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Toxicity, hypoglycemic and antioxidant potentials of *Massularia acuminata* Stem

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Abstract
Massularia acuminata is mostly employed in herbal decoctions for the treatment of diabetes, and as an aphrodisiac. Its toxicity index and acclaimed hypoglycemic potency have not been scientifically validated, which necessitates this study. High amount of saponins, alkaloids and cardiac glycosides were detected; extract and fractions produced cytotoxicity to brine shrimp (LC_{50}, 1.26-21.54 µg/mL), and LD_{50} (111.8 mg/Kg) for intraperitoneal administration of extract using albino mice. Treatment of alloxan-induced diabetic rats with extract caused a significant reduction ($P<0.05$) in fasting blood glucose level (45.63%) in acute study and 61.73% reduction in prolong treatment (2 weeks); extract and fractions demonstrated moderate antioxidant effects (using 2,2-diphenyl-1-picrylhydrazyl radical and ferric reducing power assays) compared to ascorbic acid. These results suggest that *M. acuminata* stem is cytotoxic and possess good hypoglycemic effects. Its toxicity in herbal preparations is of essence.

Keywords: *Massularia acuminata*, lethal dose, antidiabetic effect, saponins, antioxidant activity

1. Introduction
Massularia acuminata (G. Don) Bullock ex Hoyl. (Rubiaceae), also known as bitter chewing stick, is a medium sized shrub that grows up to 5 m high. The plant is native to tropical regions of Western Africa and known for its varied uses in trado-medical practice, especially its acclaimed potency as an aphrodisiac, antidiabetic, anti-carcinogenic, and antimicrobial agent [1-3]. The toxic potentials of *M. acuminata* stem have not been given much attention, despite its use varied in folk medicine. Recent studies have focused on both pharmacology and toxicity of medicinal plants used by humans [4, 5]. This is very important in order to achieve a safe treatment with plant products [6].
Herbal aphrodisiac drugs from *M. acuminata* have been branded and marketed. Studies have substantiated the plant's aphrodisiac and antimicrobial properties [7-9]. Oriola *et al*. [10] and Adamu *et al*. [11], have respectively reported the isolation of phenolic glycosides and a triterpenoid saponin from the leaves and root bark of *M. acuminata*. Therefore, the paucity of scientific validation of the antidiabetic efficacy of *M. acuminata* stem based on folkloric claims, in addition to its safe dose and antioxidant capacity necessitates this study.

2. Materials and Methods
2.1 Plant sample
M. acuminata stems were collected from a forest in Ini Local Government Area, Akwa Ibom State, Nigeria. The plant was authenticated by a taxonomist in the Department of Botany and Ecological Studies, University of Uyo, Nigeria (voucher No. 11397). The stems were cut in pieces, air-died, pulverized, and macerated in ethanol (70%). The extraction process was repeated to obtain a good yield. The extract was concentrated through vacuum using rotary evaporator at 40 °C, and the final yield was 7.12 g.

2.2 Phytochemical evaluation
Standard methods for phytochemical screening of alkaloids, flavonoids, saponins, tannins, cardiac glycosides and anthraquinones were used to evaluate the stem extract of *M. acuminata*. Alkaloids determination was done using Mayer's and Dragendoff's reagents; the persistent frothing and sodium bicarbonate tests were used for saponins; Raymond's test, Legal test, and Killer Killiani test were employed to evaluate the presence of cardiac glycosides; alkaline reagent test for flavonoids; ferric chloride and lead acetate tests for tannins; Liebermann-Burchard test for terpenoids, and Borntrager's test for the presence of anthraquinones [12-14].

2.3 Brine shrimp lethality assay
The cytotoxic activity of *M. acuminata* extract and fractions were evaluated using brine

shrimp lethality assay where 3 graded doses (10, 100 and 1 000 µg/mL) were employed. The assay was done according to the procedure of Carballo *et al*. [15] with some modifications. Brine shrimps (*Artemia salina*) were hatched from its eggs with properly aerated filtered seawater for 48h. Ten active nauplii were drawn through a dropper and placed in Petri dishes containing 5mL of seawater and 5mL of each extract dilution. The control for this assay was 10mL of seawater containing 10 brine shrimps. The numbers of survivors were counted after 24h. The experiment was performed in triplicate and mean results noted. The percentage of mortality was then determined. The LC_{50} of the test samples after 24 h was determined by a plot of the percentage of the dead nauplii against the logarithm of sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis.

2.4 Animals

The animals (Swiss albino rats and mice) of both sexes were obtained from University of Uyo animal house. The animals were housed in standard cages and were maintained on a standard pelleted feed (Guinea Feed) and water *ad libitum*. All animal treatments were strictly in accordance with international ethical guidelines concerning the care and use of laboratory animals and all the experiments were carried out under the approval of the ethical committee of the University of Uyo. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U. S. National Institutes of Health (NIH Publication No. 85-23, revised 1996)" for studies involving experimental animals and the procedures as documented by Kilkenny *et al*. [16] for reporting animal research.

2.5 Determination of median lethal dose (LD_{50})

The LD_{50} of the extract was determined using albino mice. The extract was administered intraperitoneally (i.p.). This involved the administration of different doses of the extract (100, 125 and 150 mg/kg) to groups of three mice each. The animals were observed for physical manifestation of signs of toxicity. The number of deaths in each group within 24 h was recorded [17].

2.6 Induction of diabetes

The animals (male rats) were fasted for 24 h and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of alloxan monohydrate (140 mg/kg) in ice cold 0.9% saline (NaCl) solution. The animals were given 2 mL of 5% dextrose solution using orogastric tube immediately after induction to overcome the drug induced hypoglycemia. Seventy two hours later, rats with blood glucose levels (BGLs) above 200 mg/dL were considered diabetic and selected for the experiment.

2.7 Evaluation of anti-diabetic activity

The animals were randomly divided into five groups of five rats each and treated as follows:

Group A: diabetic rats were given normal saline (10 mg/Kg/day) for 14 days

Group B: diabetic rats received 5 mg/kg of glibenclamide daily for 14 days

Group C: diabetic rats were administered with 44.72 mg/kg of *M. accuminata* daily for 14 days.

Group D: diabetic rats given 67.08 mg/kg of *M. accuminata* daily for 14 days.

Group E: Diabetic rats were administered 89.44 mg/kg of *M. accuminata* daily for 14 days.

The fasting BGLs of all the rats were recorded at regular intervals during the experimental period. For acute study, the BGLs were monitored after 1, 2, 3, 5 and 7 h of administration of a single dose of the extract and at the end of 1, 7 and 14 days for prolonged treatments. The BGLs were monitored in the blood of the diabetic rats by tail tipping method. The blood was dropped on the dextrostix reagent pad. This was inserted into microprocessor digital blood glucometer and the readings were recorded.

2.8 DPPH radical scavenging activity

The DPPH free radical scavenging of *M. acuminata* extract, fractions and ascorbic acid prepared in methanol at various concentrations (20–100 µg/mL) were evaluated according to the method of Shekhar and Anju [18]. One millilitre of 0.1 mM DPPH solution in methanol was added to 3 mL the solutions prepared with the extract and standard, and stirred for 1 min. Each mixture was kept in the dark at room temperature for 30 min and the absorbance was recorded against a blank at 517 nm. The assays were carried out in triplicate and the results were expressed as mean values ± standard error of mean. Lower absorbance of the reaction mixture indicated higher free radical activity. Percentage scavenging activity was calculated using the expression:

$$\% \text{ scavenging activity} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.9 Ferric reducing antioxidant power assay

The reducing power of the extract, fractions, and ascorbic acid were determined according to the method of Oyaizu [19]. Various concentrations (20-100 µg/mL) of extract, fractions and ascorbic acid were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% (w/v) of potassium ferricyanide water solution (2.5 mL). The mixture was incubated at 50 °C for 20 min. Aliquots of trichloroacetic acid [2.5 mL, 10%, aqueous solution (w/v)] were added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride solution [0.5 mL, 0.1% (w/v)]. After 30 min of incubation at room temperature in the dark, the absorbance of the solution was measured at 700 nm. The experiment was performed in triplicate and the average absorbance was noted for each measurement. Higher absorbance indicated higher reducing power. The ferric-reducing capacity of the extract and standard compound were expressed graphically by plotting the absorbance against concentration.

2.10 Statistical analysis

Data are reported as mean ± SEM and were analyzed statistically using one way ANOVA followed by Tukey-kramer multiple comparison. The significance of the difference between the mean of the control and that of the treated groups was considered at $p<0.05$.

3. Results & Discussion

Phytochemical analysis revealed the presence of saponins, alkaloids, and cardiac glycosides in high amount (Table 1). Similarly, Yakubu *et al*. [7] reported high contents of saponins and alkaloids in *M. acuminata* stem. This endowed rich saponin content of *M. acuminata* stem has been is exploited medicinally and for its use as chewing stick [3].

The fractions of *M. acuminata* were evaluated for brine shrimp lethality at different concentrations (Table 2). LC_{50} value for extract, dichloromethane, ethyl acetate, butanol and aqueous fractions, and standard compound, etoposide, were 2.7667, 21.538, 3.540, 1.255, 1.8495, and 7.4625 µg/mL respectively. The extract and fractions exhibited positive lethality to brine shrimps than etoposide (10, 100 and 1000 µg/mL), except the dichloromethane fraction. Similarly, the LD_{50} (111.8 mg/Kg) of *M. acuminata* extract was established by intraperitoneal administration using albino mice (Table 3). The LD_{50} results showed that the mice could not tolerate considerably high doses of the crude extracts. According to Lorke [17], 10% and 20% of LD_{50} are adopted as effective doses. As a result, the minimum effective doses (11.18 and 22.36 mg/kg body weight) of *M. acuminata* stem extracts may be safe. Yakubu and Babasoji [5], showed that doses of 250-1000 mg/Kg of *M. acuminata* aqueous stem extract hampered the normal functioning of the liver of male rats, but lower doses were safe. The established toxicity levels of *M. acuminata* stem may be a consequence of its high saponins content. This raises a concern on its safety and implications for its use as medicines. An agent that produces adverse effect in experimental animal studies is assumed to pose a similar threat to humans [20].

A dose-dependent reduction in blood glucose levels (BGLs) was observed in alloxan-induced diabetic rats treated with ethanol stem extract of *M. acuminata*. After a single dose of the extract was administered to the alloxan-induced diabetic rats, there was a significant ($P<0.05$) reduction in BGLs of the diabetic rats within the period of acute study compared to the control group (Table 4). The maximum effect was observed at 7 h where the extract (89.44 mg/Kg) exerted a comparable effect with glibenclamide, as evident by the percent reduction in BGLs of diabetic rats (45.6% and 46.5% respectively) compared to control. During the prolonged study (14 days), the extract produced a sustained significant ($P<0.05$) reduction in BGLs of the diabetic rats compared to control (Table 5). The hypoglycemic effect of the highest dose of the extract was relatively less than that of glibenclamide, on day 14 (percent reduction in BGLs of 61.7% and 74.29% respectively). The observed biological effect of *M. acuminata* extract on these diabetic rats may be due to its major alkaloid and saponins components. However, a synergy with some minor and main constituents cannot be excluded.

The DPPH radical scavenging activities and ferric reducing power of *M. acuminata* extract and fractions are depicted in Figure 1 and 2 respectively. The plot in Figure 1 indicates the scavenging ability as percent inhibition at various concentrations; the scavenging effect was concentration dependent. This was demonstrated by the ability of constituents inherent in both extract and fractions to act as hydrogen atoms or electrons donor in the conversion of the stable purple coloured DPPH to the reduced yellow coloured DPPH-H. Ascorbic acid (100 µg/mL) showed the highest percent inhibition (83.7%), followed by the extract (63.3%), ethyl acetate fraction (57.7%) and butanol fraction (46.2%). The relative low antiradical scavenging activity of *M. acuminata* extract compared to ascorbic acid may be attributed to the deficiency of phenolic compounds, such as flavonoids, tannins, and anthraquinones in the extract (Table 1).

In Figure 2, the FRAP assay indicated the reducing potential of the antioxidants in the extracts and fractions against the oxidative consequences of reactive oxygen species. The reducing potential (as recorded by the absorbance values at 700 nm) of ascorbic acid (0.7) doubled that of the extract (0.386), though ethyl acetate fraction (0.44) demonstrated better reducing effects compared with the butanol fraction (0.384) and ethanol extract at 100 µg/ml. This implicates the plant constituents' ability to reduce the (Fe^{3+}) to (Fe^{2+}) by electron transfer.

3.1 Tables

Table 1: Phytochemical analysis of *M. acuminata* stem

Test	Intensity
Saponins	+++
Tannins	+
Flavonoids	-
Cardiac Glycosides	+++
Alkaloids	+++
Anthraquinones	+

+++ = High Concentration; ++ = Moderate intensity; + = Trace element; - = Not detected

Table 2: Cytotoxicity of extract and fractions of *M. acuminata* stem on brine shrimp nauplii

M. acuminata	Concentration (µg/mL)	No. of brine shrimps	No. of survivors	Mortality (%)
Extract	10	30	10	66.7
	100	30	07	76.7
	1000	30	05	83.3
Dichloromethane fraction	10	30	17	43.3
	100	30	16	46.7
	1000	30	11	63.3
Ethyl acetate fraction	10	30	11	63.3
	100	30	09	70.0
	1000	30	02	93.3
Butanol fraction	10	30	13	56.7
	100	30	09	70.0
	1000	30	08	73.3
Aqueous fraction	10	30	12	60.0
	100	30	11	63.3
	1000	30	05	83.3

Table 3: Acute toxicity of *M. acuminata* hydro-ethanol stem extract

Dose (mg/kg)	Animals per group	Outcome
100	3	All survived
125	3	All Dead
150	3	All Dead

Table 4: Antidiabetic effects of *M. acuminata* on blood glucose level of alloxan-induced diabetic rats (acute study)

		Blood glucose level (mg/dL) in hours					
Treat-ments	Dose (mg/kg)	0 h	1 h	2 h	3 h	5 h	7 h
Control	10 mL	366.0±1.24	373.4±3.14	383.0±2.05	382.2±4.9	363.4±2.65	384.0±1.30
Glib.	5 mL	318±484 (13.11%)	242±4.79 (35.19%)	221±6.43 (42.30%)	215±8.63 (43.75%)	214±7.27 (41.11%)	206±4.69 (46.35%)
Extract	44.72	324.6±4.61 (11.31%)	262±4.93 (29.62%)	240.2±5.11 (37.28)	233.8±5.27 (38.83%)	256.2±5.94 (29.50%)	242.4±5.02 (36.88%)
	67.08	346.2±4.02 (5.41%)	284.4±5.31 (23.84%)	233.4±7.51 (39.06%)	229.2±79 (40.03%)	236.2±3.35 (35.0%)	222.8±4.55 (41.98%)
	89.44	275±3.56 (24.86%)	244.8±5.57 (34.44%)	224.2±2.39 (41.46%)	216.8±6.87 (43.31%)	213±7.92 (41.39%)	208.8±5.15 (45.63%)

Value in parentheses: Percent reduction of BGLs of diabetic rats compared to control; Glib: Glibenclamide.

Table 5: Antidiabetic effects of *M. acuminata* on blood glucose level of alloxan-induced diabetic rats (prolong treatment)

		Blood glucose level (mg/dL) in days			
Treatments	**Dose (mg/kg)**	**Day 0**	**Day 1**	**Day 7**	**Day 14**
Control	10 mL	366.0±1.24	410.4±8.63	395.0±2.86	364.8±1.77
Glib.	5 mL	318±484 (13.11%)	198±3.50 (51.75%)	127.0±4.112 (67.85%)	93.8±2.13 (74.29%)
Extract	44.72	324.6±4.61 (11.31%)	225.4±7.51 (45.08%)	189.2±7.79 (52.10%)	172.4±7.20 (52.74%)
	67.08	346.2±4.02 (5.41%)	220.4±4.58 (41.30%)	189.2±7.79 (52.10%)	157.8±7.29 (56.7%)
	89.44	275±3.56 (24.86%)	207±4.83 (49.56%)	173.2±4.89 (56.15%)	139.6±3.35 (61.73%)

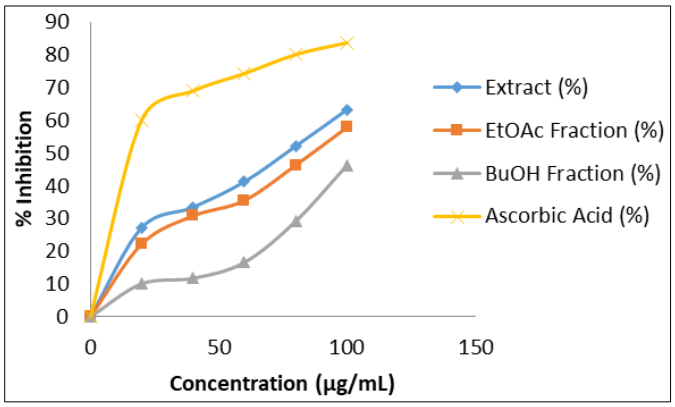

Fig 1: DPPH radical scavenging activity of *M. acuminata* stem

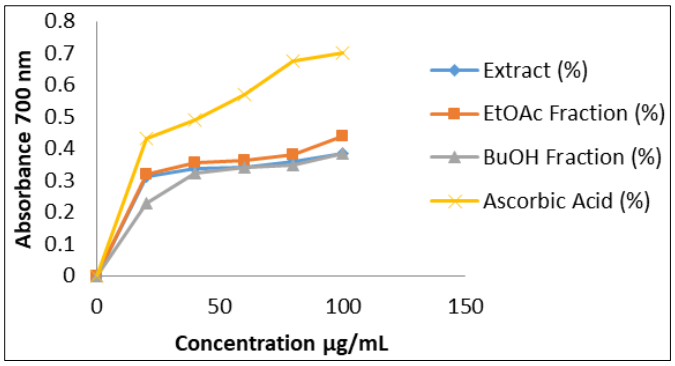

Fig 2: Ferric reducing antioxidant power assay of *M. acuminata* stem

4. Conclusions

This study substantiates the use of *M. acuminata* stem in the traditional management of diabetes, however the toxicity of this plant is of immense concern as evident by relatively low LC_{50} and LD_{50} values, in both *in vitro* and *in vivo* studies.

5. Acknowledgement

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6. Animal right ethics

All experimental procedures involving animals were conducted in accordance with international ethical guidelines concerning the care and use of laboratory animals and all the experiments were carried out under the approval of the ethical committee of the University of Uyo, Nigeria.

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