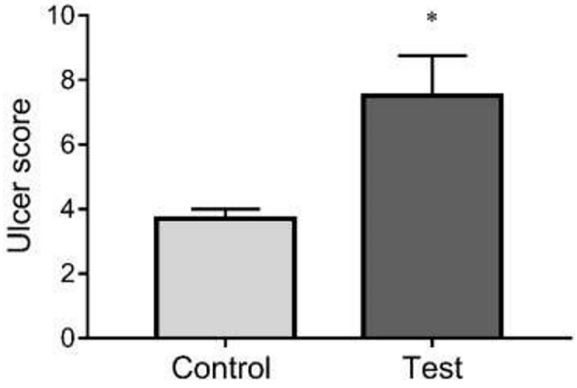

Figure 4:
Comparison of gastric ulcer score between the control and test groups. *Values are mean ± SEM, n = 5. * p< 0.05 vs control.*

Gastric pH in the control and the test group: The pH value for the control group was 4.10 = 0.37 while the pH value for the test group was 2.94 ± 0.14. The pH value of the test group was significantly higher than that of the control group (Figure 5).

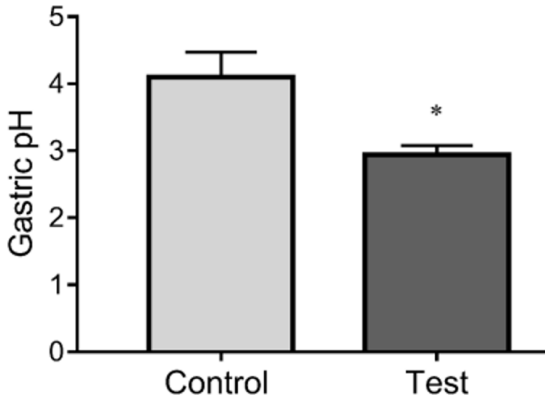

Figure 5:
Comparison of gastric pH between the control and test groups. *Values are mean ± SEM, n = 5. *p< 0.05 vs control.*

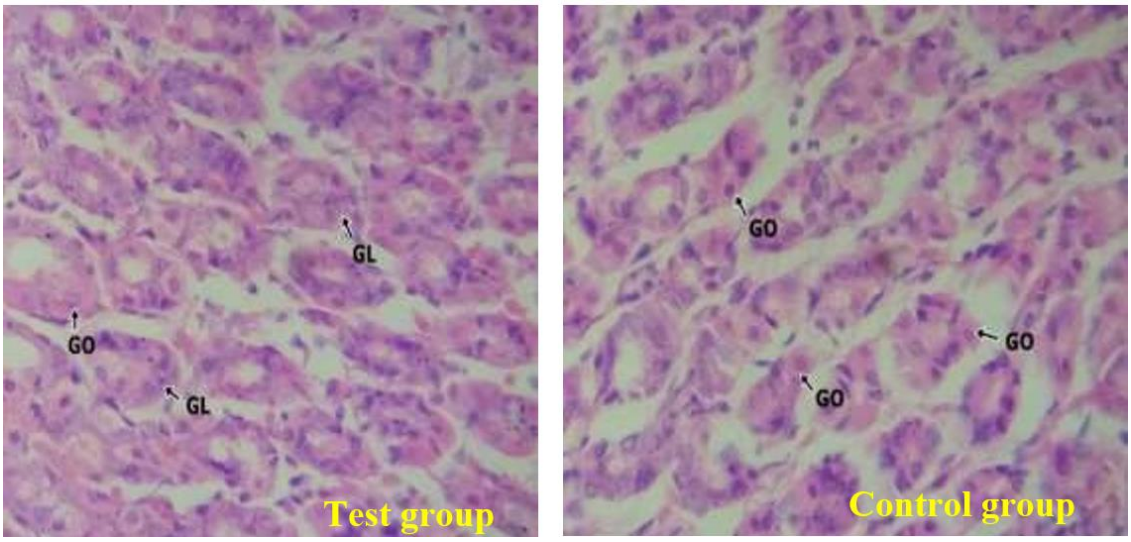

Plate 1:
Cross section of the stomach (test group and control group) X400

DISCUSSION

Gastric ulcer disease is common both in humans and animals. It occurs as a result of the imbalance between protective and aggressive factors present in the gastric mucosa. Every day, the gastric mucosa is exposed to factors that can cause injury such as gastric acid, certain types of food, pepsin, bacterial agents, bile acids, and certain drugs (Ezomike *et al*., 2020). Ekong *et al*., (2009) had shown some gastric lesions following the consumption of calabash chalk in rats. However, no attempt was made to elucidate the causative factors that could cause the gastric ulcer following the consumption of calabash chalk. This study therefore, investigated the aggressive factors that could provoke gastric ulceration, notably free hydrogen ions, low mucus secretion, and high pepsin secretion. There was also no significant difference in mean basal gastric acid secretion in the test group when compared to the control group. When Histamine was administered, the gastric acid output was significantly increased in both the control and the test group. When Cimetidine was administered, the gastric acid output of the animals in both groups was reduced as expected. However, the pH of the test group was significantly lower than the control group, suggesting a significant increase in free hydrogen ions in the test group than in the control. These free hydrogen ions may cause damage to the gastric mucosa.

The adherent mucous in the test group was significantly reduced when compared with the control group. The reduced mucous in the test group may be due to damage caused to the epithelial cells by the toxic elements and organic pollutants present therein (Ekong *et al*., 2009). Histology of the stomach from the test group showed edema, inflammation, erosion of the surface epithelium, and prominent goblet cells when compared to the control group. All these would have a negative effect on the ability of the epithelial cells to secrete mucous (Ekong *et al*. 2012).

Pepsin concentration in the test group was significantly higher when compared to the control group. Pepsin solubilises gastric mucous thus exposing the gastric mucosa to acid attack (Pearson *et al*., 1986). The significantly increased pepsin and pH and the significantly reduced gastric mucous worked synergistically to cause significantly increased gastric ulcerations observed in the test group when compared to the control group. The liberal consumption of calabash chalk should therefore be discouraged.

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Niger. J. Physiol. Sci. 39 (June 2024): 39 – 43
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Niger. J. Physiol. Sci.

Full length Research Article

Assessing the Interplay between Dyslipidemia and Bone-Related Markers in Postmenopausal Women.

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Summary: Dyslipidemia, marked by abnormal lipid levels, contributes to cardiovascular disease risk and affects bone health, particularly in postmenopausal women. Hormonal changes during menopause disrupt lipid and bone metabolism, increasing the likelihood of cardiovascular and bone-related disorders. This study explores the relationship between dyslipidemia and bone-related markers in postmenopausal women to understand its implications for bone and cardiovascular health. A cross-sectional study was conducted on 100 women: 60 postmenopausal (PMP), 20 premenopausal (PRM), and 20 reproductive-age (RWA) women. Fasting blood samples were collected and analyzed for lipid profile, alkaline phosphatase (ALP), inorganic phosphate, calcium, and vitamin D using standard laboratory techniques. Statistical analysis was performed using ANOVA and posthoc tests. Postmenopausal women showed significantly elevated levels of total cholesterol, LDL-C, and triglycerides, along with higher ALP and inorganic phosphate levels compared to premenopausal and reproductive-age women ($p<0.05$). Calcium and vitamin D levels were lower in the PMP group. Dyslipidemia in postmenopausal women is associated with disrupted bone metabolism, indicating an increased risk of cardiovascular and bone-related disorders. Comprehensive health assessments are recommended for early intervention.

Keywords*: menopause, dyslipidemia, vitamin D, osteoporosis, cardiovascular risk*

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Manuscript received- January 2024; Accepted: April 2024

DOI: https://doi.org/10.54548/njps.v39i1.6

INTRODUCTION

Menopause, defined as the cessation of menstruation for 12 consecutive months due to estrogen deficiency, marks a significant physiological transition in women's lives. It typically occurs between the ages of 48 and 52, primarily influenced by genetic factors rather than socio-economic status or previous ovulation history (Araujo *et al.*, 2023; Pardhe *et al*., 2017; Soares, 2019). Beyond its reproductive implications, menopause impacts various bodily systems, including urogenital, psychogenic, and cardiovascular functions. The transition encompasses perimenopause, menopause, and postmenopause, each characterized by hormonal shifts and physiological changes (Soares, 2019; Achie *et al*., 2021).

During menopause, ovarian follicles decline in number, accompanied by a reduction in granulosa cell activity, the primary producers of estrogen and inhibin. Decreased estrogen and inhibin levels lead to elevated follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production, disrupting the hypothalamic-pituitary-ovarian axis and causing irregular menstrual cycles until cessation (Faulds *et al*., 2012). Additionally, menopause triggers significant changes in vaginal tissue, marked by mucosal atrophy due to decreased estrogen levels, resulting in dryness and fragility (Sarmento *et al*., 2021). Surgical procedures, such as hysterectomy with bilateral oophorectomy, or medical treatments for conditions like endometriosis or breast cancer, can induce menopause prematurely (Sarmento *et al*., 2021).

Dyslipidemia, characterized by an imbalance in cholesterol and lipid levels, poses a risk factor for cardiovascular diseases. In Nigeria, dyslipidemia has become an increasingly prevalent health issue, reflecting the global rise in non-communicable diseases. Urbanization, changes in dietary habits, and sedentary lifestyles have contributed to the growing incidence of lipid disorders. Recent studies have shown that dyslipidemia affects approximately 20-40% of adults in Nigeria, with a higher prevalence in urban areas compared to rural settings (Ayogu *et al*., 2021; Ekpenyong *et al*., 2012). Factors contributing to dyslipidemia include diet, tobacco use, genetics, and hormonal changes, such as those occurring during menopause (Kopin & Lowenstein, 2017). Additionally, menopause has been identified as a significant factor contributing to dyslipidemia in Nigerian women, exacerbating their risk of cardiovascular diseases (Igweh *et al*., 2005; Nie *et al*., 2022). The increasing prevalence of dyslipidemia highlights the need for targeted public health interventions and lifestyle modifications to mitigate the risk of cardiovascular complications. Contrary to the perception of bones as static structures, they function dynamically as organs, playing crucial roles in mobility, organ protection,

and hematopoiesis. Bone remodeling, a continuous process influenced by hormonal regulation, involves the resorption and formation of bone tissue. Bone markers, detectable in blood and urine, serve as indicators of bone turnover and can aid in diagnosing bone disorders like osteoporosis and Paget's disease (Song *et al*., 2023; Schini *et al*., 2023). Postmenopausal women face heightened risks of bone disorders and low serum calcium levels, with dyslipidemia potentially exacerbating these risks.

Imbalances in bone resorption and formation, detectable through bone markers, are associated with various diseases. Postmenopausal women represent a group that is especially susceptible to bone-related disorders as a result of hormonal changes (Faulds *et al*., 2012; Adewole *et al*., 2021). Despite previous research on bone markers and metabolic bone diseases, gaps persist in understanding the relationship between post-menopause, bone markers, and dyslipidemia. Existing literature has explored the utility of bone-associated biomarkers in diagnosing and monitoring bone disorders. However, gaps remain regarding the interplay between bone markers, dyslipidemia, and postmenopausal status. Given the increased risk of bone disorders and dyslipidemia in postmenopausal women, elucidating these correlations is crucial for preventive and therapeutic interventions. Therefore, this study aims to explore the relationship between dyslipidemia and bone-related markers in postmenopausal women to better understand the implications for bone and cardiovascular health.

MATERIALS AND METHODS

Experimental Design: A cross-sectional study was undertaken involving 100 women aged 50 years and above, selected randomly from the Owo metropolis, Ondo State, Nigeria. The research spanned from January to August 2022. Among the participants, 60 were PMP aged between 50 and 60 years, while 40 were tagged controls, comprising 20 PRM and 20 RWA. Data collection involved obtaining medical histories and personal information through a comprehensive questionnaire, following approval from the hospital's ethical committee. All participants provided informed consent prior to inclusion in the study.

Consent and Ethical Clearance: All participants in this study received detailed explanations of the research protocols at the clinic, followed by the requirement to sign written consent forms. Ethical clearance, bearing reference number FMC/OW/380/VOL.CL/200, was obtained from the Ethical Review Committee of the Federal Medical Center, Owo, and ensuring adherence to ethical standards throughout the study.

Inclusion and Exclusion Criteria: The study included women aged 50 years and above categorized as postmenopausal women, while women aged 30 to 50 years were considered either premenopausal or within the reproductive age range. Participation required informed consent from the participants. Participants with known comorbidities including hypertension, HIV, hepatitis, cancer, or those undergoing oral anticoagulant treatment, with bleeding tendencies, and breastfeeding mothers were excluded from the study. Additionally, individuals who did not provide consent were also excluded.

Samples Collection and Storage: Blood samples were collected from each participant following standard procedures. 5 milliliters (5ml) of fasting blood samples were collected into sterile lithium heparin bottles. After gentle mixing, samples were centrifuged at 4000 revolutions per minute (rpm) for 5 minutes to obtain serum. Serum was stored at -20°C until analysis for vitamin D, calcium, uric acid, lipid profile, inorganic phosphate, and alkaline phosphatase (ALP).

Analytical Methods: Using reagents provided by Randox Laboratories Ltd. (UK), standard enzymatic methods were used to determine serum levels of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C), as well as fasting plasma glucose, ALP, phosphorus, uric acid, calcium (Ca2+). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedwald equation (Friedwald *et al*., 1972). Additionally, ELISA kit from Melsin Medical Company, USA, was used to assess the serum levels of vitamin D. Every participant had their height and weight measured, and their body mass index (BMI) was calculated using the guidelines provided by Atere *et al*. (2020).

Statistical Analysis: The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 25.0 0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was employed to compare variables within the groups, while correlation analysis was used to assess associations between variables. Quantitative values were presented as mean ± standard deviation (mean ± SD). The level of significance was set at a 95% confidence interval, with p-values ≤ 0.05 considered statistically significant.

RESULTS

Figure 1 depicts the demographics of the subjects investigated. The results showed that the mean age (years) of RWA was 33.9±2.0, PRM was 44.4±2.2, and PMP was 57.8±7.4. The subjects' age, BMI, and SBP showed significant differences ($p<0.05$). Table 1 compares atherogenic indices and bone-related biomarkers in premenopausal and postmenopausal women with reproductive age. PMP and PRM individuals had significantly higher mean TC, HDL-C, and LDL-C levels than the RWA group ($p<0.05$). PMP and PRM participants had considerably greater mean values of ALP, inorganic phosphate, and uric acid compared to the RWA group. However, FBS, calcium (Ca^{2+}), and vitamin D were significantly lower ($p<0.05$). The PMP group had significantly greater mean levels of ALP, inorganic phosphate, and uric acid compared to the PRM group ($p<0.05$). However, Ca^{2+}, FBS, and vitamin D levels were lower. Additionally, bone-associated markers (ALP, inorganic phosphate, Ca2+, and Vitamin D) linked with atherogenic indices (TC, TG, and LDL-C) in postmenopausal women. Inorganic phosphate had a statistically significant positive correlation with TC, TG, and LDL-C, however vitamin D had a negative link with TC. Finally, TG showed significant positive correlation with inorganic phosphate only among premenopausal women (Figure 3).

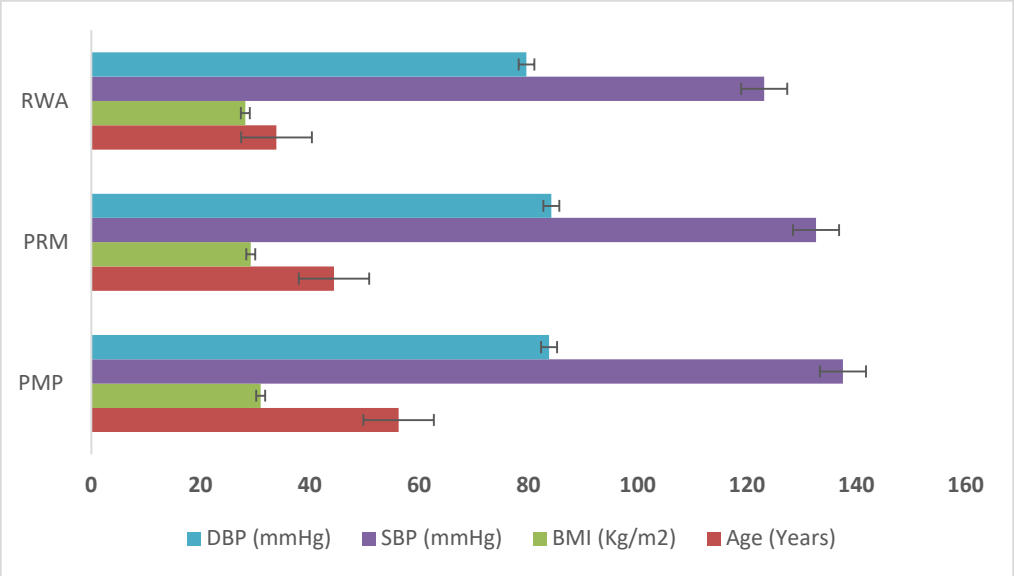

Figure 1:
Demographic Characteristics of the Participants
Key: RWA= Reproductive women age, PRM = Premenopausal, PMP= Postmenopausal, DBP= diastolic blood pressure, SBP= systolic blood pressure, BMI= body mass index

Table 1:
Comparison of atherogenic indices and bone related markers in postmenopausal women, premenopausal women and reproductive women age

Parameters	RWA (n=20)	PRM (n=20)	PMP (n=60)	p-value
HDL-C (mg/dl)	40.10±10.10[a]	50.12±11.10[b]	50.49±10.59[c]	0.000*
LDL-C (mg/dl)	100.25±30.30[a]	110.15±34.15[b]	157.93±19.47[c]	0.000*
TG (mg/dl)	60.14±20.31[a]	45.56±14.92[b]	83.99±19.53[c]	0.000*
TC (mg/dl)	170.22±40.44[a]	180.36±35.40[b]	208.22±21.25[c]	0.001*
FBG (mg/dl)	77.24±7.13[a]	87.24±20.30[b]	102.49±18.73[c]	0.001*
ALP (U/L)	330.47 ± 68.45[c]	187.37 ± 49.80[b]	138.90 ± 36.74[a]	0.000*
Inorganic phosphate (mmol/L)	4.41 ± 0.84[b]	3.65 ± 0.54[a]	3.31 ± 0.63[a]	0.000*
Uric Acid (mg/dl)	5.07 ± 1.08[c]	3.51 ± 0.99[b]	2.77 ± 0.36[c]	0.000*
Calcium (mg/dl)	6.75 ± 0.88[a]	7.88 ± 0.76[b]	10.33 ± 1.29[c]	0.000*
Vitamin D (ng/mL)	13. 96 ± 3.18[a]	22.34 ± 4.18[b]	30.40 ± 10.03[c]	0.000*

* Significant at p≤0.05
a = postmenopausal women; b = premenopausal women; c = reproductive age women
**Values were represented with Mean± SD. Mean values were compared using one-way ANOVA with level of significance set at p<0.05. Values in the same column with the same superscript are not statistically different at p<0.05 using the Post-Hoc test.*
Key: n=sample size, HDL-C= High density lipoprotein Cholesterol, LDL-C= Low density lipoprotein, TG= Triglycerides, TC= Total Cholesterol, FBG- Fasting blood glucose, RWA= Reproductive women age, PRM = Premenopausal, PMP= Postmenopausal

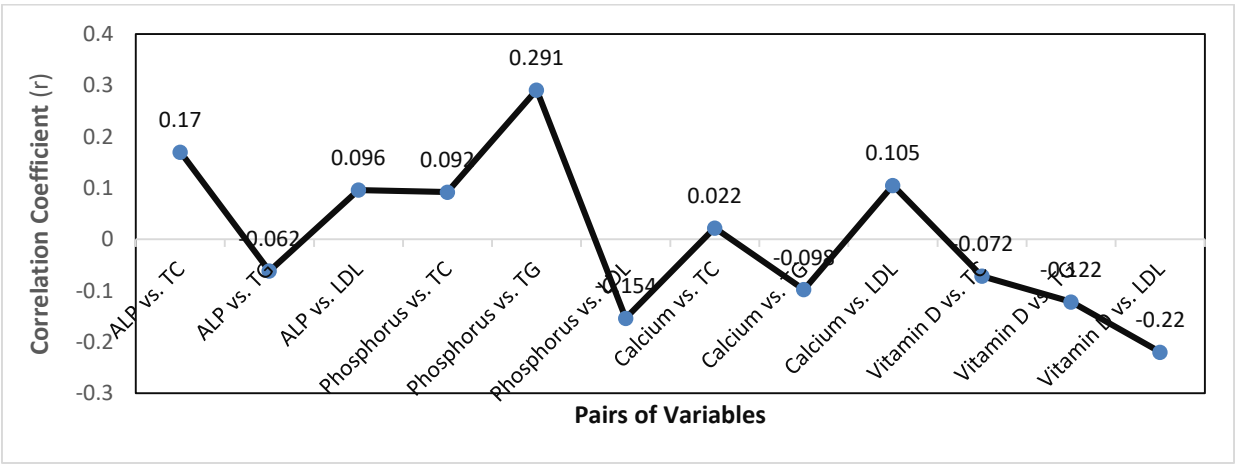

Figure 2:
Correlation of mean Bone associated-markers (ALP, Inorganic phosphate, Ca^{2+}, Vitamin D) with atherogenic indices (TC, TG and LDL-C) in Postmenopausal Subjects

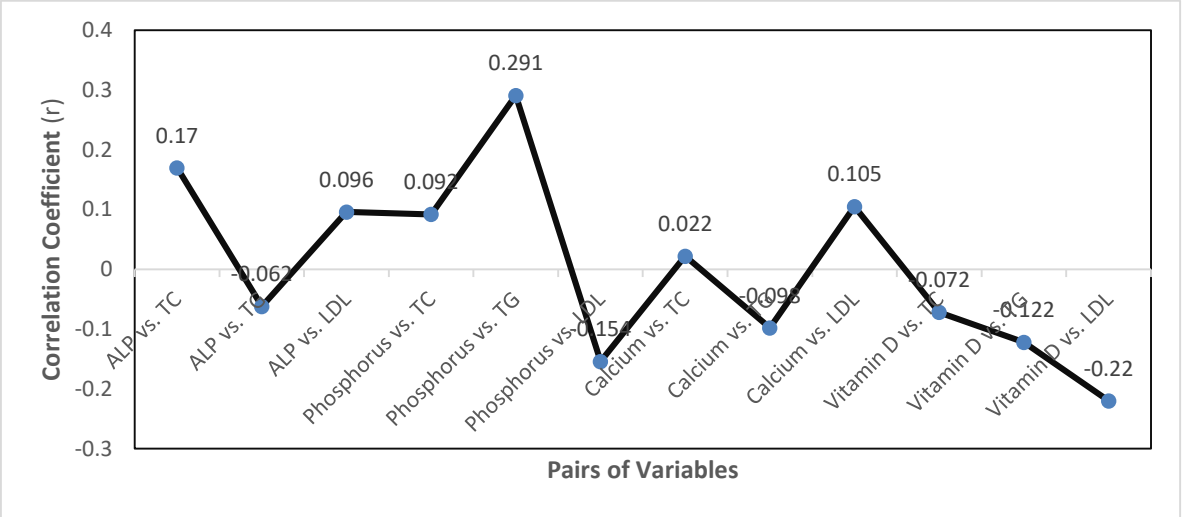

Figure 3:
Correlation of mean Bone associated-markers (ALP, Inorganic phosphate, Ca^{2+}, Vitamin D) with atherogenic indices (TC, TG and LDL-C) in Pre-menopausal Subjects

DISCUSSION

Menopause represents a significant physiological milestone in a woman's life, marked by profound hormonal changes and associated physiological alterations. The decline in estrogen levels during menopause plays a pivotal role in various metabolic and biochemical shifts, particularly affecting bone mineral metabolism (Faulds *et al*., 2012; Araujo *et al*., 2023). This study highlights the multifaceted impact of menopause on lipid metabolism, bone health, and associated biomarkers.

Consistent with existing literature, our findings demonstrate a transition towards a more atherogenic lipid profile during menopause, characterized by elevated plasma levels of TC, LDL-C, and TG, coupled with decreased levels of HDL-C (Fernandez & Murillo, 2016; Otsuki *et al*., 2017; Nandhini *et al*., 2022). The observed alterations in lipid parameters highlight the metabolic consequences of estrogen deficiency post-menopause, predisposing women to increased cardiovascular risk.

Moreover, our results shed light on the intricate relationship between menopause, bone-related biomarkers, and dyslipidemia. Postmenopausal women exhibited significantly higher levels of bone turnover markers, including ALP, inorganic phosphate, and uric acid, compared to their counterparts and reproductive-age women. Concurrently, lower levels of Ca2+, FBG, and vitamin D were observed among PMP women, indicating potential disruptions in bone homeostasis and metabolic regulation (Black & Rosen, 2016; Pardhe *et al*., 2017).

The correlation analysis further elucidates the interplay between bone-associated markers and atherogenic indices among postmenopausal women. Inorganic phosphate levels exhibited significant positive correlations with TC, TG, and LDL-C, underscoring the potential influence of bone metabolism on lipid homeostasis. Conversely, vitamin D levels displayed a negative correlation with total cholesterol, suggesting a possible protective role against dyslipidemia in postmenopausal women (Manninen *et al*., 1992; Kim *et al*., 2022; Romandini *et al*., 2023). Vitamin D may enhance lipid metabolism by promoting calcium absorption and regulating adipocyte function, thereby reducing total cholesterol levels. Its anti-inflammatory properties can also mitigate dyslipidemia, highlighting its potential protective role against cardiovascular risk in postmenopausal women through improved lipid profiles (Kim *et al*., 2022; Romandini *et al*., 2023).

These findings emphasize the importance of comprehensive metabolic evaluation in postmenopausal women, considering the complex interrelationships between bone health, lipid metabolism, and cardiovascular risk. Strategies aimed at mitigating dyslipidemia and preserving bone health post-menopause warrant further exploration, encompassing lifestyle modifications, pharmacological interventions, and targeted therapeutic approaches (Libby & Theroux, 2005; Suresh & Naidu, 2006; Bristow *et al*., 2019).

In conclusion, this study highlights the significant interplay between dyslipidemia and bone-related markers in postmenopausal women. The observed alterations in lipid profiles, characterized by elevated total cholesterol and LDL-C levels, alongside disrupted bone metabolism, emphasize the increased risk of cardiovascular and bone disorders. These findings highlight the need for comprehensive health assessments and targeted interventions to mitigate dyslipidemia and enhance bone health in postmenopausal women. Future research should focus on developing specific therapeutic strategies to address the unique metabolic challenges faced by this population.

Acknowledgments
The authors express their gratitude to all study participants and the medical personnel at the Gynecology & Obstetrics clinic at the FMC, Owo.

Authors' contributions: *The ADA conceived of the research and drafted the first manuscript. OMO, KYA and RMO conceptualized and evaluated the intellectual content of the final manuscript. Each author made a contribution to the final draft of the manuscript and bears the responsibility for ensuring the integrity and precision of this review*

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