Niger. J. Physiol. Sci. 39 (June 2024): 77 – 85
www.njps.physiologicalsociety.com

Niger. J. Physiol. Sci.

Full length Research Article

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

Brai B.I.C., Amosun, B.E., Komolafe T.R., Joseph R.O. and Komolafe K.
Department of Biochemistry, Federal University Oye-Ekiti, 370112, Ekiti State, Nigeria

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*

**Authors for correspondence: kayode.komolafe@fuoye.edu.ng, Tel: +234 8033339286*

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).

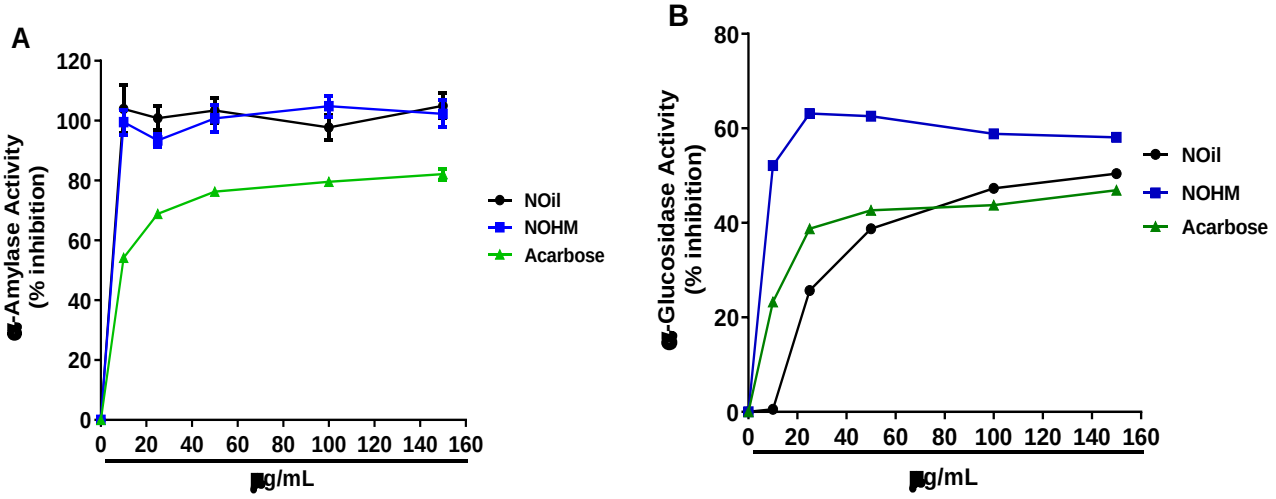

Figure 1:
In vitro inhibition of α-amylase- (A) and α-glucosidase- (B) activities by Neem oil (NOil) and its cyclohexane-methanol extract (NOHM).
† Each value represented mean ± SEM (n = 3)

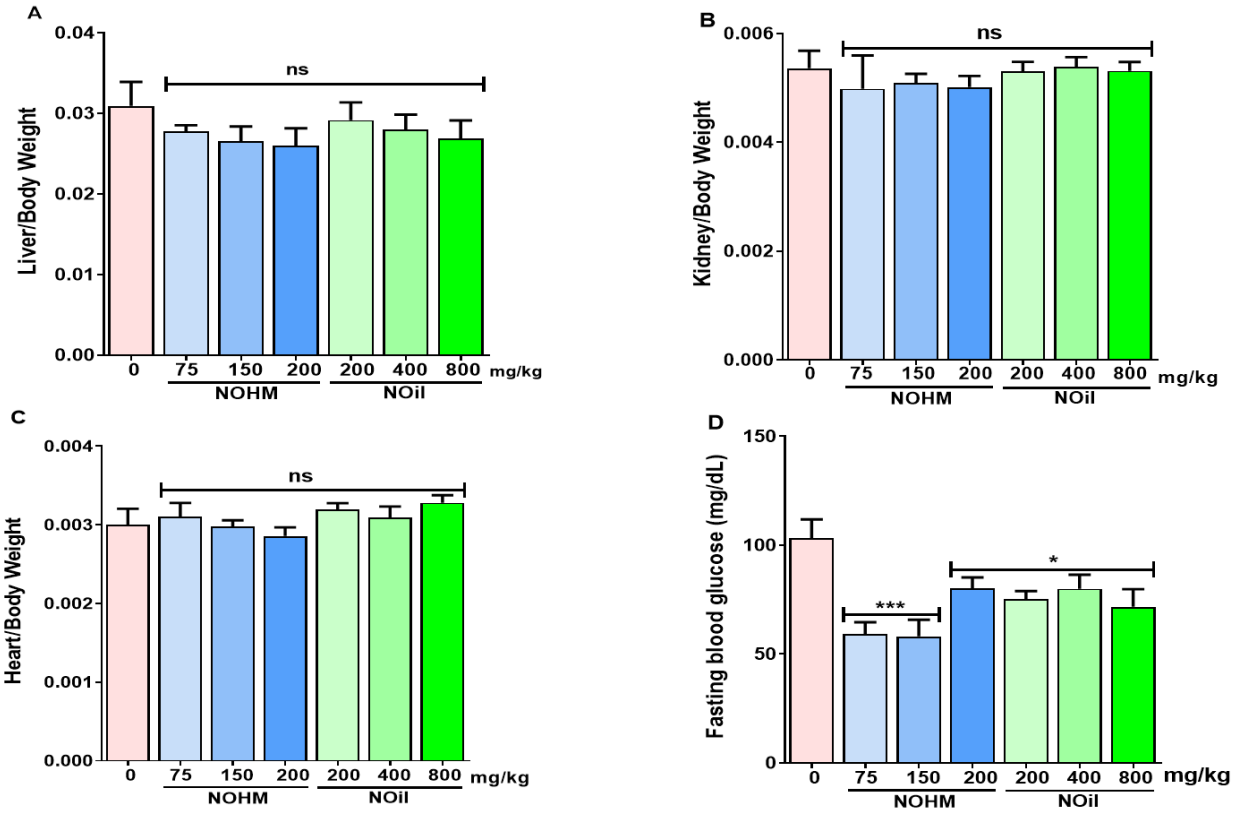

Figure 2:
Influence of Neem oil (NOil) and its cyclohexane-methanol extract (NOHM) on the organ/body weight ratios (A, B, C) and fasting blood sugar (D) of rats.
Values are expressed as mean ± SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water. *p<0.05;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

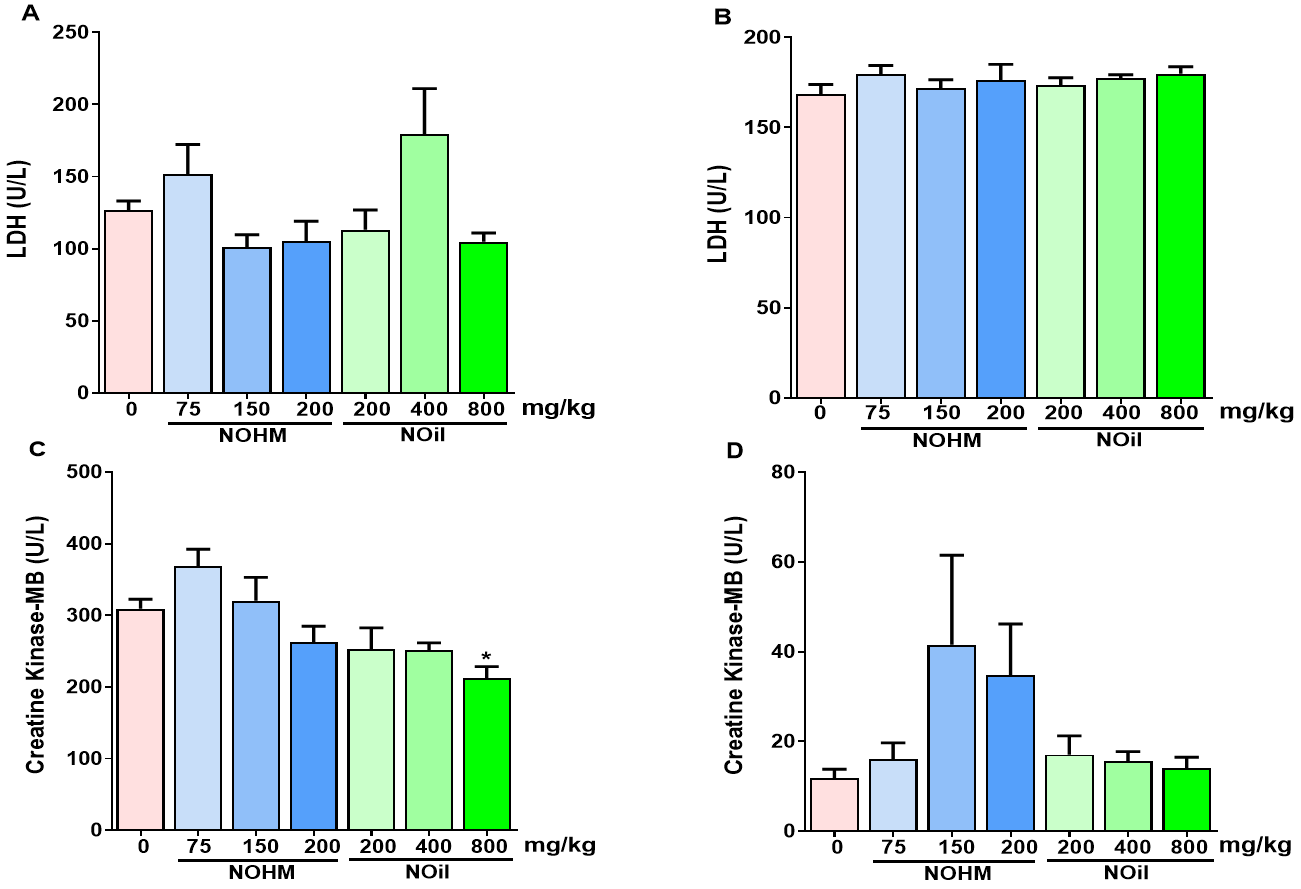

Figure 3:
Serum and cardiac activities of lactate dehydrogenase (A, B) and creatine kinase-MB (C, D) respectively in rats treated with Neem oil extract (NOil) and its cyclohexane-methanol extract (NOHM).
Values are expressed as mean ± SEM (n=6). Bars with different superscripts are not statistically 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.

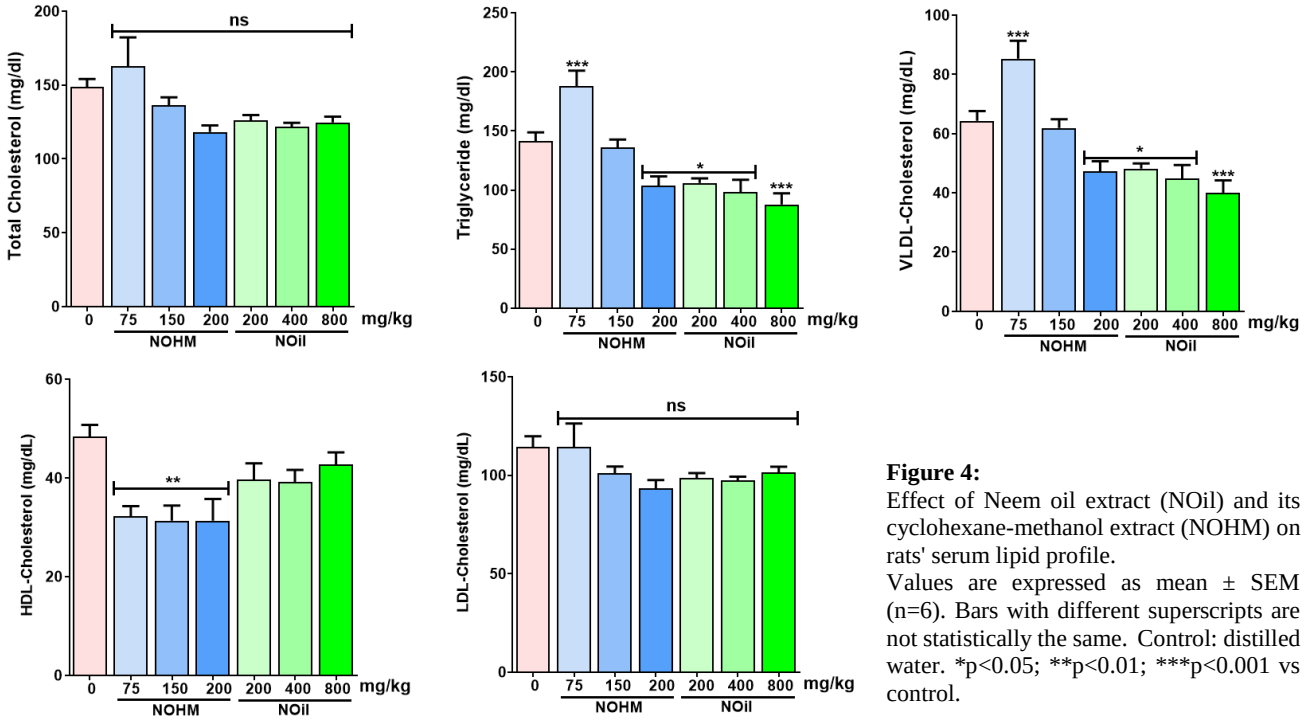

Figure 4:
Effect of Neem oil extract (NOil) and its cyclohexane-methanol extract (NOHM) on rats' serum lipid profile.
Values are expressed as mean ± SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control.

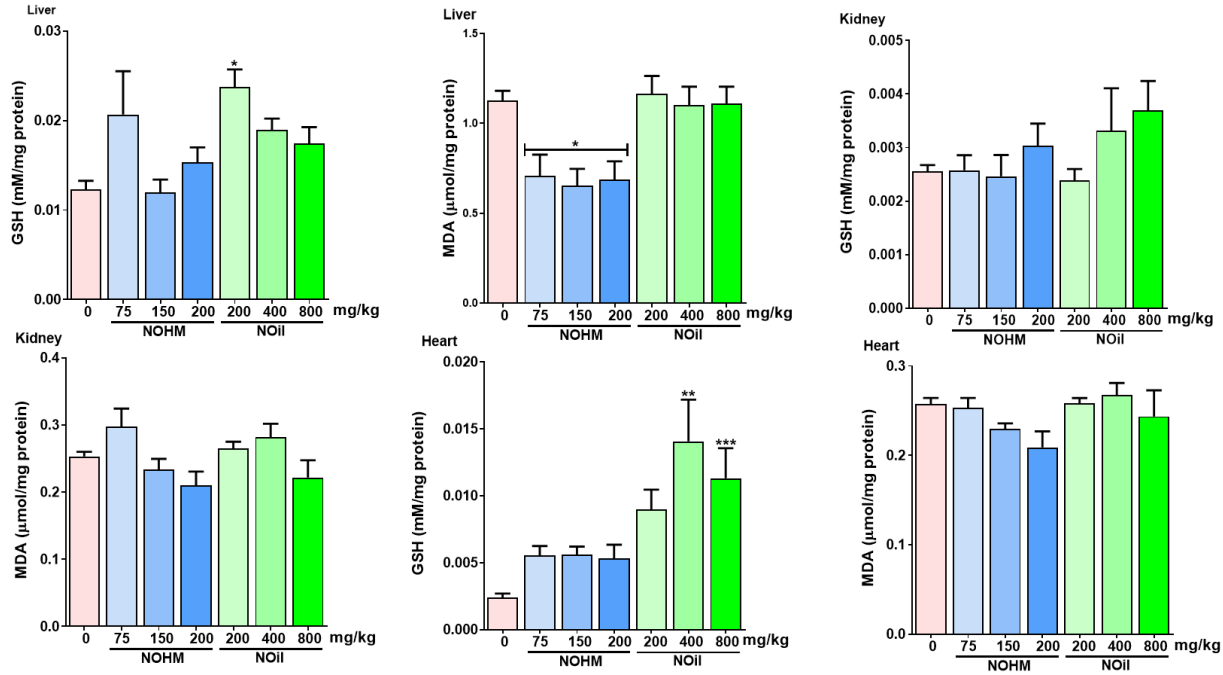

Figure 5:
Tissue antioxidant profile of rats treated with NOil and NOHM
Values are expressed as mean±SEM (n=6). Bars with different superscripts are not statistically the same. Control: distilled water. *p<0.05; **p<0.01; ***p<0.001 vs control.

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.*

REFERENCES

Abdel-Baky, E.S., Abdel-Rahman, O.N. (2020). Cardioprotective effects of the garlic (Allium sativum) in sodium fluoride-treated rats. *The Journal of Basic and Applied Zoology*. 81, 7.

Akinmoladun, A.C., Olaleye, M.T., Komolafe, K., Adetuyi, A.O., Akindahunsi, A.A. (2015). Effect of homopterocarpin, an isoflavonoid from Pterocarpus erinaceus, on indices of liver injury and oxidative stress in acetaminophen-provoked hepatotoxicity. *J Basic Clin Physiol Pharmacol.* 26, 555-62.

Ali, M.S., Jahangir, M., Hussan, S.S., Choudhary, M.I. (2002). Inhibition of alpha-glucosidase by oleanolic acid and its synthetic derivatives. *Phytochemistry*. 60, 295-9.

Alzohairy, M.A. (2016). Therapeutics Role of *Azadirachta indica* (Neem) and Their Active Constituents in Diseases Prevention and Treatment. *Evidence-based complementary and alternative medicine* : eCAM. 2016, 7382506-06.

Apostolidis, E., Kwon, Y.I., Shetty, K. (2007). Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. *Innovative Food Science & Emerging Technologies*. 8, 46-54.

Attanzio, A., Restivo, I., Tutone, M., Tesoriere, L., Allegra, M., Livrea, M.A. (2022). Redox Properties, Bioactivity and Health Effects of Indicaxanthin, a Bioavailable Phytochemical from Opuntia ficus indica, L.: A Critical Review of Accumulated Evidence and Perspectives. *Antioxidants* (Basel). 11.

Bandaranayake, W.M. (2006). Quality control, screening, toxicity, and regulation of herbal drugs.In Modern Phytomedicine: (eds I. Ahmad, F. Aqil and M. Owais). https://doi.org/10.1002/9783527609987.ch2

Basnet, S., Ghimire, M.P., Lamichhane, T.R., Adhikari, R., Adhikari, A. (2023). Identification of potential human pancreatic α-amylase inhibitors from natural products by molecular docking, MM/GBSA calculations, MD simulations, and ADMET analysis. *PLoS One*. 18, e0275765.

Bernfeld, P. (1951). 'Enzymes of Starch Degradation and Synthesis.' in, Advances in Enzymology and Related Areas of Molecular Biology.

Cesa, S., Sisto, F., Zengin, G., Scaccabarozzi, D., Kokolakis, A.K., Scaltrito, M.M., Grande, R. *et al*. (2019). Phytochemical analyses and pharmacological screening of Neem oil. *South African Journal of Botany*. 120, 331-37.

Chen, Y.F., Kobayashi, S., Chen, J., Redetzke, R.A., Said, S., Liang, Q., Gerdes, A.M. (2008). Short term triiodo-L-thyronine treatment inhibits cardiac myocyte apoptosis in border area after myocardial infarction in rats. *Journal of Molecular Cellular Cardiology*. 44, 180-7.

Chipiti, T., Ibrahim, M.A., Singh, M., Islam, M.S. 2015. In vitro α-amylase and α-glucosidase inhibitory effects and cytotoxic activity of Albizia antunesiana extracts. *Pharmacognosy magazine.* 11, S231-S36.

Cromwell, W.C., Otvos, J.D. (2004). Low-density lipoprotein particle number and risk for cardiovascular disease. *Current Atherosclerosis Reports*. 6, 381-7.

Deng, Y.X., Cao, M., Shi, D.X., Yin, Z.Q., Jia, R.Y., Xu, J., Wang, C. *et al*. (2013). Toxicological evaluation of neem (*Azadirachta indica*) oil: acute and subacute toxicity. *Environmental Toxicology and Pharmacology*. 35, 240-6.

Garavaglia, J., Markoski, M.M., Oliveira, A., Marcadenti, A. (2016). Grape Seed Oil Compounds: Biological and Chemical Actions for Health. *Nutrition and Metabolic Insights*. 9, NMI.S32910.

Ghosh, D., Mondal, S., Ramakrishna, K. (2019). Acute and sub-acute (30-day) toxicity studies of Aegialitis rotundifolia Roxb., leaves extract in Wistar rats: safety assessment of a rare mangrove traditionally utilized as pain antidote. *Clinical Phytoscience*. 5, 13.

Gowda, S., Desai, P.B., Kulkarni, S.S., Hull, V.V., Math, A.A.K., Vernekar, S.N. (2010). Markers of renal function tests. North *American Journal of Medical Sciences*. 2, 170-73.

Gupta, S., Kataria, M., Gupta, P.K., Murganandan, S., Yashroy, R.C. (2004). Protective role of extracts of neem seeds in diabetes caused by streptozotocin in rats. *Journal of Ethnopharmacology*. 90, 185-9.

Ihedioha, J.I., Noel-Uneke, O.A., Ihedioha, T.E. (2013). Reference values for the serum lipid profile of albino rats (Rattus norvegicus) of varied ages and sexes. *Comparative Clinical Pathology.* 22, 93-99.

Injac, R., Radic, N., Govedarica, B., Perse, M., Cerar, A., Djordjevic, A., Strukelj, B. (2009). Acute doxorubicin

pulmotoxicity in rats with malignant neoplasm is effectively treated with fullerenol C60(OH)24 through inhibition of oxidative stress. *Pharmacological Reports.* 61, 335-42.

Kazeem, M.I., Adamson, J.O., Ogunwande, I.A. (2013). Modes of inhibition of α -amylase and α -glucosidase by aqueous extract of Morinda lucida Benth leaf. *BioMed Research International*. 2013, 527570-70.

Komolafe, K., Akinmoladun, A., Olaleye , M. (2013a). Methanolic leaf extract of Parkia biglobosa protects against doxorubicin-induced cardiotoxicity in rats. *International Journal of Applied Research in Natural Products*. 6, 39-47.

Komolafe, K., Akinmoladun, A., Olaleye, M. (2013b). Methanolic leaf extract of Parkia biglobosa protects against doxorubicin-induced cardiotoxicity in rats. International Journal of Applied Research in Natural Products. 6 39-47.

Komolafe, K., Komolafe, T.R., Fatoki, T.H., Akinmoladun, A.C., Brai, B.I.C., Olaleye, M.T., Akindahunsi, A.A. 2021. Coronavirus Disease (2019) and Herbal Therapy: Pertinent Issues Relating to Toxicity and Standardization of Phytopharmaceuticals. *Revista Brasileira de Farmacognosia.* 31, 142-61.

Li, K., Yao, F., Xue, Q., Fan, H., Yang, L., Li, X., Sun, L., Liu, Y. (2018). Inhibitory effects against α-glucosidase and α-amylase of the flavonoids-rich extract from Scutellaria baicalensis shoots and interpretation of structure-activity relationship of its eight flavonoids by a refined assign-score method. Chemistry Central Journal. 12, 82.

Manuwa, T.R., Akinmoladun, A.C., Crown, O.O., Komolafe, K., Olaleye, M.T. (2017). Toxicological Assessment and Ameliorative Effects of Parinari curatellifolia Alkaloids on Triton-Induced Hyperlipidemia and Atherogenicity in Rats. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. 87, 611-23.

Obidike, I., Salawu, O. (2013). 'Screening of Herbal Medicines for Potential Toxicities, New Insights into Toxicity and Drug Testing ' in Sivakumar Gowder (ed.), New Insights into Toxicity and Drug Testing (IntechOpen).

Oboh, G., Ademosun, A.O., Ademiluyi, A.O., Omojokun, O.S., Nwanna, E.E., Longe, K.O. (2014). In Vitro Studies on the Antioxidant Property and Inhibition of α-Amylase, α-Glucosidase, and Angiotensin I-Converting Enzyme by Polyphenol-Rich Extracts from Cocoa (Theobroma cacao) Bean. *Pathology Research International*. 2014, 549287.

Ogundipe, D.J., Akomolafe, R.O., Sanusi, A.A., Imafidon, C.E., Olukiran, O.S., Oladele, A.A. (2017). Ocimum gratissimum Ameliorates Gentamicin-Induced Kidney Injury but Decreases Creatinine Clearance Following Sub-Chronic Administration in Rats. *Journal of Evidence-based Complementary & Alternative Medicine*. 22, 592-602.

Olaleye, T.M., Komolafe, K., Akindahunsi, A. (2013). Effect of methanolic leaf extract of Parkia biglobosa on some biochemical indices and hemodynamic parameters in rats. *Journal of Chemical and Pharmaceutical Research*. 5 213-20.

Padhye, S., Banerjee, S., Ahmad, A., Mohammad, R., Sarkar, F. (2008). From here to eternity - the secret of Pharaohs: Therapeutic potential of black cumin seeds and beyond. *Cancer Therapy.* 6, 495-510.

Porwal, M., Khan, N.A., Maheshwari, K.K. (2017). Evaluation of Acute and Subacute Oral Toxicity Induced by Ethanolic Extract of Marsdenia tenacissima Leaves in Experimental Rats. *Scientia Pharmaceutica.* 85(3), 29; https//doi.org/10.3390/scipharm85030029

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K.M., Yoga Latha, L. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. African Journal of Traditional, Complementary, and Alternative Medicines. 8, 1-10.

Subapriya, R., Nagini, S. (2005). Medicinal properties of neem leaves: a review. *Current Medicinal Chemistry - Anticancer Agents.* 5, 149-6.

Suganthy, N., Muniasamy, S., Archunan, G. (2018). Safety assessment of methanolic extract of Terminalia chebula fruit, Terminalia arjuna bark and its bioactive constituent 7-methyl gallic acid: In vitro and in vivo studies. *Regulatory Toxicology and Pharmacology*. 92, 347-57.

Varnham, A. (2014). Seed Oil: Biological Properties, Health Benefits and Commercial Applications Nova Science Publishers, United Kingdom.

Varshney, R., Kale, R.K. (1990). Effects of calmodulin antagonists on radiation-induced lipid peroxidation in microsomes. *International Journal of Radiation Biology*. 58, 733-43.

Vermaak, I., Kamatou, G.P.P., Komane-Mofokeng, B., Viljoen, A.M., Beckett, K. (2011). African seed oils of commercial importance — Cosmetic applications. *South African Journal of Botany.* 77, 920-33.

Winston, D., Maimes, S. (2007). Adaptogens: Herbs for strength, stamina and stress relief Healing Arts Press: Rochester, Vermont.

Xu, J., Fan, Q.J., Yin, Z.Q., Li, X.T., Du, Y.H., Jia, R.Y., Wang, K.Y. *et al*. (2010). The preparation of neem oil microemulsion (*Azadirachta indica*) and the comparison of acaricidal time between neem oil microemulsion and other formulations in vitro. *Veterinary Parasitology*. 169, 399-403.

Zhang, Y.-Q., Xu, J., Yin, Z.-Q., Jia, R.-Y., Lu, Y., Yang, F., Du, Y.-H. *et al*. (2010). Isolation and identification of the antibacterial active compound from petroleum ether extract of neem oil. *Fitoterapia*. 81, 747-50

.