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Toxicological and Nutritional Evaluations of Milk Bush (Thevetia neriifolia) Seed Oil-Based Diet in Albino Rats

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Abstract
The seed oil of milk bush *Thevetia neriifolia* have been analyzed to provide some physical data for the oil and growth performance and toxicity evaluation in rats using the seed oil-based. The fatty acid composition of the oil was identified using Gas Chromatography (GC) and Gas Chromatography – Mass Spectrophotometer (GC-MS). The oil consist of 97.583% fatty acid of which the most abundant is the monounsaturated ($C_{18}H_{34}O_2$) Oleic acid (52%), and the others are saturated fatty acids ($C_{18}H_{36}O_2$) Stearic acid (25%) and the other Palmitic acid ($C_{16}H_{32}O_2$). The physicochemical studies of the seed oil showed: acid value of 0.515 ± 0.27; 117.125±2.38 mgKOH/g saponification value and 74.145 ± 0.784 iodine value. Growth performance was investigated in Wistar albino rats following a feeding period of 6weeks, using 5 %, 10 % and 15% of the oil compounded feed. We observed a significant decrease in body weight gain and feed intake in 10% and 15% oil-based feed group compared to control. The enzymatic antioxidant, biomarkers of kidney/liver toxicity and histopathology of visceral organs were evaluated. There was a significant ($p < 0.05$) increase in serum AST, and ALT of treated groups with respect to control group. Which explain its toxic nature.
Keywords: chemical composition, characteristics, feed formulation, growth performance, toxicological evaluations

Introduction

Several seed oils of several members of the Compositae family were shown to possess unusual properties such as a high yield of oil which contained trans, trans conjugated diene and dienoic acid (Irvine 1962; Tsuyeshi 1967; Aiyelaagbe et al 1996). The relatively high proportion of the oils, suggested that they might have considerable industrial utility. Feeding experiments (Atteh *et al.,* 1990; Atteh *et al.,* 1995) and thermal studies (Ibiyemi *et al.,* 1995) have shown that the oil has a very good replacement value for orthodox domestic vegetable oils. There is relatively minimal data on the toxicity of this oil, hence we carried out a comprehensive assay of the fatty acid composition and animal model nutritional study to evaluate the suitability of oil for consumption and possible toxicity. *Thevetia neriifolia* plant is a dicotyledon which belongs to *Aponaceae* family. It is an evergreen Compositae shrub with milky sap. It is native to West Indies, Mexico and Brazil. The common names are yellow oleader (nerium), gum bush, bush milk, exile tree in India, Cabalonga in Puerto Rico, Olomi ojo by Yurubas in Nigeria. The shrub reaches a height of about 3 to 3.9 meters. The plant is perennial; the leaves are linear, narrow sword like and green. The fruit when unripe is hard and green but gradually turns black as it ripens. The fruits have varying masses (2- 6.1 g) which are dispersed by man and propagated by seed or stem. The seed contain about 60-64 % oil on dry matter basis. The plant produces a highly poisonous white latex sap (Ibiyemi *et al*., 1988). *Thevetia neriifolia* seed contains toxic compounds which are mostly cardiac glycosides and their free aglycones such as thevetic, digitoxigenin etc (Sticker 1970; Sticher 1971; Gupta e*t al.,* 1974; Abe *et al* 1994) and has been shown to be toxic. The defatted seed cake however has a high level of toxicity (Vohra *et al*.1961; Verma 1964; Pahwa and Chatterjee 1990; Uber-Bucek *et al* 1992; Bose *et al*. 1999) and it is most likely that the attention given to toxins has distracted interest from proper research of the oil and protein that would have promoted its industrial and domestic potentials.

In spite of the toxicity of the plant, it has been found useful in tradomedicine. The latex is used as an analgesic for toothache and the wood is used as axe handle. The seed consist of 60-65 % oil and 35 - 40% protein (Ibiyemi *et al*., 2002). The physical properties of the oil particularly the saponifiable and unsaponifiable matters shows that it can be used by commercial soap making industries.

To the best of our knowledge the toxicity and nutritional potential, growth performance of the oil of milk bush and the toxicity has not been evaluated. The above informed our interest in this very oily seed *also* to evaluate the chemical characteristics and probable suitability of the oil as supplement in animal feed production, especially as the cultivation of the shrub is relatively easy and the yield is very good. The shrub or tree can grow outside in warmer climates but in frost prone areas best brought back inside for winter and it tolerates most kinds of soil as long as they are well drained and is situated in full sun in a sheltered area. Useful as a landscaping plant in warmer climates as it does not need much maintenance.

Materials and methods

Plant identification and preparation

The mature seeds were harvested from shrubs in University of Ibadan, Ibadan and identified by Mr D Esimekhuai at the Herbarium of the Department of Botany, University of Ibadan, Ibadan Nigeria. A voucher specimen was deposited at the Departmental Herbarium. The fleshy fruits were dried and the soft portion removed to obtain the hard light brown seeds. The air dried seeds were crushed carefully to obtain the soft oily creamy pulp encased in dark brown seed coat. These were coarsely powdered using the dry cup of domestic Kenwood blender and subjected to Soxhlet extraction using *n*Hexane for 6 hours in the Laboratory to obtain the oil.

Physicochemical Properties

The GC of the oil (as their methyl ester) was taken and the GC-MS was recorded on JEOL MSRoute. Column and the flow rate of the carrier gas (helium) was 1-2 mls/min and injection temperature 250^{O}C. Peaks were identified using authentic standards from computer literature search using fragmentation pattern. The percentage of each fatty acid component was calculated as the peak area percent of the total of all fatty acids. The various physicochemical properties such as acid value, saponification value and iodine value were determined as described by A.O.A.C (1990).

Feed components and formulation

Soybean oil marked as Grand vegetable oil, a product of Grand Cereals and Oil mills Limited, Bukuru, Jos, Nigeria, yellow maize (*Zea mays*) grains and soy beans were purchased from Bodija Market, Ibadan, Nigeria. Vitamin mix was from BASF Aktiengesellschaft, Ludwigshafen, Germany. The composition of test feed per kg diet is as shown on Table 1. Briefly, Corn starch was carbohydrate source, it was cleaned, sundried and milled. Soybean was first dehusked before milling and it was used as protein source. Components of the mineral mix was from Sigma-Aldrich Co. Ltd., Poole Dorset, UK The different components of the diet were thoroughly mixed, made into pellets for easy handling by animals and was thoroughly oven dried to prevent mould growth was stored in air tight bags at 4^{O}C to prevent microbial contamination and auto-oxidation of the oil. (Oladiji et al. 2007)

Proximate analysis of the feeds

The 5%, 10%, 15% milk bush oil feed, 100% pure commercial oil and commercially purchased rat chow from Ladokun feeds were analyzed for ash and mineral content using muffle furnace at 550^{O}C for 4 h. Moisture content was determined by drying in the oven at 100^{O}C until a constant weight was obtained (at least 24 h). Total dietary fiber was determined by an enzymatic gravimetric method, (AOAC, 1990). Crude oil content was assayed by extraction with *n*-hexane in a Soxhlet extractor (AOAC 1990). Nitrogen was determined by standard micro kjeldahl method (AOAC 1990) using a digestion apparatus. The crude protein content was thereafter calculated by multiplying nitrogen by a factor of 5.71, which takes into account the nonprotein nature of part of the nitrogen and has been approved for calculating the crude protein content of soybeans (Pellet 1980, Petzke et al 1997)

Animal Used

Thirty male albino rats (Wistar strain) weighing between 70 g and 80 g were obtained from the animal house in the Department of physiology university of Ibadan. On arrival, the rats were transferred and allowed to acclimatized, been maintained on the standard normal diet with water *ad libitum* in the Biochemistry department animal house under normal room temperature before the commencement of the experiment.

Experimental Design.

Animals were distributed randomly into five different groups of six animals each. The control group received diet (commercially available rat feed), group A compounded feed using Grand soybean oil purchased from Bodija market, Ibadan, group B compounded diet with 5 % of the milk bush seed oil, group C compounded diet with 10 % of the milk bush seed oil and group D compounded diet with 15 % of milk bush seed oil.

Sample collection

Animals were sacrificed by cervical dislocation and blood was obtained using 2ml syringe by cardiac puncture into clean bottles and allowed to clot. These were spun at 3000 rpm for 10 minutes; the supernatant (serum) was removed and stored at 4^{o}C. The liver and kidney were quickly removed, weighed, washed with 1.15% KCl, homogenized in 56 mM Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride and the homogenate was centrifuged at 10.000 rpm for 15 minutes to obtain post mitochondrial fraction (PMF) at 4^{o}C.

Biochemical analysis

The concentration of protein in the serum and PMF was determined using the method Lowry et al. 1951 with BSA as standard. The extent of lipid peroxidation was determined by estimating the thiobarbituric acid reactive substances (TBARS) formed following the method of Varshney and Kale 1990. Microsomal catalase (CAT) activity was determined by using hydrogen peroxide, briefly, the reaction mixture contained phosphate buffer (0.01 M, pH 7.0), tissue homogenate and 2 M H_2O_2. The reaction was stopped by the addition of dichromate-acetic acid reagents (5%potassium dichromate and glacial acetic acid were mixed in a ratio of 1:3) Claiborne,

1989. Cholesterol was determined according to the method of Richmond 1973 by absorbance measurement at 490 nm when cholesterol reacts with $FeS0_4$ in glacial acetic acid is treated with H_2SO_4. Triglycerides was estimated after enzymatic hydrolysis with lipases by measuring quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under catalytic influence of peroxidase (Tietz 1990)

Urea estimation was done using kit supplies by Dialab production and Vertrieb vonchemisch Technischen. Urease hydrolysis urea to ammonia and carbondioxide and the ammonia reacts with alkaline hypochlorite and sodium salicylate to produce a colored complex which is measured spectrophotometrically at 546 nm. Enzyme parameters Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were assayed using Randox kits.

Statistical Analysis

Data was expressed as mean ± S.D of six determinations, except for the proximate analysis and were analyzed using one way analysis of variance (ANOVA) and complimented with student t-test. Values for $p < 0.05$ were considered to be statistically significant.

Result and discussion

The yield calculated from the oil extraction was approximately 58.03 % oil on dry matter basis. This was slightly lower than 61 - 63% previously reported in the literature, but was not significantly different. We obtained oil, faintly whitish in color, with no characteristic smell and semi-solid at room temperature. The oil consists of mostly saturated and monounsaturated fatty acids of which the most abundant is Oleic acid, followed by Stearic acid and Palmitic acid. This seemed comparable with consumable vegetable oils as palm oil (*Eleaeis guineensis*) and peanut oil (*Arachis hypogeae*) The physicochemical studies of the seed oil showed: acid value of 0.515 ± 0.27; 117.125±2.38 mgKOH/g saponification value and 74.145 ± 0.784 iodine value. This was similar to earlier values reported by Ibiyemi *et al*. 2002. The saponification value compared favourably with most food oils and indicates that the oil might be useful for soap making, candle production and as chemical feedstock for lubricants. Data obtained from the change in body weight, change in weight of visceral organs and feed intake of animal on the compounded milk bush oil meal, commercially sold vegetable oil and regular rat chow are shown on Tables I and II. There was a significant decrease in the weight of the visceral organs when compared with the control. (Table I) The groups fed with compounded diets with milk bush oil and Grand vegetable oil showed significant decrease in the weight of the kidney when compared with the control. Furthermore there was 11.07 % significant ($p < 0.05$) decrease in the body weight of rats fed with 15 % of the seed oil when compared with the control. Also, there was a significant decrease in the feed intake when control group was compared with 10 % and 15 % seed oil compounded diet feed groups. To relate feeding experiment result to a distinguished single component is difficult as antinutritive factors in foods and feed intake should also be considered. Due to this, we have used the same food stuff for compounding the feed except the oil. The feed intake was greatly reduced in the groups on the compounded diets and this culminated in reduced body weight, except for the animals on pure commercially sold vegetable oil. There was a significant ($p < 0.05$) decrease in feed intake when control group was compared with 10 % and 15 %. Groups on bush milk oil.

Previous study by Pahwar and Chatterjee 1990 reported the toxic effects of yellow oleander seed kernels in the roof rat (Rattus rattus Linn) and the main signs of poisoning observed were hind limb paralysis, rolling of the body on the long axis, circular flailing of the tail, muscular twitch, tetanic convulsions, tremors, collapse and death. We did not observe any abnormal behavior or decreased psychomotor activity in animals fed the bush milk seed oil based diet

The result of serum ALT, AST, ALP, Urea and protein are shown on Table III. Bush milk oil elevated on serum AST and it was significant ($p < 0.05$) when control group was compared with rats fed with 15 % (Group E) of the seed oil. A non significant decrease in AST was obtained when control group was compared with rats treated with 5 % and 10 % of the *T. neriifolia* seed oil (Groups C and D). The result for ALT showed that there was a significant increase in serum ALT activities when 5 %, 10 % and 15 % were compared with the control. The significant rise in these marker enzymes portrays a slight degree of impairment to the liver. There was a significant increase in serum AST, ALT and ALP when control (Group A) was compared with the test animals on pure grand vegetable oil diet (Group B). Urea was significantly increased in the control rats compared to test animals on pure Soybean oil feed (Group B), whereas the urea values was significantly increased in the 10% and 15% bush milk oil (Groups D and E) when compared with control. There were no significant changes in serum protein concentration in the animals used in this study.

Transaminases play an important role in protein and amino acid metabolism in hepatocytes and striated muscle cells. However, when cells are injured or necrosis occur, these enzymes may leak or escape from these cells into the blood stream, where their present activities are consequently increased. Therefore, the determination of these transaminases in the serum is often used as one of the essential marker of liver damage. This is because they are cytoplasmic in location and are released into circulation after cellular damage (Sallic et al, 1991). The elevated values obtained for AST, ALT and ALP compared to Grand vegetable oil group indicates that the commercially sold oil is not hepatotoxic as the *T.nerilolia* oil and the hepatotoxicity increases with

increase in the concentration of the oil in the formulated meal.

Liver cholesterol was significantly decreased in the animals on the 10% and 15% Bush milk diet (Groups D and E) relative to control and in the 5 % (Group C) relative to the test diet group with pure soybean vegetable oil. The observed decrease in liver cholesterol might be due to the smaller size of the internal organs in animals on bush milk diet and the general reduction in bodyweight which we observed in this study. Cholesterol has been shown to reduce fatty acid oxidation, which in turn, increases the levels of hepatic and plasma triglyceride (Meittinin and Tarpila 1997). Liver triglyceride level was significantly reduced in the test diet (Soybean oil) group as well as in the 10% and 15% *T neriifolia* oil (Groups D and E) compared to control. Only in the 10% (Group D) bush milk oil group was the heart triglyceride level significantly reduced compared to animals on the test feed (Group B) with market sold vegetable oil. Lipid peroxidation (LPO) was only significantly reduced in the rats on 5% bush milk oil (Group C) compared to control in the liver, while the value obtained for kidney of animals on pure vegetable oil (Group B) increased by about 150%. There was a non significant increase in catalase activity in the kidney of test animals relative to control while we observed a non significant decrease in the liver of animal on soybean oil, 5% and 10% (Group C and D) bush milk seed oil based diet..

An overabundance of free radicals in the cell leads to uncontrolled chain reactions and lipid peroxidation, resulting in various pathological conditions that may include cardio vascular diseases, such as preeclampsia, atherosclerosis and cancer. An increase in the level of the LPO product (malondialdelyde) which is an index of the level of oxygen free radical, hence a decrease in LPO may lead to a reduction in the arterial wall cholesterol content. Lipid peroxidation contributes to the development of and the end product of this process, malondialdehyde (MDA), and 4 – hydroxynonenal (HNE) can cause damage to proteins and DNA. Concentration of malondialdehyde (MDA), an index of lipid peroxidation was significantly reduced in the liver of rats on 5% (Group C) bush milk oil relative to control and it was lower than the commercially sold vegetable oil (Group B). Oxygen free radicals contribute to physiological endothelial cell injury, which appears to be an important component in the response to injury. Animals on 5% and 10% bush milk oil in (Groups C and D) had similar catalase enzyme activity as control animals. Histopathological photomicrograph (data not included because of simplicity) of the liver and the heart did not show any significant lesions, only in the 5% milk bush oil (Group C) did we have generalized fatty acid degeneration.

Observed increment in the activities of ALT, ALP and AST in the serum in the present study may be attributed to the leakage of these enzymes from the liver into the serum indicating the possible hepatotoxic effect of the test diet. We also had reduction in MDA in the liver of rats on 5% *T. neriifolia* oil (Group C) but higher values we obtained at higher concentration of the bush milk oil based diet. Although we did not observe any significant lesion in the visceral organs, this oil should be used with caution, especially when consumed over a long period. Also there was reduction in feed intake, which subsequently led to reduced body weight. The ingestion of *Thevetia neriifolia* seed caused significant reductions in the rats' weights, increased BUN, SGOT and LDH significantly and histopathological studies showed inflammatory and degenerative changes in the liver and kidney (Pawha and Chatterjee 1990). Severe to moderate fatty metamorphosis, congestion, hepatocytolysis, nuclear degeneration, pyknosis, and necrosis were major changes observed in the liver of rats on ingestion of bush milk seed (Pawha and Chatterjee 1990). Atteh *et al* 1995 and Atteh *et al* reported that the oil was safe for chicken feed. Our results have not shown that the consumption of the oil for prolonged period is safe in rats especially in the 10% and 15% (Groups D and E) bush milk oil compounded feed. The result of the chemical composition of the oil shows great similarity with conventional vegetable oil. Our study has shown the chemical composition of bush milk oil, the need to further purify the *T neriifolia* seed oil for dietary purposes and the oil should be used with caution at above 5% replacement in rat feeds.

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Table 1. Effect of milk bush (*Thevetia neriifolia*) seed oil on weight of organs of rats fed with varying percentage of the oil.

Treatment	Weight of liver	Weight of kidney	Weight of heart
Group A	3.00 ± 0.00	1.03 ± 0.10	0.51 ± 0.23
Group B	2.73 ± 0.29*	0.64 ± 0.14*	0.25 ± 0.09
5 % seed oil C	2.96 ± 0.90	0.78 ± 0.07*	0.40 ± 0.25
10 % seed oil D	2.96 ± 0.09	0.78 ± 0.10*	0.34 ± 0.08

The results are present as mean ± S.D of six rats in each group. *P < 0.05 is significant when compared with the control

Table II. Effect of milk bush (*Thevetia neriifolia*) seed oil on body weight and feed-intake of rats fed with varying percentage of the oil.

Treatment	Initial body weight	Final body weight	% Weight gain	Feed intake
Control A	92.33 ± 10.32	140 ± 25.15	44.67 ± 51.6	112.74 ± 10.88
Veg. oil B	81.67 ± 4.08	161 ± 27.02	79.33 ± 97.1	69.56 ± 16.72
5% TNO C	86.67 ± 6.06	142 ± 25.15	55.33 ± 63.8	62.84 ± 18.83
10% TNO D	87.50 ± 5.24	103 ± 16.43*	15.50 ± 17.7	59.60 ± 19.86*
15% TNO E	85.83 ± 3.77	107 ± 10.95*	21.17 ± 24.7	56.11 ± 16.03*

The results are present as mean ± S.D of six rats in each group *P < 0.05 is significant when compared with the control.

Table 1V Proximate analysis of commercial rat chow (control) and test diet with vegetable oil

Treatment	% moisture	%Fat	%crud fibre	%Ash	%Protein
Control diet	8.83	4.88	9.22	7.38	18.39
Test diet	7.83	3.96	5.56	7.30	18.19

Table III. Effect of milk bush (*Thevetia neriifolia*) seed oil on serum AST, ALT, ALP, Urea and Protein rats fed with varying percentage of the oil.

GROUP	AST (U/I)	ALT (U/I)	UREA	ALP (U/I)	PROTEIN
Control A	14.50±1.73*	2.25±0.30*	164.62±14.9*	48.85±5.70*	5.54±o.8
Veg. oil B	20.25±10.72	6.25±6.13	234.12±23.44	53.79±21.27	4.75±1.34
5% seed oil C	24.05±3.56	18.75±4.99*	216.47±36.25	70.29±33.6*	5.51±0.9
10% seed oil D	41.75±14.54	17.5±4.43*	252..89±33.05*	101.73±16.54*	7.08± 1.8
15% seed oil E	53.54±17.10*	20.25±6.24	263.97±5.60*	119.13±17.70*	5.75±1.25

The results are present as mean ± S.D of six rats in each group. *P < 0.05 is significant when compared with the control

Figure I Effect of bush milk oil based diet on liver and kidney cholesterol

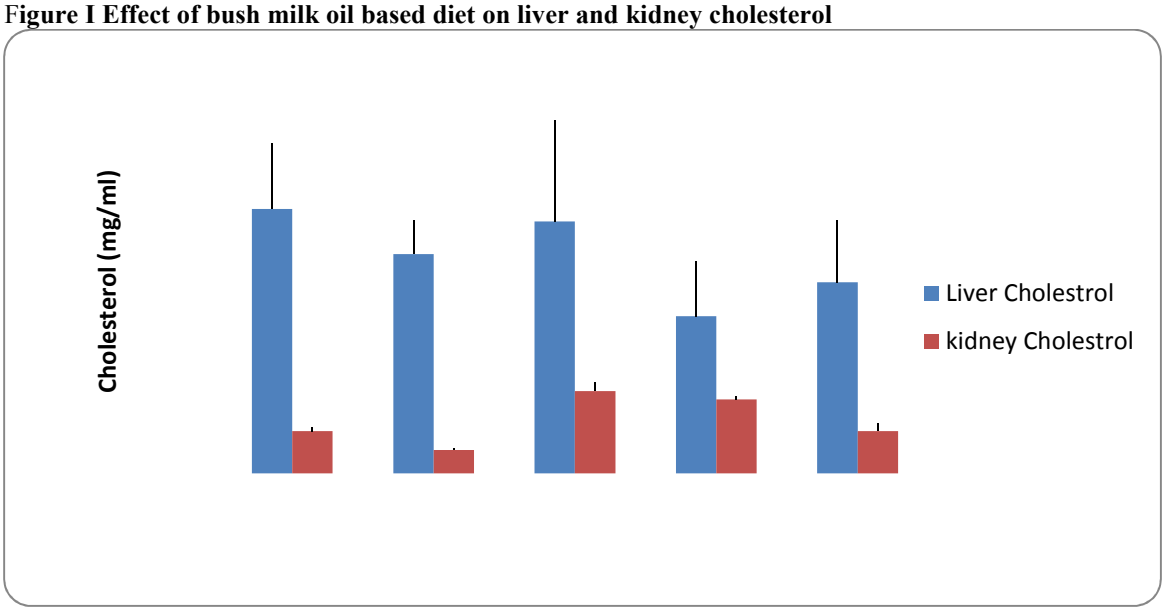


Figure II. Effect of milk bush (*Thevetia neriifolia*) seed oil on liver and heart Triglyceride level

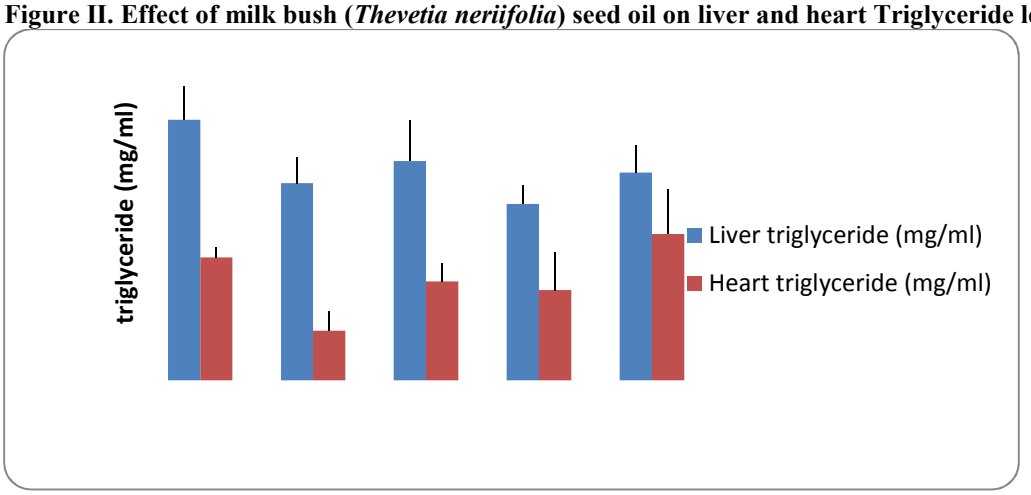


Figure III. Effect of milk bush (*Thevetia neriifolia*) seed oil on liver and kidney MDA level

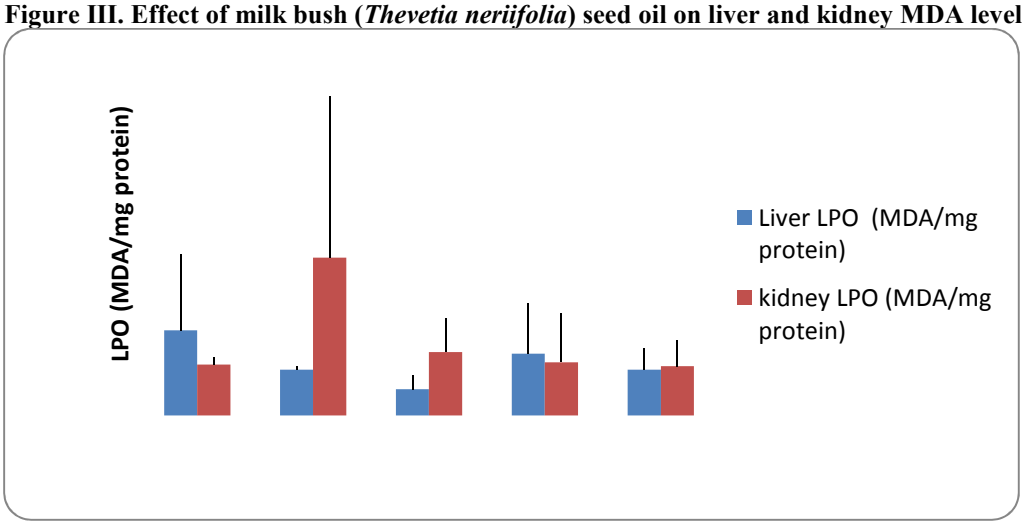
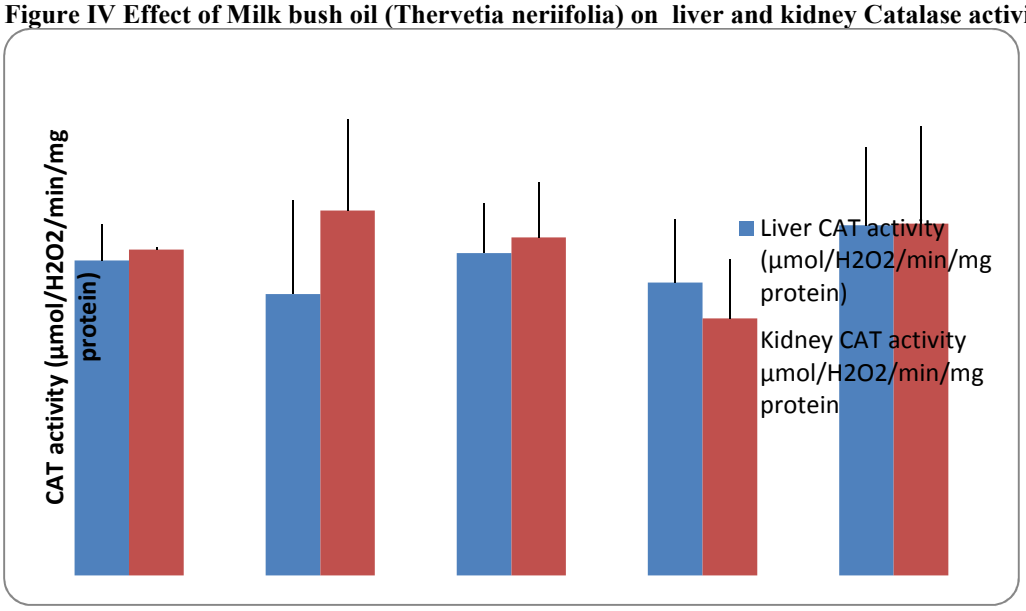


Figure IV Effect of Milk bush oil (Thervetia neriifolia) on liver and kidney Catalase activity



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