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Research Article

Evaluation of the Effects of Honey on Lipopolysaccharide-Induced Depressive-Like Behavior and Oxidative Stress in Swiss Mice.

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Abstract
Depression constitutes over 12.3% of the global disease burden. Recent evidence has suggested oxidative stress as one of the major culprits in the neuropathology of depression as the monoamine theory is now known to be insufficient to account for the pathogenesis of the illness. Honey has been documented to be rich in phenolic compounds and flavonoids, which are potent antioxidants. Hence, this study was designed to examine the potential of honey in the prevention of oxidative stress and depressive-like behaviour in a lipopolysaccharide-induced mouse model of depression.
Thirty Swiss male mice, assigned into 6 groups (n=5), were used for this study. Group 1 received normal saline only while Group 2 received lipopolysaccharide, LPS (830 µg/kg) only. Groups 3-5 were pre-treated with honey (50, 100, and 200 mg/kg) for 7 days plus LPS (830 µg/kg) on the 7th day. Group six received imipramine (10 mg/kg) for seven days plus LPS (830 µg/kg) on the 7th day. The animals were then subjected to behavioural assessment and sacrificed with their brain harvested for lipid peroxidation (MDA) and glutathione (GSH) evaluation on the 8th day. Honey (50, 100, 200 mg/kg) significantly ($p < 0.05$) reduced the LPS-induced increase in immobility time in the forced swimming test, tail suspension test, and increased the number of lines crossed compared to the LPS-induced decrease in the number of lines crossed in LPS-treated only mice in the open field test. Honey significantly suppressed MDA levels when compared to the LPS-treated only treated mice. Honey protected the brain against lipopolysaccharide-induced behavioural despair, ambulatory deficits, and lipid peroxidation associated with depressive-like behaviours.

Key Words: Lipopolysaccharide, Natural Honey, Malondialdehyde, Depression

INTRODUCTION

Depression is classified as a mood disorder characterized by feelings of sadness, anger, and loss of interest that interferes with the everyday activities of affected persons (Sekhon and Gupta, 2021), which can progress into emotional and physical problems leading to chronic medical illness affecting overall wellbeing (Ranji Cui, 2015). Depression affects more than 450 million of the global population constituting about 12.3% of the global disease burden which may increase to 15% in 2020 (WHO, 2021). It is also expected to be one of the leading causes of premature deaths globally by 2020 as number two after cardiovascular diseases.
Depression is reported to result from the derangement in the level of monoamine neurotransmitters classically serotonin (5-HT) in the brain. However, these monoamine theories of depression are insufficient to explain the pathogenesis of depression (Bajpai *et al*., 2014). Recent evidence from preclinical and clinical studies has led to the suggestion that there are alternative theories implicating reactive oxygen species (ROS) in the pathogenesis of depression, with oxidative stress leading to neuroinflammation and neurodegeneration suggested to play a central role in the development of depressive behaviours. (Bhatt *et al*., 2020).
The redox imbalance in the brain is reported to be capable of activating a vicious cycle of oxidative stress and neuroinflammation leading to the development and onset of depressive disorders (Michel *et al*., 2004, Michel *et al*., 2007, Sarandol *et al*., 2007, Maes *et al*., 2009, Michel *et al*., 2010, Bajpai *et al*. 2014, Belleau *et al*., 2019, and Zhang *et al*., 2019), and the brain high lipid content and energy demand increasing its susceptibility to oxidative stress damage (Hulbert, 2007 and He, 2017).
The current major treatment of depression and drug development is focused on the drugs that prevent the lowering and maintains the balance of monoamines in the brain (Racagni and Popoli, 2010). However, response to treatment and improvement is disappointing and complicated by the unwanted side effects and tolerance to the drugs on chronic treatment (Onasanwo *et al*., 2010 and Correl *et al*., 2015). Therefore, exploring the oxidative stress theory of depression, the search for novel and natural products for the treatment of depression with pharmacological efficacy is imperative.

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Honey is a natural product produced by honeybee Apis mellifera from the nectars of different flowering plants (Samarghandian *et al*., 2017). Honey is composed of carbohydrates, proteins, vitamins, minerals, and organic acids. In honey are polyphenols such as flavonoids and phenolic compounds which are reported to be the main antioxidants in honey (Cianciosi *et al*., 2018).

Traditionally, it has been used as food, as a sweetener, and as an antimicrobial agent (Samarghandian *et al*., 2017). Honey's antioxidant (Ahmed and Othman, 2013), cardioprotective, neuroprotective (Aziz *et al*., 2014 & Syarifah-Noratiqah *et al*., 2018) and anti-inflammatory effects (Gasparrini *et al*., 2017) are now being expounded in modern literature. The health benefits of honey, as expounded by the increasing evidence of its nutraceutical potential, are due to the presence of phenolic and flavonoid bioactive compounds (Ahmed and Othman, 2013). In the past two decades, preclinical and clinical evidence has suggested that oxidative stress is strongly involved in the pathogenesis of depression as an alternative theory to the monoamine theory of depression (Bajpai *et al*., 2014, Belleau *et al*., 2019, Zhang *et al*., 2019 and Bhatt *et al*., 2020). However, there is a paucity of information on the role of honey in oxidative stress and depression, and the dose at which the antidepressant-like effect of honey could be seen is yet to be expounded. As a result, this study assessed honey's potential antidepressant-like activity in lipopolysaccharide-induced oxidative stress and depressive-like behaviour in Swiss mice.

MATERIALS AND METHODS

Drugs and reagents: Lipopolysaccharides (LPS, *Escherichia coli* 055:B5), Thiobarbituric acid (TBA), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), ammonium molybdate were all products of Sigma Chemical Co. (St. Louis, MO, USA). Imipramine and other chemicals and reagents used were of analytical grade.

Honey and lipopolysaccharide preparation: Honey was purchased from the University of Ibadan Teaching and Research Farm and further confirmed at the Department of Crop Science and Environmental Protection of the University of Ibadan. This is followed by the qualitative assessment of the phenolic compound present in the honey. Three random doses of 50, 100, and 200 mg/Kg b.w p.o were selected for this experiment, following confirmation of the 200 mg/kg to be the equivalent of two and a half teaspoons of the honey sample. With 100 mg/kg being half and 50 mg/Kg being a quarter of 200 mg/Kg. Sterile water was used as a dissolution vehicle for the honey.

Lipopolysaccharide (LPS) serotype 0111: B4 was purchased from Sigma Aldrich Company, the stock solution was prepared with normal saline as a dissolution vehicle, and a dose of 830 µg/kg, was prepared for intraperitoneal administration as described by Park *et al*., (2011).

Experimental animal: Thirty male mice (20-30 g,) were obtained from Central Animal House, University of Ibadan, Ibadan for this study, and housed in the Department of Physiology, University of Ibadan Animal House. The animals were given access to water and rodent chow ad libitum and maintained under normal light and dark cycles. Experimental protocol followed standard procedure following the Principle National Institution of Health, and the experiment was approved by the University of Ibadan Animal Care and Use Research Ethics Committee with ethical approval number UI-ACUREC/130-1019/25.

Experimental design: The animals in this study were grouped into six groups of 5 mice per group;

Group 1-received only distilled water (Control).

Group 2- received a dose of 830 µg/kg of LPS only.

Group 3 -received LPS (830 µg/kg) and a dose of 50 mg/kg of Honey

Group 4- received LPS (830 µg/kg) and a dose of 100 mg/kg of Honey

Group 5 - received LPS (830 µg/kg) and a dose of 200 mg/kg of Honey

Group 6 - received Imipramine dose of 10 mg/kg

The duration of the experiment was 8 days, the animals were pretreated with honey for 7 days, followed by LPS administration on the seventh day. The animals were then subjected to behavioural assessments 24 hours after the LPS administration and sacrificed for glutathione and lipid peroxidation levels.

Behavioural assessment

Forced swimming test: The Forced Swimming Test (FST) is the most widely used in vivo model for assessing antidepressant activity and was performed according to the method of Porsolt *et al*, (1977). The apparatus consists of a clear plexiglass cylinder (20 cm high and 12 cm diameter) filled to a 15cm depth with water. The water was kept at a temperature of 34±1°c during the experiment.

All the animals used for the FST were made to swim individually for fifteen minutes each 24 hours before the test day. The pretest was done to expose the animals to the new swimming environment.

On the test day, the animals were forced to swim for 6 minutes during which the period of immobility is measured using a stopwatch. The period of immobility is not measured during the first minute. The animal is assumed to be immobile when it stopped moving to escape and makes only movement that is necessary to keep its head above water.

Tail Suspension Test: The Tail Suspension Test (TST) was performed according to the method described by Steru *et al*., (1985). The mice were individually suspended 60cm above the surface of a table with the adhesive tape placed 1cm away from the tip of the tail. Immobility duration was recorded for the last five minutes during the 6 minutes test. Mice were considered immobile only when they hung passively and were completely motionless.

Open Field Test: The open field is an arena with walls to prevent escape. Commonly, the field is marked with a grid and square crossings. The center of the field was marked with a different colour to differentiate it from the other squares and the test was conducted as described by Sebenhener and Wooten, (2015).

Behavioural patterns measured in the open field test include:

Line crossings – Frequency with which the rodent crosses grid lines with all four paws (a measure of locomotor activity),

sometimes divided into activity near the wall and activity in the center.
All technicians recruited for the behavioural protocols were blinded to the experimental design and no animals were excluded from the behavioural experiments.

Biochemical analysis: The brains of the animals were harvested on the 8th day after anesthetizing the animals with an intraperitoneal injection of 100 mg/kg b.w of Ketamine, the brain was rinsed in ice-cold isotonic tris-KCl solution. The weight of the brain was taken before homogenizing at 20oC in phosphate buffer saline (PBS) and centrifuged. The supernatant was collected for the estimation of Malondialdehyde as described by Garcia *et al*., (2005), and Glutathione as described by Beutler, (1963).

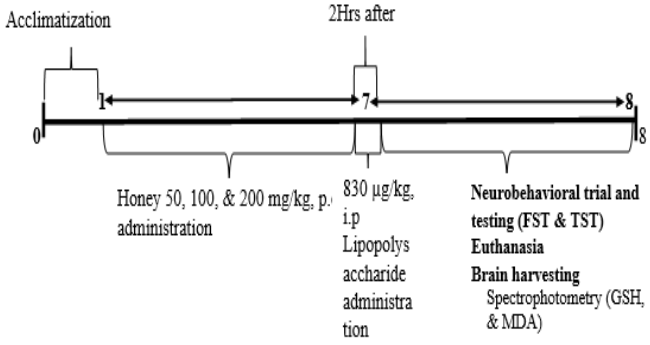

Figure 1:
Schematic diagram of experimental design

Qualitative assessment for phenolic presence in honey: To confirm the potential of honey procured for possessing phenolic compounds which are said to be responsible for the health benefits of honey, a qualitative assessment was carried out as described by Alvarez *et al*., 2016 and the results are presented in figure 2.

Statistical analysis: All data are expressed as Mean ± Standard Error of Mean (SEM) and statistical analysis to estimate differences within and between the groups was carried out using one-way analysis of variance (one way-ANOVA) followed by Tukey post-hoc test. All statistical analysis was carried out using graph pad prism version 7.0, with statistical significance expressed at the level P <0.05.

RESULTS

Phytochemicals	Status in Honey
Saponins	++ve
Tannins	+ve
Flavonoids	+ve
Cardiac glycosides	-ve
Anthraquinones	-ve
Terpenoids	++ve
Steroids	-ve
Alkaloids	-ve
Phenol	+ve

INTERPRETATIONS

+ve : Present

++ve : Abundant

-ve : Absent

Figure 2:
Qualitative phytochemical screening of honey

Effects of honey treatment on immobility time in the forced swimming and tail suspension tests: As shown in figure 3a, In the forced swimming test measured in seconds (F (5, 30) = 33.87; P<0.0001), LPS significantly increased immobility time as seen in the group that received LPS only (78.1±2.71) when compared to the control group (35.20±6.69) and the imipramine + LPS group (18.90±3.48). However, at the highest dose of 200 mg/kg (39.80±3.89), honey effectively reduces immobility time in the forced swimming test when compared with the LPS-only group.
In figure 3b, the mobility time of the tail suspension test was measured in seconds (F (5, 24) = 19.72; P<0.0001), at 100 mg/kg and 200 mg/kg (67.92±3.01; 57.12±5.32) honey significantly reduced the immobility time when compared to the control, LPS only and imipramine + LPS groups (110.30±13.53, 120.70±9.38 and 116.50±8.97) respectively. While the group that received the lowest dose of 50mg/kg (81.72±11.02) produces reduced immobility time, it is insignificant.
In both tests of despair, FST and TST the results showed that honey significantly improved response to behavioural despair in mice with depression at the highest dose.

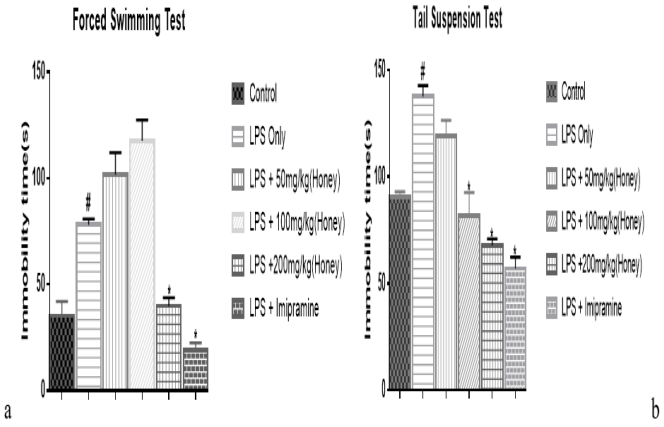

Figure 3a & 3b:
The effect of Honey on the immobility time in the Forced Swimming Test (FST) and Tail Suspension Test (TST) in lipopolysaccharide-induced depressive-like behaviour in mice. Data were expressed as Mean ± SEM (n=5). #indicate p<0.0001 LPS versus control, *indicate p<0.0001 Honey versus LPS.

Effect of honey on ambulatory behaviour in lipopolysaccharide-induced depression in mice: In figure 4, the effect of honey on ambulatory behaviour in the depression mice model was evaluated with the number of lines crossed in the open field test, (F (5, 24) = 21.54).
The LPS-only group (42.4±8.29) showed a significant decrease in the number of lines crossed when compared to the control group (125±12.23). However, honey at the highest dose of 200 mg/kg b.w, p.o (92±2.49) significantly increased the number of lines after LPS administration compared to the LPS-only or LPS + imipramine groups (Fig. 4).

Effect of honey on glutathione level in the lipopolysaccharide-induced depression mice model: Figure 5 showed the glutathione level (µmol/mg of protein) in the mice as assessed and evaluated (F (5, 18) = 8.31), the group

that received LPS only (1.72±0.59) showed a significant reduction in the level of brain glutathione when compared to the control (5.58±0.54). Honey and imipramine treatments (1.97±0.14, 2.05±0.65, 2.01±0.61 and 1.58±0.49) showed no significant improvement in the glutathione level when compared to the control.

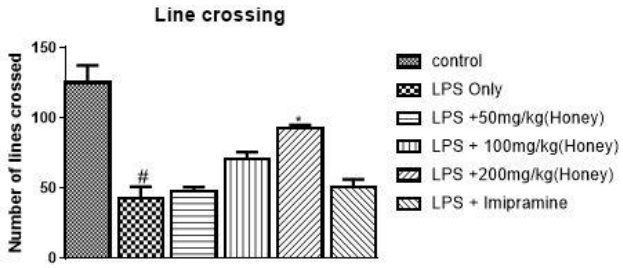

Figure 4:
The effect of Honey on the number of lines crossed in the open field test in lipopolysaccharide-induced depressive-like behaviour in mice. Data were expressed as Mean ± SEM (n=5). # indicate p<0.0001 LPS versus control, * indicate p<0.0001 Honey versus LPS.

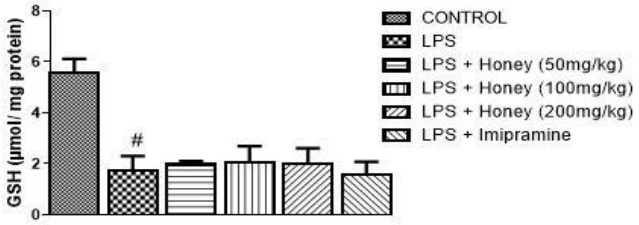

Figure 5:
The effect of Honey on the glutathione concentration in lipopolysaccharide-induced depressive-like behaviour in mice. Data were expressed as Mean ± SEM (n=5). #indicate p<0.0001 LPS versus control.

Effect of honey on lipid peroxidation in the lipopolysaccharide-induced mice model of depression: Figure 6 showed brain membrane lipid peroxidation was assessed with malondialdehyde level and evaluated (F (5, 18) = 7.99). Results of the MDA (ŋmol/mg of protein) evaluation showed LPS significantly increase MDA level in the mice brain of LPS only group (1.29±0.03) when compared to the control group (0.74±0.13), while honey treatment at 200 mg/kg (0.66±0.07) and imipramine (0.60±0.13) treatment showed a significant reduction in the level of MDA.

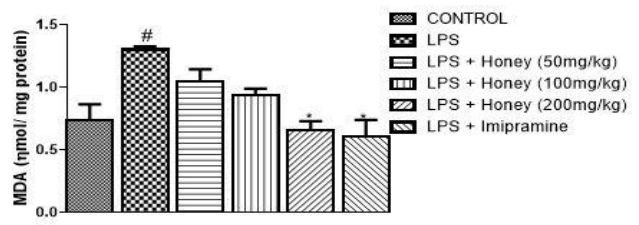

Figure 6:
The effect of Honey on the malondialdehyde levels in lipopolysaccharide-induced depressive-like behaviour in mice. Data were expressed as Mean ± SEM (n=5). #indicate p<0.0001 LPS versus control, *indicate p<0.0001 Honey versus LPS.

DISCUSSION

Depression is one of the leading causes of disability in the 21st century, despite many advances in the development of drugs for the treatment of depression. The current monoamine theory of depression is believed to be insufficient to explain the cause and nature of depression even though the current treatment regime for depression is largely defined by the monoamine hypothesis, which includes serotonin, norepinephrine modulators, and electroconvulsive therapy, which leaves patients with undesirable side effects. Also, the current treatments do not guarantee full recovery (Bajpai *et al*., 2014). Lipopolysaccharide, is a bacterial endotoxincapable of producing an immune system response, resulting in the production of reactive oxygen species (ROS). Several studies have shown that oxidative stress generated by LPS can cause the depletion of the antioxidant system, leading to ROS reacting with cell macromolecules like fatty acids and DNA protein. The brain is a vulnerable target of ROS due to its high metabolic rate, leading to neurochemical alterations and subsequent depressive-like behaviour and cognitive changes in mice (Bhatt *et al*., 2020). Some studies have observed an increase in the levels of biomarkers of oxidative stress and neurodegeneration and a decrease in the levels of antioxidants in patients with depression. This is corroborated by the same biomarkers measured in animal models of depression (Maes *et al*., 2009; Maes *et al*., 2011).

In this study, we explored the oxidative stress hypothesis of depression and assessed the potential of honey as a source of antioxidants in the management of depression using different doses of honey in a lipopolysaccharide (LPS) model of depression as described by Zhang *et al*., 2019 in comparison to the tricyclic antidepressant imipramine.

Following treatment with honey, imipramine, and lipopolysaccharide, we subjected the animals to tests of despair: forced swimming test, tail suspension test, used in the screening of antidepressant drugs, and an open field test used to assess locomotor behaviour. In the tests of despair, forced swimming test, and tail suspension test, we checked the animals' responses and adaptations to stressful situations. This assesses how soon the animals will give up under stressful situations in both tests (Vincent *et al*., 2010).

In both tests of despair, honey at the highest dose (200 mg/kg) improved the animals' responses to the tests, showing the potential of honey in ameliorating some depressive-like behaviours, especially given up during stressful situations observed in humans. Our results, which showed that lipopolysaccharide significantly increased the immobility time in both tests of despair forced swimming and tail suspension tests, align with the study of Zhang *et al*., 2019, which reported the same effect for intraperitoneal injection of lipopolysaccharide in mice. Due to the paucity of information on the role of honey in depressive-like behaviour, the only similarities found is that other sources of antioxidants like plant extracts were able to ameliorate the depressive-like behaviour associated with lipopolysaccharide injection, as found in the 2019 work of Zhang and his colleagues (Zhang *et al*., 2019).

One of the characteristics of depressive-like behaviour is the reduction in explorative movements for outdoor activity in both humans with depression and animals in experiments (Oyekunle *et al*., 2010). In this experiment, locomotion was assessed using the Open Field Test (OFT). While lipopolysaccharide reduced ambulatory behaviour in the animals used in this study, the highest dose of honey ameliorated this behavioural deficit. This corroborated the work of Oyekunle *et al*. (2010), which reported the potential of honey to modulate novelty behaviours in an anxiety study. Also, it appears, that we are the first to report the link between lipopolysaccharide-induced ambulatory deficits and the ability of honey to protect against this depressive-like behaviour in mice.

Following the aim of this experiment, the role played by oxidative stress in depression was assessed to corroborate the results of the neurobehavioral experiments by evaluating endogenous antioxidant and membrane lipid peroxidation to assess oxidative stress activities.

GSH is an endogenous, non-enzymatic antioxidant produced in the liver. GSH protects the brain against oxidative stress-induced damage, maintains plasma Vitamin E and C, participates in immune cell regulation and regeneration and cellular energy metabolism. The levels of GSH can be reduced by psychological or physiological stress, infection, and exposure to environmental toxicants. (Cruzat *et al*., 2018 and Bhatt *et al*., 2020).

Maes *et al*. (2011) reported that decreased levels of GSH are implicated in the pathogenesis of depression and major depressive disorders as a major quencher and detoxifier of free radicals. We found a corresponding reduction in the levels of glutathione in lipopolysaccharide-induced depressive-like behaviour in mice, as reported by Maes *et al*. (2011). Overall honey could not prevent the depletion of reduced glutathione in the brain of the mice used in this study. However, there is a need for further study to assess the long-term effect of honey administration on the reduced glutathione level.

Michel *et al*. (2012) reported the link between depression and increased levels of malondialdehyde (MDA), a product of oxidative degradation of cell membranes due to the stealing of electrons from the membrane lipids, leading to oxidative damage of the cell (Su *et al*., 2020). MDA is formed by reactive oxygen species interactions with cell membrane fatty acids, and the MDA formed is highly reactive and toxic to DNA. Once the antioxidant defense system is depleted, ROS reacts with membrane lipids, leading to the formation of MDA and hydrogen peroxide, another potent oxidant, leading to a vicious cycle of MDA formation and oxidative damage to neurons (Galecki *et al*., 2007). MDA levels are reported to be elevated in patients with depression, which indicates increased lipid peroxidation in major depressive disorder (Sarandol *et al*., 2007, Talarowska *et al*., 2012, Rybka *et al*., 2013).

Elevated MDA levels are reported to affect visual-spatial and auditory-verbal working memory efficiency in people with depressive behaviour. Also, the level of MDA is reported to correspond to the severity of depression and the intensity of symptoms (Michel *et al*., 2012). In this study, mice in the lipopolysaccharide group showed a significantly increased level of MDA when compared to mice in the control and imipramine-treated groups. This aligns with the work of Hakimi *et al*. (2019), who reported an increase in the level of MDA in lipopolysaccharide-treated animals. However, this was reversed by honey treatment, which may be a result of the potential of honey to donate electrons due to the presence of phenolic compounds in honey that are known antioxidants (El-Seedi *et al*., 2020).

According to Michel *et al*., (2012), malondialdehyde is responsive to antioxidants provided through diet and honey contains flavonoids and phenolic acids that can contribute electrons to mop up free radicals, suggesting the potential of honey consumption in malondialdehyde breakdown.

As a result, the antioxidant capacity of the honey used in this study may have contributed to the reduction in MDA levels in the mice brain observed in this study.

CONCLUSION

In this study, exploring the oxidative stress theory of depression, honey consumption at 200 mg/kg which was equivalent to two and a half teaspoonfuls improves performance on the test of despair in forced swimming and tail suspension tests. This dose also improves locomotor deficits, which are depressive-like behaviours induced by lipopolysaccharide. Also, honey prevented membrane lipid peroxidation in the mice brain, possibly through its antioxidant capacity. Hence, honey consumption is a potential dietary source of antioxidants in preventing depressive-like behaviour in rodents, according to this study.

Authors Contributions:

All authors contributed to the conceptualization and the design of the study. Ismaheel Akinwale Adeniyi, carried out data collection, data analysis for the study, and wrote the manuscript. Alfred Tobi Omolowo and Oyetola Oyebanjo, carried out the experimental procedure. Oyetola Oyebanjo, carried out biochemical assay and proofread the manuscript. Samuel Adetunji Onasanwo, provided the funds, reagents and laboratory space for the experiments.

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