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Research Paper

Pharmacognostic profile and antidiabetic activity of methanol extract of *Annonamuricata* root in mice.

OkechukwuOluoma Chinecherem[1], Ifebi Hope Morris Nchekwube[*1], Ezea Charity Chinasa[1], Ogbue Cyril Onyeka[1], Agwunobi Stella Chisom[2]and Eze Petra Somkenechkwu[2]

1. *Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, NnamdiAzikiwe University Awka, Anambra State*
2. *Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka.*

ABSTRACT
Diabetes and its related complications remain a major clinical problem. There is current interest in the search for novel antidiabetic agents from medicinal plants Annonamuricatais used in folkloric medicine for the management of diabetes but its antidiabetic potentials has not yet been validated.
This study investigated thepharmacognosticparameters, phytochemical constituents andantidiabetic potentials ofA. muricata root. The plant material was cold-macerated in methanol to obtain the crude extract,Pharmacognostic profile, phytochemical analyses, and acute toxicity were determined using standard procedure. The hypoglycemic activity was studied in normoglycemic rats.Theantidiabetic activities of the extract were also determined usingalloxan induced diabetic mice model. Diabetes was induced using 50mg/kg alloxan. The crude extracts were administered (p.o.) daily at the dose of 100, 200, and 400 mg/kg for 14 days. The fasting blood glucose was monitored for 7 days. Glibenclamide was used as a reference drug.
The microscopic examination revealed the presence of epidermis, trichomes and starch grains. Acute toxicity test in rats gave an LD_{50} of 5000 mg/kg. Results show that the extract produced a dose-dependent significant ($p < 0.05$) lowering of the fasting blood glucose of the diabetic mice after 7 days, normalizing the fasting blood glucose within the periods.The phytochemical analysis revealed the presence of alkaloids, saponins, flavonoidsand terpenoids.
It has been demonstrated from the study, that the rootofA. muricatapossess antidiabetic activity which may be due to phytochemicals present which supports its ethnomedicinal use in diabetes management.
KEYWORDS: *Glibenclamide, Diabetes mellitus, Alloxan monohydrate, Annonamuricata, Crude extracts*

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I. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease caused by an absolute or relative lack of insulin and or reduced insulin activity, which results in hyperglycemia and abnormalities in carbohydrate, protein and fat metabolism. Despite the great efforts that have been made in the understanding and management of DM, the incidence of the disease has been increasing unabated [1, 2]. Currently, there is a growing interest in herbal remedies due to the side effects associated with the oral hypoglycemic agents for the treatment of DM [3]. Marles and Farnsworth [4] estimated that more than 1000 plant species are being used as folk medicine for DM. The hypoglycemic properties of these plants and indeed the medicinal properties of plants used by traditional medical practitioners may be due to one or more of the many arrays of chemical constituents of the plant material. These include complex carbohydrates, alkaloids, glycopeptides, peptides and amines, tannins, cyanogens, terpenoids, steroids, flavonoids, lipids, coumarins, inorganic ions, sulphur compounds, among others.

The World Health Organization (WHO) has recently recommended the use of medicinal plants for the management of DM and further encouraged the expansion of the frontiers of scientific evaluation of hypoglycemic properties of diverse plant species (Dirks , 2004). Current estimates has showed that over 70%

of the global population applies resources derived from traditional medicine for the management and abation of diabetes mellitus and its complications [5].

*A. muricata*is an evergreen, terrestrial, erect tree reaching 5–8 m in height and features an open, roundish canopy with large, glossy, dark green leaves. The edible fruits of the tree are large, heart-shaped and green in color, and the diameter varies between 15 and 20 cm [6]. Extensive phytochemical evaluations on different parts of the *A. muricata* plant have shown the presence of various phytoconstituents and compounds, including alkaloids[7], megastigmanes[8],flavonoltriglycosides[9], phenolics[10],cyclopeptides and essential oils [11]. The crushed seeds are believed to have anthelmintic activities against external and internal worms and parasites. In tropical Africa, the plant is used as an astringent, insecticide and piscicide agent and to treat coughs, pain and skin diseases. In India, the fruit and flower are employed as remedies against catarrh, while the root-bark and leaves are believed to have antiphlogistic and anthelmintic activities [12]. In Malaysia, the crushed leaf mixture of*A. muricata* together with *A. squamosa* and Hibiscus rosa-sinensis is used as a juice on the head to protect against fainting [13]. In South America and tropical Africa, including Nigeria, leaves of *A. muricata* are deployed as an ethnomedicine against tumors and cancer [12]. In addition, the anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive and antispasmodic effects are also attributed to the leaves, barks and roots of *A. muricata*[14]. The crushed seeds are believed to have anthelmintic activities against external and internal worms and parasites. In tropical Africa, the plant is used as an astringent, insecticide and piscicide agent and to treat coughs, pain and skin diseases. In India, the fruit and flower are employed as remedies against catarrh, while the root-bark and leaves are believed to have antiphlogistic and anthelmintic activities [12]. In Malaysia, the crushed leaf mixture of *A. muricata* together with *A. squamosa* and Hibiscus rosa-sinensis is used as a juice on the head to protect against fainting[13]. In South America and tropical Africa, including Nigeria, leaves of*A. muricata* are deployed as an ethnomedicine against tumors and cancer [12]. In addition, the anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive and antispasmodic effects are also attributed to the leaves, barks and roots of *A. muricata*[14]. In addition to ethnomedicinal uses, the fruits are widely employed for the preparation of beverages, candy, ice creams, shakes and syrups [15].Nwokocha*et al* [16] reported the evaluation of the antihypertensive properties of *A. muricata* leaves.

The current study reports the anti-diabetic effects of methanol root extract of A. muricataon alloxan induced diabetic mice. This is, to the best of our knowledge, the first report of the antidiabetic activity of the root of this plant.

II. MATERIALS AND METHODS

2.1 Plant material

Fresh roots of *A, muricata* were collected from Abagana town in Njikoka Local Government Area, Anambra State. The plant was identified and authenticated by Mr. A. Ozioko, a taxonomist with the International Centre for Drug Discovery and Development (InterCEDDNsukka). The voucher specimen (PCG/529/B/033) has been deposited in our laboratory. Fresh roots were collected in bulk, shade dried and pulverized in a mechanical grinder to obtain fine powder.

2.2 Preperation of the plant extract

Five hundred grams (500 g) of dried and pulverized leaves were macerated twice in 2.5 L of 95% methanol for 48 h with intermittent shaking. Filtrations were done using cheese-cloth and then Whatman No. 1 filter paper. The combined filtrate was concentrated using a vacuum evaporator at 40 ºC to afford the crude methanol extract (crude extract) which was stored in the refrigerator and used for the experiments.

2.3 Physico-chemical analysis

The physico-chemical analysis of the plant was carried out using standard methods. The total ash, acid insoluble ash, water soluble ash, sulphated ash , alcohol soluble extractive , water soluble extractive and moisture content of the samples were carried out in triplicates according to standard methods [17].

2.4 Phytochemical analysis

The phytochemical screening was carried out on the crude extract and fractions of *A. murica* leaves according to standard methods to identify the classes of bioactive compounds present [18,19].

2.5 Experimental Animals

Wistar albino mice (either male or female, i.e without consideration of the sex) weighing between 16 – 33.1 g were purchased from the Animal House of Pharmacology and Toxicology of NnamdiAzikiwe University, Awka,. The animals were housed in standard animal cages, fed with commercial feed and water *ad libitum*.

Acclimatization was for seven days. The care and handling of animals was in line with the National Institute of Health Guide for care and use of laboratory Animals (Pub No. 85-23 revised)

2.6 Chemicals

Alloxan monohydrate was manufactured by Sigma, while Glibenclamide was from NGC. All other chemicals used in our study were of analytical grade. All laboratory reagents were freshly prepared and freshly distilled water was used when required.

2.7 Acute-toxicity and lethality (LD_{50}) test

Acute toxicity analysis of the extracts was performed using Lorke's method. This method has two phases (Phase 1 and Phase 2).

Phase 1: Nine adult albino mice were weighed, marked and randomized into three groups of three mice each. Each group of animals were administered different doses (10, 100 and 1000 mg/kg) of the extracts. The mice were observed for 24 hours for signs of toxicity as well as mortality.

Phase 2: Four mice were weighed, marked and randomized into four groups of one mouse each. Dose selection was based on result obtained in Phase 1. Observation for 24 hours for obvious signs of toxicity and death was recorded accordingly. The LD_{50} was calculated using the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced mortality.

2.8 Anitdiabeticstudy [20]

Hypoglycemic study in normoglycemic mice

The acclimatized rats were kept fasting overnight for 12 hrs before the experiment with water ad libitum. Twenty five normoglycemic rats were randomized into five groups (n = 5) and treated according to the following experimental protocols:

Group 1: 10 ml/kg Distilled water (control),
Group 2: 5mg/kg Glibenclamide
Group 3: 100 mg/kg of crude extract
Group 4: 200 mg/kg of crude extract,
Group 5: 400 mg/kg of crude extract

Blood sample was withdrawn from the tail vein and fasting blood glucose (FBG) was measured with a glucometer (One touch) at 0, 1, 3, 6 and 9 hrs.

Antidiabeticstudy onalloxan induced diabetic mice

Twenty five (25) albino mice were fasted for 12 h prior to induction of hyperglycemia. Diabetes was induced using freshly prepared solution of alloxan monohydrate (i.p. at 120 mg/kg body weight) as previously reported . After the induction, the diabetic animals were randomly divided into seven groups (n = 5) and treated orally according to the following protocols:

Group 1: 10ml/kg distilled water
Group 2: 5mg/kg glibenclamide
Group 3: 100mg/kg crude extract
Group 4: 200mg/kg crude extract
Group 5: 400mg/kg crude extract

Treatment was done for 7 days. The fasting blood glucose (FBG) levels were determined on days 0, 3, and 7, and the percentage reduction in the fasting blood glucose (FBG) concentration calculated for each of the treatment groups.

2.9 Statistical analysis

Data obtained were subjected to one way analysis of variance (ANOVA) for determining the significant difference using the SPSS software (version 21). The significance between the various groups and the control was analyzed by post hoc using Dunnett t-test. A probability p value < 0.05 was considered to be significant. Results are expressed as mean ± SEM.

III. RESULTS

Result of the macroscopic and microscopic evaluation of the root of *A. muricata*

Macroscopic evaluation of the root of *A, muricata* revealed the presence of useful features that will be used to differentiate it from other plants. The macroscopic evaluation revealed that the root is dark brown in colour, brittle, sour taste, odourless, rough texture with a tap root of about 4m long. The microscopic analysis of root of *A.muricata* showed the presence of fragmentnts of bordered pitted vessels and epidermis, medullary ray, starch granules,prism shappedcalcium oxalate crystals,tricomes and fibres associated with a bordered pitted vessel (Figure)

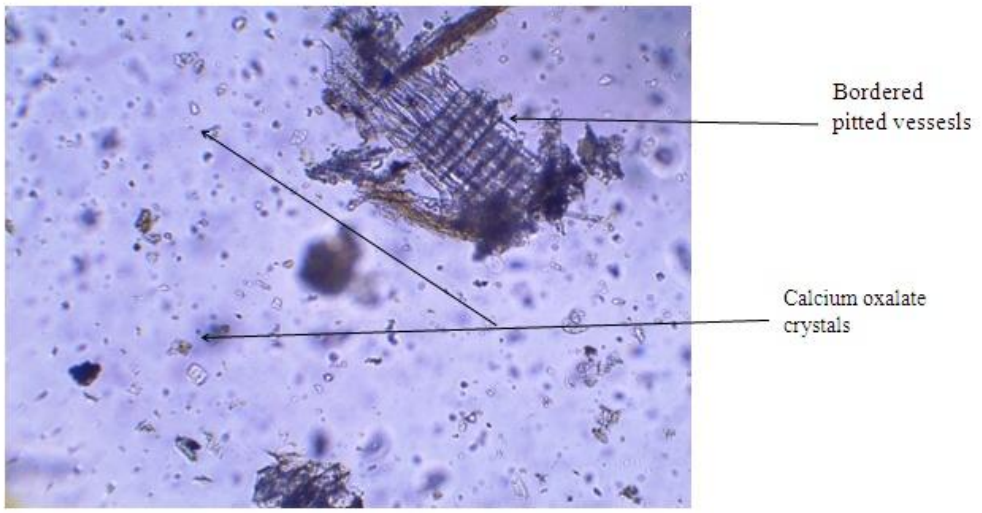

Figure 1: Chemomicrograph of the root showing bordered pitted vessels and calcium oxalate crystals.

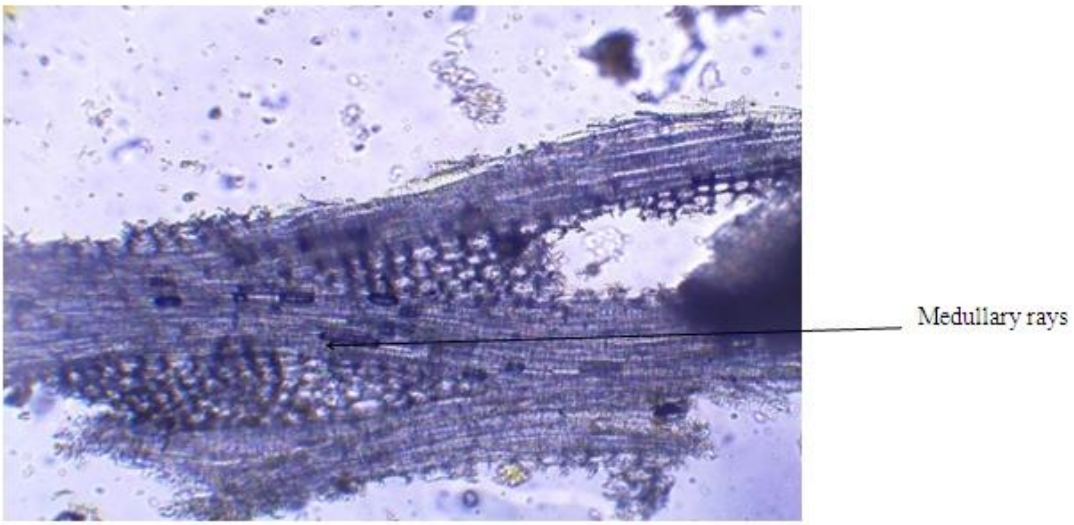

Figure 2: Chemomicrograph of the root showing medullary rays

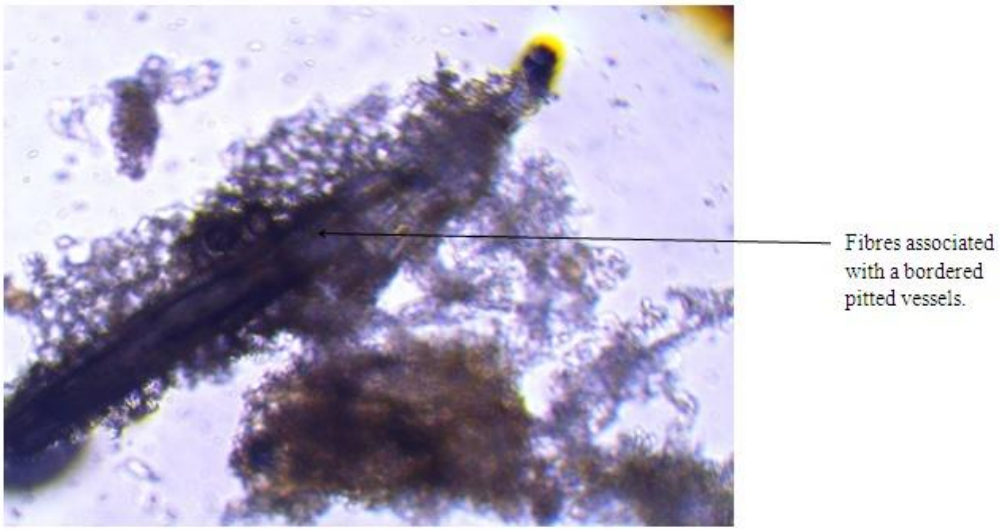

Figure 3: Chemomicrograph of the root showing Fibres associated with a bordered pitted vessel.

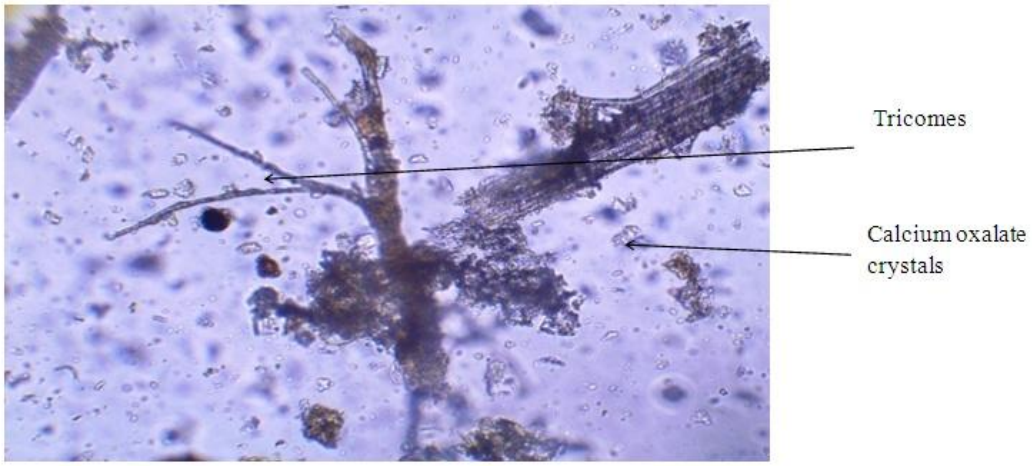

Figure 4: Chemomicrograph of the root showing epidermis with tricome and calcium oxalate crystals.

Figure 5: Chemomicrograph of the root showing group of fibres and starch granules.

Results of Physico-chemical analysis of the root of *A. muricata*

The proximate composition of the root of *A. muricata* showed the percentage composition of the total ash, water soluble ash, acid-insoluble ash, Sulphated ash , Alcohol soluble extractive , Water soluble extractive and moisture content as presented in Table 1. The total ash obtained was 11.5% and its used as a measure of purity. Moisture content was 10.0% and it's used as a measure of stability.

Table 1: Results of proximate composition of the root of *A. muricata*

Physico-chemical analysis	**Composition(%)**
Total Ash	11.5
Sulphated ash	8.7
Alcohol soluble extractive	9.1
Water soluble extractive	12.0
Acid Insoluble Ash	7.5
Water Soluble Ash	9.5
Moisture Content	10.0

Results are expressed as mean ± SEM (n = 3).

Result of phytochemical analysis of *A.muricata root*.

The phytochemical analysis revealed that the crude extract is rich inflavonoids, proteins, terpenoids, alkaloids and cardiac glycosides. The result of the phytochemical analysis is presented in Table 2 below.

Table 2: Result of the Phytochemical analysis of *A. muricata*

S/N	PHYTOCHEMICAL	CRUDE EXTRACT
1	Alkaloids	++
2	Saponins	+
3	Steroids	-
4	Tannins	+
5	Flavonoids	++
6	Terpenoids	++
7	Cardiac glycosides	++
8	Proteins	+
9	Reducing sugars	-

(-) = Not Present.
(+) = Present in small concentration.
(++) = Present in moderately high concentration.
(+++) = Present in high concentration.

Result of Acute toxicity test

In the acute toxicity and lethality test, results (Table 3) in mice gave an $LD_{50}$of $\geq$ 5000 mg/kg.

Table 3: Results of acute toxicity (LD_{50}) test

PHASE ONE	DOSE (Mg/kg)	Mortality
	10 100 1000	0/3 0/3 0/3
PHASE TWO		
	1600 2900 5000	0/1 0/1 0/1

Result of antidiabetic study

The normoglycemic mice which received different doses of the crude extract (100 mg/kg, 200 mg/kg and 400mg/kg) and glibenclamide showed no significant ($p>0.05$) reduction in their glycemic levels after 9 hours when compared with the positive control group that received glibenclamide 5 mg/kg. The results are shown in Table 4 below:

The crude extract of *A. muricata*in studied doses (100, 200 and 400 mg/kg) showed a significant ($p<0.05$, $p<0.01$) reduction of the blood glucose levels in 3 and 7 days when compared to the control group (distilled water 10 ml\kg and glibenclamide 5 mg/kg). The blood glucose level was normalized by 200 and 400mg/kg of crude extract in 7 days with a percentage glucose reduction of 52.18 and 68.31 % respectively. The results of the effect of crude extract on blood glucose level of alloxan-nicotinamide induced diabetic mice are shown in Table 5 below.

Table 4: Effect of A. muricata root on the fasting blood glucose of normoglycemic rats

Treatment	Basal (mg/dl)	1hr (mg/dl)	3hrs (mg/dl)	6hrs (mg/dl)	9hrs (mg/dl)
10ml/kg D/w	58.75± ±3.27	69.00 ±4.00ns	62.00 ±5.02ns	57.82 ±4.12	52.60 ±3.79
5mg/kg Glibenclamide	98.50 ±6.30	82.57 ±6.25ns	91.50 ±3.09ns	81.50 ±3.22	90.75 ±7.30
100mg/kg Crude extract	105.55 ±4.20	91.00 ±3.89ns	92.17 ±3.19ns	88.05 ±3.05ns	94.27 ±3.03
200mg/kg Crude extract	79.00 ±4.00	74.02 ±3.216ns	61.45 ±7.91ns	73.62 ±1.46	69.00 ±0.04
400mg/kg Crude extract	102.52 ±8.00	98.60 ±7.85ns	89.97 ±5.28ns	95.02 ±9.93ns	99.62 ±2.15

Results are expressed as mean ± SEM (n=4). *$p < 0.05$ as compared with control group.

Table 5: Effect of A. muricata root on alloxan-induced diabetic mice

Groups	Treatment	Basal (mg/dl)	0hr (mg/dl) (Hyperglycaemia)	Day 3 (mg/dl)	Day 7 (mg/dl)
1	10ml/kg Distilled water	79.77± 5.40	178.70±8.29	175.67±4.26^{ns} (1.68%)	183.20±6.81^{ns} (0.00 %)
2	5mg/kg Glibenclamide	75.58±3.92	138.43±21.18	68±40.62* (50.83%)	49.00±1.73** (64.57%)
3	100mg/kg Crude extract	63.21±3.92	121.13±5.20	102.90±7.51^{ns} (15.70%)	73.62±5.49* (39.10%)
4	200mg/kg Crude extract	75.01±2.74	140.71±17.61	110.45±18.35^{ns} (21.32%)	67.43±7.67* *(52.18%)
5	400mg/kg Crude extract	70.20±3.34	194.30±15.12	139±54.48** (28.62%)	61.61±2.01** (68.31%)

Results are expressed as mean ± SEM (n = 5). *$p < 0.05$, ** $p < 0.01$, as compared with control group.Figures in parenthesis represents percentage reduction of fasting blood glucose from 0 hr (Hyperglycaemia0.

IV. DISCUSSION

Macroscopic analysis of plants is useful to discriminate morphologically similar plant and also to distinguish between the desired plant species and plant part in the field during the plant sampling. The macroscopic examination of *Annonamuricata*revealed the physical appearance ofthe root..This morphological features of the root are seen with the naked eyes, taste and smell. This gives an idea of the plant but cannot be relied on solely to identify the plant. Alternatively, Microscopic approach also utilizes techniques such as light microscopy to analyze characteristics such as the presence or absence of particular cell types will help to distinguish between the desired plant species and plant part at ultrastructural level. Microscopic and macroscopic methods are also useful in assisting the pharmacologist to gain a standard. Thus, the microscopic and macroscopic evaluation of *Annonamuricata*root will serve as diagnostic tools for its differentiation from other plants and will also help in detection of adulteration with related species.

Ash value determinations furnish a basis for judging the identity, quality, purity and cleanliness of a sample compound and it also gives information relative to its adulteration with inorganic matter. There are limits for variations in total ash or the acid insoluble ash values present in a drug.

The total ash obtained was 11.5 % and this measures the total amount of ash remaining after ignition. This includes both 'physiological ash' which is derived from the plant tissue itself, and 'non-physiological ash [21]. The water soluble portion of the total ash is referred to as water soluble ash. The water soluble ash obtained was as low as 9.5 % which implies minimal portion of total ash constituents were soluble in water. The determination of water-soluble or alcohol-soluble extractive value is used as means of evaluating drugs, the constituents of which are not readily estimated by other means and these extractive values are highly required as pharmacopoeial standards [22].The sulphated ash value indicates the presence of non-volatilized residual substance and was obtained as 8.7 %. The moisture content is not too high (10 0%), it falls within the limit of the general requirement of 8-14% indicating less of microbial degradation [23].

Plants having anitdiabetic activity have been ascertained to be rich in alkaloids, flavonoids, terpenoids, steroids and saponins[24] which are known to be bioactive against diabetes. They (phytochemicals) are also considered as a class of hypoglycemic molecules that may act in part by inhibiting glycogen phosphorylase, a key enzyme in glycogen hydrolysis [25]. Saponins possess hypoglycemic activity, which may be due to inhibition of liver glycogen hydrolysis [26], and may contribute to the observation of the hypoglycemic activity

of the plant extract. Flavonoids, tannins and saponins have also been reported by Tiwari and Rao[27] to possess hypoglycemic properties via an inhibitory action on the sodium glucose transporter 1 (S-GLUT1). Phytochemical screening of the extract of *Annonamuricata* showed the presence of various chemical constituents mostly alkaloid, flavonoids, saponin, terpenoid, tannin, cardiac glycosides, and proteins. Cardiac glycosides, flavonoids and alkaloids were present in a moderately high amount in the extract. All these have potential health promoting effect, at least under some circumstances [28]. The literature reports reveal that flavonoids and terpenoids present in the plant extract is known to possess antidiabetic activity [29]. Among this bioactive component, highest concentration of flavonoids were found and thought to be the chief ingredient helping to produce antidiabetic activity [30]Flavonoids have also been reported to suppress glucose level significantly and the typical flavonoid, luteolin, has been found to be a strong inhibitor of α-glucosidase[31]. Alkaloids have also been severally reported to have antidiabetic activity. For example, alkaloids isolated from *Catharanthusroseus* leaves have shown to induced antidiabetic and antioxidant properties in mouse pancreatic β-cells [32]. Generally alkaloids have been said to inhibit α-glucosidase and decrease glucose transport through the intestinal epithelium [24,33]. Saponin containing Kalopanaxpictus has been shown to exhibit hypoglycemic activity in streptozotocin-induced diabetic rats. In other studies, saponins have been shown to lower blood glucose levels in elderly diabetic patients [34].

Acute toxicity studies through oral administration of the aqueous leaf extracts of *S. liberica* in low and high doses (that is from 100 to 5000mg/kg body weight) did not produce significant changes in the animals behaviour, such as in breathing, cutaneous effect, sensory and nervous system responses or, on gastro-intestinal effects. No adverse effects were observed during the experimental period and no death occurred, which may indicate that the administration of the herb in the doses indicated presented little toxicity in the animals studied. The oral acute toxicity of the crude leaf extract of *A. muricata* calculated to be 5000 m/kg body weight According to expert recommendations, a chemical with a large LD_{50} ($\geq$5000 mg/kg) is practically non toxic and may not likely cause any toxicity on short term exposure [35].

The results of the present study have shown that the root of *A. muricata* do not have any significant effect on the blood glucose of normal animals.*Acanthopanaxsenticosus*saponins which were extracted from leaves when administered into mice (100, 200 mg/kg, intraperitoneally) decreased experimental hyperglycaemia without affecting plasma glucose levels in normal mice as reported by Sui *et al*[37]. This may have some beneficial clinical implications in normoglycaemic patients who may be taking the leaf extract for other medicinal purposes since the blood glucose would not likely be significantly reduced.

The study demonstrated that various doses of A. muricata (100, 200 and 400 mg/kg) showed antidiabetic effect against alloxan-induced diabetic animals. This shows that the extract contained the antihyperglycaemicconstituents. The crude extract showed a dose dependent significant ($p<0.01$) glucose level reduction with dose of 400mg/kg giving the highest activity . The crude extract of *A. muricata*at 100, 200 and 400 mg/kgdecreased glucose level by 39.10, 52.18 and 68.31% respectively after 7 days . The effect of the 400 mg/kg of the crude extract was comparable to that of the the reference drug (glibenclamide 5mg/kg) which reduced the blood glucose level by 64.57 %.The blood sugar decreasing effect of the *A. muricata* root extract may be associated with the presence of bioactive compounds such as flavonoids, phenols, alkaloids, saponins, and tannins that have been shown to have hypoglycemic activity [37].Thus bioactive compounds are effective antidiabetic drugs in preclinical and clinical studies [38].As reported by Ghule*et al* [39]flavonoids have been shown to have insulinomimetic effect hence stimulate lipogenesis as well as glucose transport in the adipocytes tissues hence decreasing the blood glucose level. Flavonoids stimulate transport of glucose in the adipocytes and lipogenesis, hence decreasing blood glucose. Flavonoid glycosides of Psidiumguajava such as strictinin, isostrictinin and pedunculagin manage diabetes by improving the sensitivity of insulin [40].

V. CONCLUSION

This study demonstrated that *A. muricata* posses hypoglycaemic effects in alloxan-induced diabetic mice, thus scientifically validating its continued use in the management of diabetes mellitus. The antidiabetic activity was due to cumulative effect of phytochemicals present in the plant extract including alkaloids, tannins, flavonoids, and saponins. However, further research should be done focusing on isolating the bio-active molecules responsible for the hypoglycaemic effect of thepant through bioassay guided fractionation.

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