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Gliclazide and *Crassocephalum rubens* leaf extract inhibit glucose-induced mitochondrial permeability transition pore (MPTP) opening in isolated pancreas mitochondria of Wistar rats

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ABSTRACT

Introduction: Mitochondrial permeability transition pore (MPTP) has been implicated in a wide variety of diseases such as cancer, neurodegenerative diseases, and diabetes. *Crassocephalum rubens* is a leafy vegetable consumed in different parts of Africa for the management of symptoms of diabetes mellitus, inflammation, malaria, and blood pressure. The present study evaluated the modulatory effects of aqueous leaf extract of *C. rubens* (ACR) and gliclazide on MPTP in the pancreas of Wistar albino rats *in vitro*.

Methods: Pancreatic mitochondria were isolated from experimental animals using standard protocols. Furthermore, MPTP was induced using various concentrations (15, 22.5, 30, and 37.5 mmol/L) of glucose and $CaCl_2$ (3 µM). Alterations in MPTP and ameliorative potential of different concentrations of ACR (8, 24, 40, 56 µg/mL) and gliclazide (0.054 mg/mL) were monitored spectrophotometrically via changes in absorbance at 540 nm for 12 minutes, under sodium succinate energized condition.

Results: It was observed that 30 mmol/L, 37.5 mmol/L D-glucose, and Ca^{2+} significantly induced MPTP opening by 0.635, 5.10, and 9.95 folds, respectively, an effect that was reversed by gliclazide and ACR, in a none-dose dependent manner. In addition, ACR at 56 µg/mL in conjunction with Ca^{2+} opened the MPTP.

Conclusion: Data from this study suggest that gliclazide and ACR, especially at the lower concentrations, possess significant inhibitory effects against MPTP opening in the pancreas of male Wistar albino rats and, therefore, could be useful in protecting beta-cell death usually associated with diabetes mellitus, as well as other conditions in which MPTP opening is implicated.

Implication for health policy/practice/research/medical education:
This study demonstrated that aqueous extract of Crassocephalum rubens leaves has inhibitory effect on mitochondrial permeability transition pore (MPTP) opening, especially at low doses. The extract could be useful in managing diseases in which MPTP opening is implicated.

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Introduction

Diabetes mellitus is a chronic disease that adversely affects the quality of life of patients as well as the well-being of their families and society at large. It is among the highest causes of adult mortality, with huge economic implications worldwide. In 2017, about US$ 727b was the global expenditure on diabetes (1). The prevalence of the disease has increased steadily, rising from 285 million people in 2009 to 463 million people in 2019. Furthermore, about 578 million people and 700 million people are expected to have diabetes by 2030 and 2045, respectively (2). The disease is a metabolic disorder characterized by a chronic increase in fasting blood glucose level (≥200 mg/dL) and an alteration in lipids, carbohydrates, and protein metabolism, in which insulin action is either diminished or completely lost due to change in its secretion, reduction in its activity, or a combination of these two factors (3).

Diabetes mellitus can be classified into several types; however, there are two main forms: type 1 and type 2 diabetes mellitus (4). A commonly shared comorbidity of both types of diabetes is beta-cell death (5), which is a key event in the pathogenesis of both diseases (6). Much as mitochondria are involved in beta-cell function and survival, their critical role in triggering beta-cell apoptosis has been firmly established (7). Studies have shown that beta-cells are more susceptible to oxidative stress as a result of relatively low expression of antioxidant enzymes, such as glutathione peroxidase and catalase (6). This could then contribute to beta-cell dysfunction and eventual death in the conditions of persistent hyperglycemia-induced mitochondrial reactive oxygen species (mtROS) production (8).

An important means through which mitochondria mediate apoptosis is via the induction of mitochondrial permeability transition pore (MPTP) opening, which is characterized by an abrupt increase in the permeability of the inner mitochondrial membrane to solutes lesser than 1.5 kDa in size (9). Opening of this pore is involved in pathways of apoptosis, and it has become a pharmacological target in drug development (10). A wide variety of triggers such as Ca^{2+}, ROS, and inorganic phosphate (Pi) are activators of MPTP opening (11). In addition, studies have shown that high glucose concentration is a potent trigger of mitochondrial dysfunction and MPTP opening (12,13).

The use of plants as medicine has been well documented over the years, and with availability of modern equipment and means of analyses, the active principles of these plants have been isolated, and their therapeutic potential described, leading to the development of drugs (14). Earlier studies have reported the modulatory effect of plants on MPTP opening, usually triggered by Ca^{2+} (15-17). This modulatory effect is usually ascribed to the presence of health promoting bioactive compounds in the plants. The bioactive compounds account for the ability of plants to mitigate oxidative stress, a major culprit in MPTP opening (18). *Crassocephalum rubens* is a traditional leafy vegetable consumed in different parts of the world. Extracts of the leaves were reported to contain polyphenolic compounds such as flavonoids and phenolic acids (19). The effects of this plant in the management of diabetes, cancer, inflammation, liver dysfunction, and other ailments have been well documented (4,20,21). However, its effect on MPTP opening, triggered by either Ca^{2+} or glucose, has not been documented. This study, therefore, aims to evaluate the effects of aqueous leaf extract of *C. rubens* (ACR) and gliclazide, a standard antidiabetic drug, on MPTP in the pancreas of Wistar rats as a way of delineating part of their mechanism of antidiabetic actions.

Materials and Methods

Chemicals and Reagents

Mannitol, D-sucrose, D-glucose, sodium succinate, bovine serum albumin (BSA), ethylene glycol tetraacetic acid (EGTA), 2-[4-(2-hydroxyethyl)-piperazin-1-yl]-ethanesulfonic acid (HEPES), gliclazide, $CaCl_2$, rotenone, potassium hydroxide pellets, and spermine were purchased from Sigma- Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Plant materials and authentication

The leaves of *C. rubens* were obtained from a farm in Ado-Ekiti, Ekiti State, Nigeria. The leaves were identified and authenticated at the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria, where a voucher number was deposited for the plant at the Herbarium Department with voucher number FHI 112047

Processing of *Crassocephalum rubens* leaf extract

The *C. rubens* leaves were air-dried at room temperature and ground to powder. Twenty grams of the sample was soaked in 200 mL of distilled water for 24 hours and then filtered using a Whatmann filter paper (No. 1). The filtrate was concentrated to dryness and preserved at 4˚C until needed.

Experimental animals

Five male albino Wistar rats (weighing between 180 and 200 g) were acquired from the Animal Care Facility, Afe Babalola University, Ado-Ekiti, Nigeria. The rats were allowed to acclimatize for 15 days, with unrestricted access to water and rat chow.

Isolation of mitochondria from pancreas

Low ionic strength mitochondria were isolated in accordance with a previously described method (22). The animals were sacrificed by cervical dislocation, dissected, and the pancreas was immediately excised and trimmed to remove excess tissues. It was washed in isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH, and 1 mM EGTA at pH 7.4) until a clear wash was obtained. It was then weighed and minced with a pair of scissors. A 10% suspension was prepared after homogenizing

the pancreas on ice. The suspended tissue (pancreas) in isolation buffer was implored into a refrigerated Sigma centrifuge (3-30K, Germany), where the nuclear fraction and cell debris sedimented by low-speed centrifugation at 2300 rpm for 5 minutes. The supernatant obtained was re-centrifuged at the same speed and time to remove unbroken cells. The supernatant was centrifuged at 13 000 rpm for 10 minutes to sediment the mitochondria. The brown mitochondria pellets obtained after the supernatant were discarded and washed by re-suspending in wash buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH and 0.5% BSA at pH 7.4) and centrifuged at 12 000 rpm for 10 minutes. The mitochondria were immediately suspended in a solution of ice-cold swelling buffer (210 mM mannitol, 70 mM sucrose, and 5 mM HEPES-KOH at pH 7.4), then dispensed in Eppendorf tubes in aliquot and placed on ice for immediate use.

Mitochondria swelling assay

Mitochondria swelling and mitochondrial membrane permeability transition were evaluated spectrophotometrically at 540 nm by measuring the decrease in absorbance of mitochondria suspension in the presence and absence of triggering agents: Ca^{2+} (in the form of $CaCl_2$) and glucose, for 12 minutes (23). Modulatory effects of both aqueous *C. rubens* leaf extract and gliclazide, dissolved in distilled water on mitochondrial swelling, were evaluated.

Statistical analysis

Data analyses were done with GraphPad Prism 5 to calculate the means. Significance between the treatments was expressed in real numbers as induction fold. $P < 0.05$ was considered significant.

Results

Figure 1 shows the effects of the triggering agent and spermine on the pancreas MPTP. In the absence of triggering (NTA: non-triggering agent), the pancreas MPTP was not open. However, with the addition of exogenous calcium ion in the form of calcium chloride, as a triggering agent, MPTP opening was induced but was greatly reversed by spermine, a well- known inhibitor of MPTP opening. Figure 2 shows the effects of different concentrations of glucose on pancreas MPTP. Both 30 mmol/L and 37.5 mmol/L of glucose induced MPTP opening, while lower glucose concentrations did not induce MPTP opening. In Figure 3, it was observed that different concentrations of *C. rubens* leaf extract did not induce pancreas MPTP opening in the absence of triggering agents (Ca^{2+} and 37.5 mmol/L glucose). Figure 4 shows the effect of aqueous leaf extract of *C. rubens* on pancreas MPTP in the presence of Ca^{2+}. It was observed that lower concentrations of the extract did not induce MPTP opening, even in the presence of Ca^{2+} (triggering agent). However, pancreas MPTP opening was induced in

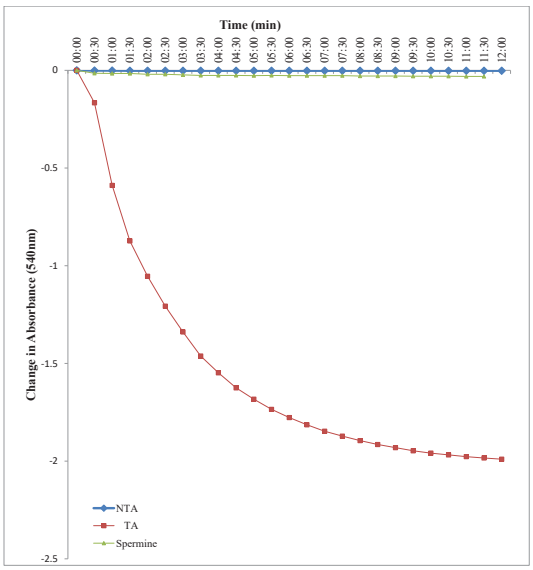

Figure 1. Effects of Ca^{2+} and spermine on pancreas mitochondrial permeability transition pore. NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}).

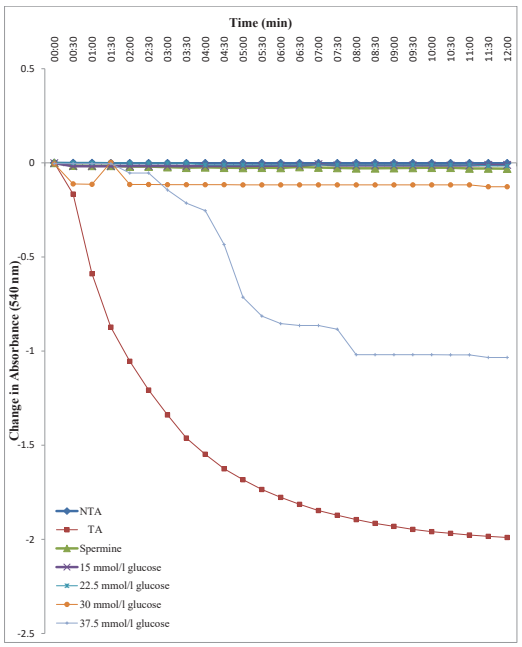

Figure 2. Effect of glucose on pancreas mitochondria permeability transition pore. NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}).

the presence of the highest concentration of the extract (56 µg/mL) and Ca^{2+}. In Figure 5, it was observed that different concentrations of *C. rubens* leaf extract in the presence of 37.5 mmol/L of glucose did not induce the opening of MPTP in the pancreas of Wistar rats. As presented in Figure 6, 0.054 mg/mL gliclazide inhibited pancreas MPTP opening in the presence of triggering agents (Ca^{2+} and 37.5 mmol/L glucose).

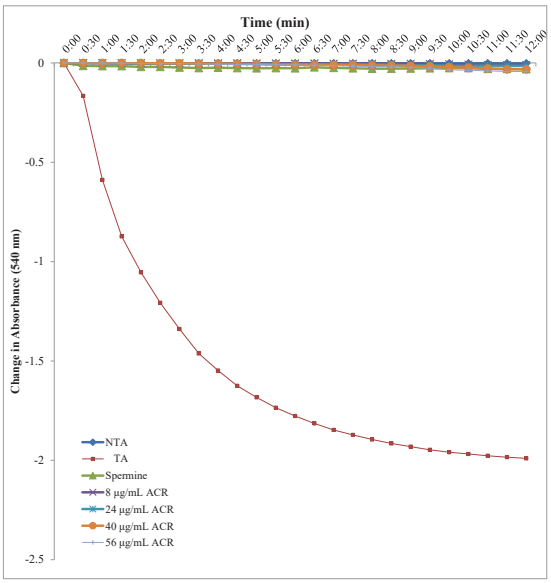

Figure 3. Effect of *Crassocephalum rubens* leaf extract on pancreas mitochondria permeability transition pore. NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}); ACR: aqueous extract of *C. rubens* leaves.

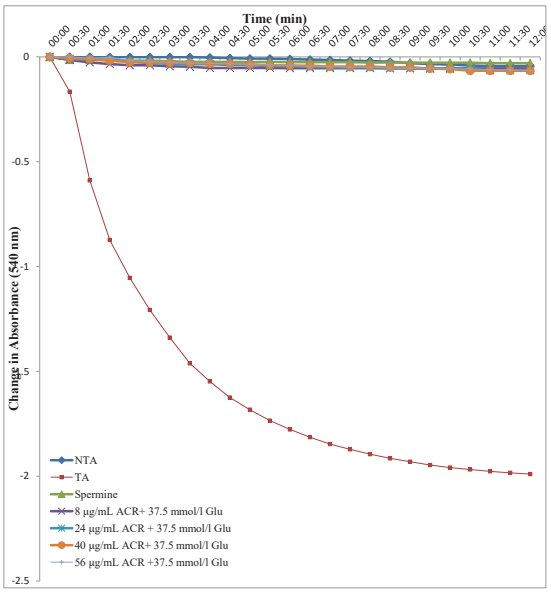

Figure 5. Effect of *Crassocephalum rubens* leaf extract on pancreas mitochondria permeability transition pore in the presence of glucose. NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}); ACR: aqueous extract of *C. rubens* leaves.

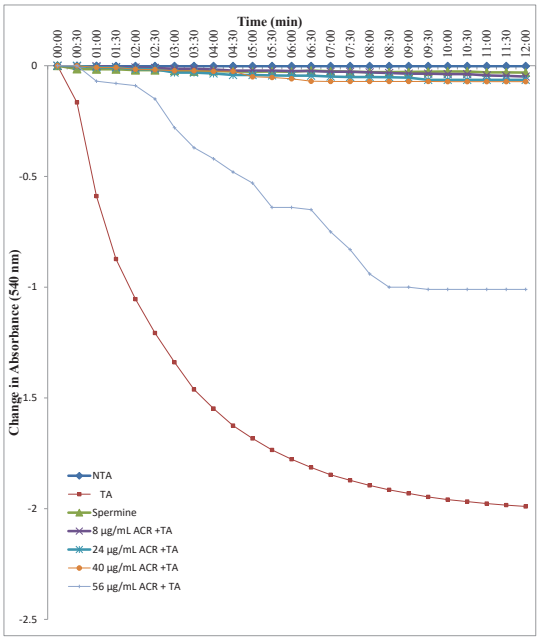

Figure 4. Effects of various concentrations of *Crassocephalum rubens* leaf extract on pancreas mitochondria permeability transition pore in the presence of Ca^{2+}. NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}); ACR: aqueous extract of *C. rubens* leaves.

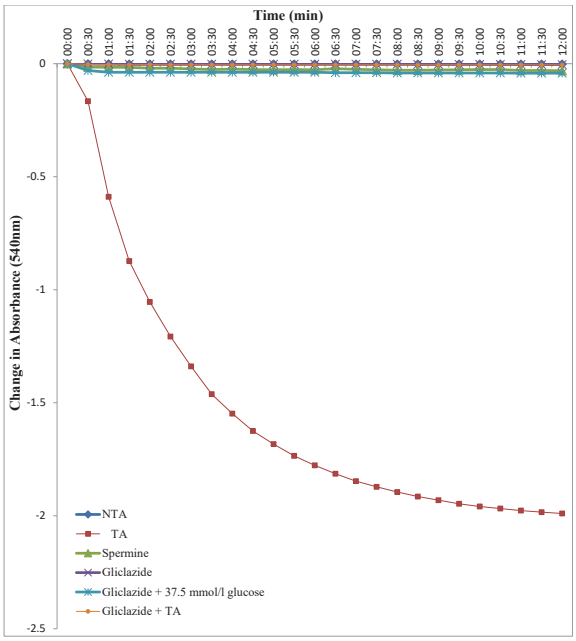

Figure 6. Effect of gliclazide on pancreas mitochondria permeability transition pore in the presence of glucose and Ca^{2+}. NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}); Gliclazide: 0.054 mg/mL gliclazide.

Table 1 shows that the induction fold of Ca^{2+}, 30 .0 mmol/L glucose, and 37.5 mmol/L glucose were 9.95, 0.635, and 5.10, respectively. It was observed that pancreas MPTP induction fold of Ca^{2+} was almost twice that of 37.5 mmol/L glucose, which was the highest concentration of glucose used in this study.

Discussion

Mitochondrial membrane permeability transition pore is a non-specific channel formed on the inner mitochondrial membrane, allowing the flow of substances ≤1.5 kDa and leading to depletion of the membrane potential of the mitochondria (Δψm), which is a critical requirement for

Table 1. Change in the absorbance of isolated pancreas mitochondria and corresponding induction fold of Ca^{2+} and glucose

Groups	Change in absorbance	Induction fold
NTA	-0.002	0.00
TA	-1.99	9.95
30 mmol/L glucose	-0.127	0.635
37.5 mmol/L glucose	-1.03	5.10

NTA: No triggering agent (absence of Ca^{2+}); TA: Triggering agent (presence of Ca^{2+}).

the production of ATP. Excess calcium in mitochondrial matrix is a potent MPTP inducer (24). Implication of MPTP in disorders such as muscular dystrophy, amyotrophic lateral sclerosis, hepatocarcinogenesis, ischemia/reperfusion injury, diabetes, and cancer has been well documented (25-27). In recent times, MPTP opening has been identified as a promising drug target (28,29). According to previous studies using pancreatic β-cell lines (MIN6 and INS-1), pancreatic MPTP opening could have both beneficial and deleterious effects. For instance, prevention of MPTP opening with cyclosporin A reduced glucose-induced secretion of insulin (30). Conversely, inhibition of MPTP opening reduced Pdx1 deficiency-induced cell death in mouse insulinoma MIN6 cells (31). However, a more recent study established the protective effect of imeglimin, a novel antihyperglycemic drug, in high glucose-induced β-cell apoptosis (32). Moreover, it was also reported that the same drug inhibited high glucose-induced MPTP opening and eventual cell death in human endothelial cells (HMEC-1) (33), thereby making inhibition of MPTP opening an important mechanism of action of Imeglimin (34).

In the present study, it was discovered that the addition of exogenous Ca^{2+} expectedly induced MPTP opening in isolated mitochondria from pancreas of Wistar rats, a situation that was reversed by spermine. This observation confirms the established effect of a known activator (Ca^{2+}) and inhibitor (spermine) on mitochondria swelling, implying that it was indeed an MPTP event (35,36).

Hyperglycemia accounts for the majority of damaging consequences of type 2 diabetes and is a marker of the disease (37). High glucose concentration has been known to induce MPTP opening (12). Moreover, it is well established in the literature that both acute and chronic hyperglycemia promotes oxidative stress-induced β-cell apoptosis in diabetes (38). In the present study, the effect of glucose on MPTP opening in the pancreas of Wistar rats was carried out directly on isolated mitochondria. It was noted that glucose at 30 and 37.5 mmol/L caused MPTP opening. This observation is similar to previous studies in which glucose concentrations ≥ 30 mmol/L induced MPTP opening. However, as alluded to earlier, the studies were not directly on isolated mitochondria, as most studies on the MPTP-inducing effect of glucose were done with mitochondria isolated from either cell lines or experimental animals that have undergone some kinds of treatment (12,26,33). It should be noted that even at 37.5 mmol/L, the MPTP inductive effect of glucose was lower than that of Ca^{2+}, as observed from the induction fold presented in Table 1. This underscores the potency of Ca^{2+} to induce MPTP.

Several studies have reported the ability of medicinal plants to modulate MPTP. These MPTP modulatory effects are usually considered as a part of the mechanism actions of such plants, usually due to the presence of important phytoconstituents (39). The polyphenolic compounds identified in the extracts of *C. rubens* (19) might be responsible for the observed modulatory effect on MPTP. This is in line with earlier studies reporting the modulatory effects of flavonoids and phenolic acids on MPTP via several mechanisms (18,40). Therefore, the modulatory effects of *C. rubens* on MPTP might be related to the presence of these phytochemicals. We observed that *C. rubens* at all the concentrations tested did not induce MPTP opening in the absence of triggering agents (Ca^{2+} and glucose) as well as in the presence of glucose (37.5 mmol/L), an indication of cytoprotection. However, in the presence of Ca^{2+}, the highest concentration of the extract (56 µg/mL) induced MPTP opening, which suggests cytotoxicity. This could suggest that the MPTP-inducing potential of the extract at the highest concentration was potentiated in the presence of Ca^{2+}. Our observation aligns with the maxim that a non-toxic substance may exhibit toxicity at a high dose while a toxic substance might be non-toxic at a low dose (41).

Previous studies have reported the modulatory effects of oral antidiabetic agents on mitochondrial function and metabolism. Biguanides were reported to modulate mitochondrial membrane permeability transition pore opening in intact cells, isolated mitochondria. Hyperglycemia is a marker of the disease. Hyperglycemia is accounted for the majority of damaging consequences of type 2 diabetes via the inhibition of complex one of the electron transport chain (37,42,43). In this study, gliclazide, which belongs to the sulfonylurea class of antidiabetic drugs, was observed to prevent MPTP opening in the presence of both glucose and Ca^{2+}. This is an indication of the ability of gliclazide to protect cells against apoptosis involving mitochondria and could be important for β-cells preservation in type 2 diabetes (44). Our result is in agreement with an earlier study which showed that gliclazide protected cells from oxidative stress-induced apoptosis in a manner involving restoration of mitochondrial membrane potential (45). This is in contrast to the proapoptotic effect of glibenclamide in human islets (46). This finding supports the outcome of earlier research in which the safety profile of gliclazide was confirmed in both experimental and epidemiological studies (47,48).

Conclusion

This study notably utilized mitochondria isolated from the pancreas of Wistar rats in evaluating the effect of *C. rubens* leaf extract as well as gliclazide on MPTP opening. It could be reasonably inferred from available results from this study that *C. rubens* could have an antiapoptotic effects via the inhibition of MPTP opening. However, moderation is important, especially at high doses of the extracts. In addition, the outcome of this study further underlines the protective effect of gliclazide in cells, especially in situations of hyperglycemia-induced oxidative stress, which is usually a prelude to MPTP opening.

Authors' contributions

OTO, OBA, and FJA provided the study design. EJN, AOB, and AJO performed experiments. AAO and AOB analyzed the data, OOA prepared the first draft, OA edited and completed the manuscript. All authors read and approved the final version of the manuscript for publication.

Conflict of interests

Authors declare no conflict of interests.

Ethical considerations

The procedures adopted in this study were in accordance with Guidelines for Care and Use of Laboratory Animals in Biomedical Research of the National Institutes of Health of the United States (NIH Publication, revised in 1985). All animal experiments were approved by the animal care committee of the Afe Babalola University Research Center, Ado-Ekiti, Nigeria. Ethical approval for the study was granted by the Ethics Committee of College of Sciences, Afe Babalola University, Ado-Ekiti, Nigeria.

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