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Evaluation of antidiabetic and anti-lipid peroxidation potentials of leaves crude and solvent fractions of *Annona muricata* Linn (Annonaceae)

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Abstract
Medicinal plants have been useful in treatment of various diseases in man and animals since time immemorial. *Annona muricata* has been known majorly for cancer management. Ethnopharmacological information revealed the use in treatment of diabetes and oxidative induce lipid peroxidation. Phytochemical screening revealed the presence of the presence of alkaloids, flavonoids, steroids, reducing sugars, and tannins. Total phenolic and flavonoid contents were estimated to be 68.54±0.51 and 55.82±0.85 respectively. Aqueous fraction showed the highest amount of phenolics and flavonoids (46.32±0.76 and 32.81±0.28) relative to chloroform fraction. However, aqueous fraction also showed the highest DPPH scavenging property in a concentration non-dependent manner. Moreover, crude extract and chloroform fraction exhibited the highest (concentration dependent) and lowest (concentration dependent) lipid peroxidation inhibition potentials. Glibenclamide (20 mg/kg), crude extract (500 mg/kg and 1000 mg/kg) and aqueous fraction (250 mg/kg and 500 mg/kg) produced a pronounced decrease in blood glucose in a dose dependent manner over time. No significant difference $P \geq 0.05$ was noted among the samples. In conclusion, crude extract of *A. muricata* exhibited excellent antioxidant, anti-lipid peroxidation using *in vitro* model as well reduced blood glucose level in albino rats in a manner similar to Glibenclamide (20 mg/kg) $P \geq 0.05$. Aqueous fraction was likewise noted to be the most active fraction for the bioassays examined.

Keywords: *Annona muricata*, diabetes, glibenclamide, lipid peroxidation, phytochemicals

1. Introduction
Natural products gotten from plants have been used to support man's existence in sustaining its health since the beginning of medicine. Over the past century, the phytochemicals in plants have been a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated significant scientific interest in the biological activities of these substances (Moghadamtousi *et al.*, 2015) [10]. Despite these studies, a restricted range of plant species has experienced detailed scientific inspection, and our knowledge is comparatively insufficient concerning their potential role in nature. Hence, the attainment of a reasonable perception of natural products necessitates comprehensive investigations on the biological activities of these plants and their key phytochemical (Moghadamtousi *et al.*, 2015) [10].

Diabetes mellitus (DM) describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (National Diabetes Data Group, 1979) [11]. Moreover, the effects of diabetes mellitus include long– term damage, dysfunction and failure of various organs (Charles *et al.*, 1996) [4].

Moreover, DM is a complex disease where the carbohydrate and fat metabolism is impaired (Altamer *et al*., 1991) [3]. Insulin affects many sites of mammalian lipid metabolism. It stimulates synthesis of fatty acid in liver adipose tissue and in the intestine. The insulin has also been reported to increase the cholesterol synthesis. The activity of lipoprotein lipase in white adipose is also increased. From this point of view, the assessment of various lipid fractions and lipid peroxide in the cases of Diabetes Mellitus may be of some help in the prognosis of patients and in preventing the possibilities of complications or secondary disorders (Jain and Gupta 1980) [6]. The occurrence of free radical induced lipid peroxidation causes considerable change in the cell membrane (Agrawal *et al*., 1985) [2]. Peroxidation of lipid membrane has been related to the pathogenesis of many degenerative diseases,

such as atherosclerosis, oxidative damage to DNA, aging, carcinogenesis, sickle cell disease and diabetes mellitus (Chattergee *et al.*, 1988) [5]. Thus, the lipid peroxide in the blood provides useful information for the prognosis of diabetes in which secondary disorders are often fatal (Tappel, 1973) [14].

Annona muricata commonly known as soursop, graviola, guanabana, pawpaw and sirsak, is a member of the Annonaceae family comprising approximately 130 genera and 2300 species (Mishra *et al.*, 2013; Leboeuf *et al.*, 1980) [9, 7]. *A. muricata* is native to the warmest tropical areas in South and North America and is now widely distributed throughout tropical and subtropical parts of the world, including India, Malaysia and Nigeria (Adewole and Caxton-Martins, 2006) [1]. It is used as natural medicine for arthritic pain, neuralgia, arthritis, diarrhea, dysentery, fever, malaria, parasites, rheumatism, skin rushes and worms, and it is also eaten to elevate a mother's milk after childbirth. The leaves are employed to treat cystitis, diabetes, headaches and insomnia. Moreover, internal administration of the leaf's decoction is believed to exhibit anti-rheumatic and neuralgic effects (Adewole and Caxton-Martins, 2006) [1].

The aim of this research is to evaluate the effect of methanol crude and solvent fractions of *A. muricata* on lipid peroxidation (using *in vitro* model), alloxan induced hyperglycemia, free radicals scavenging. This is expected to either scientifically justify or oppose the claim of usage of *A. muricata* in treatment of secondary disorder (lipid peroxidation) associated with diabetes.

2. Materials and Methods

2.1 Collection of Plants Materials

The leaves of *A. muricata* were obtained from Okada village, Ovia North-East Local Government Area, Edo State Nigeria. The plant was identified by Professor A. Gbolade of the Department of Pharmacognosy, Igbinedion University Okada, Edo State, Nigeria, where a voucher specimen (Voucher no: IUO/13/051) was given and the plant was deposited in the Departmental herbarium.

The plants were washed with large quantity of deionized water and spread on large sack to drain, after draining, foreign matters were removed from the plants materials. The plants were oven dried at 45^0C for two days and grounded into coarse powder using electric milling machine.

2.2 Extraction of Plant Materials

The grounded plants were exhaustively extracted by Soxhlet apparatus with absolute methanol (analytical grade), the extract obtained was concentrated under reduced pressure using rotary evaporator, stored in airtight container and kept in the refrigerator for further use.

2.3 Phytochemical Screening

The qualitative phytochemical test was carried out on the crude plant extract using standard procedures as described by Trease and Evans (1996) [15].

2.4 Spectrophotometric quantification of flavonoids

The total flavonoid content was determined using the Aluminium chloride colorimetric method (Lin and Tang, 2007) [8]. 1 ml of the plant sample was mixed with 0.1 ml of 10% aluminium chloride hexahydrate ($AlCl_3.6H_2O$), 0.1 ml of 1 M potassium acetate (CH_3COOK) and 2.8 ml of deionized water. After incubation at room temperature for 40 minutes, The absorbance of the reaction mixture was measured at 415 nm on a UV-Visible Spectrophotometer (Model SM23A; Microfield, England). Flavonoid contents were calculated on the basis of calibration curve of quercetin standard (2-(3,4dihydroxyphenyl -3, 5, 7-trihydroxy-4H-1-benzopyran-4-one) with 98 % purity.

2.5 Spectrophotometric quantification of phenolics

In order to measure the phenolic content, 1 ml of the plant sample mixed with 2 ml of 7.5% sodium carbonate (Na_2CO_3) and 2 ml of Folin –Cciocalteaus reagents). After incubation using water bath at 40ºC for 45min, the absorbance of the reaction mixture was measured at 765 nm on a UV-spectrophotometer.

2.6 Determination of Antioxidant Activity

The radical scavenging activity of the plant extract against 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) (Sigma-Aldrich) was determined by UV-Spectrophotometer at 517 nm. 20-100 µg/ml of crude extract and vitamin C were prepared in methanol (Analar grade). 1 ml of the extract was placed in a test tube, followed by 2 ml of 0.1mM DPPH. The radical scavenging activity was calculated using the following formula;

% inhibition={[Ab-Aa]/Ab*100…….(1)

Where Ab is the absorption of the blank sample and Aa is the absorption of the extract.

2.7 Antilipid Peroxidation Assay

Lipid peroxidation assay was carried out using slightly modified method described by Ohkawa *et al*. (1979) [12]. Liver homogenate was prepared from commercially available goat liver. The liver was washed several times in ice –cold saline solution. A 10% of liver homogenate was prepared using ice-cold KCl (0.15M) in a blender. Lipid peroxidation was initiated in 1 ml of tissue homogenate incubated with varieties concentrations of extracts (20-100 mg/ml), by the addition of 0.1ml of ferric sulphate (25µM), 0.1ml of ascorbate (100 µM) and 0.1 ml of KH_2PO_4 (10 mM) and the volume was made up to 3 ml with distilled water and incubated at 37˚ C for 1hour.then 1 ml of 5% trichloroacetic acid (TCA) and 1 ml of 0.67% TBA was added to this reaction mixture and the tubes were boiled for 30 min in boiling water bath. This was centrifuged at 3500 rpm for 10 mins.The extent of inhibition of lipid peroxidation was evaluated, through estimation of thiobarbaturic acid reactive substance (TBARs) levels.

2.8 *In vivo* Antidiabetic Assay

2.8.1 Animals

Wister rats (female sex) of the same strain (albino) weighing between 180 - 200 g were used. They were maintained under standard animal house conditions and allowed free access to food (growers mash) and water ad libtum for 2 weeks to acclimatize to the new environment.

2.8.2 Alloxan Induced Diabetes

Rats were randomly selected to different treatment groups of six animals per group. The glucose baseline test was taken before the induction of diabetes using Alloxan (dissolved in 0.9% NaCl solution). Test albino rats were injected intraperitoneally with portions of this solution at a dose of 500 and 1000 mg/kg body weight, three days after; the glucose level had risen above normal. Control group was post-treated orally with distilled water 0.5 ml/kg; reference group received

glibenclamide (20 mg/kg) orally while the test groups were treated orally with 500 and 1000mg/kg of crude as well as 250 and 500 mg/kg of chloroform and aqueous fractions of *A. muricata* orally for 9 days. The sugar level was taken at interval of three (3) days using glucometer.

2.9 Statistical analysis
Data were presented as Mean ± SEM of the respective replicates. Means of different groups were compared using ANOVA using graph pad prism 6 version computer software packages.

3. Results
The results of the entire scientific activities carried out during the course of the research are as follow:

3.1 Extraction Yield
201 g of the powdered aerial part of *A. muricata was* extracted with methanol (Anal. Grade) using Soxhlet method of extraction. This yield 50 g methanol extract corresponding to 24.9 % as indicated in Table 3.0 below.

Table 3: Percentage extraction yield of *A.muricata* leaves

Plant material	Yield (%)
A. muricata leaves	24.9%

3.2 Preliminary phytochemical screening
The result of the phytochemical screening of the leaves of *A. muricata* is shown in the Table 3.1 below.

Table 3.1: Phytochemical screening of *A. muricata* leaves.

Phytochemical	Plant sample
Saponins	+
Reducing sugar	+
Anthraquinone	_
Flavonoids	+
Steroids	+
Tannins	+
Alkaloids	+

KEYS: + = present
- = Absent

Table 3.2: Amount of total phenolics and flavonoids in *A. muricata* leaves

Sample	Phenolics	Flavonoids
Crude sample	68.54 ± 0.51^{a}	55.82 ± 0.85^{r}
Aqueous fraction	46.32± 0.76^{b}	32.81 ± 0.28^{s}
Chloroform fraction	20.65 ± 0.11^{c}	19.92 ± 0.51^{t}

The values above are mean = SEM of three replicates. n= 3. Similar alphabets indicate no significant difference at P<0.0001 down the roll using ANOVA (Graph pad 7) Holm-Sidak's multiple comparisons test (parametric) for each parameter.

3.3 DPPH radical scavenging assay
The effect of crude and solvent fractions of *A. muricata* against DPPH radical is shown in Figure 3.1 below:

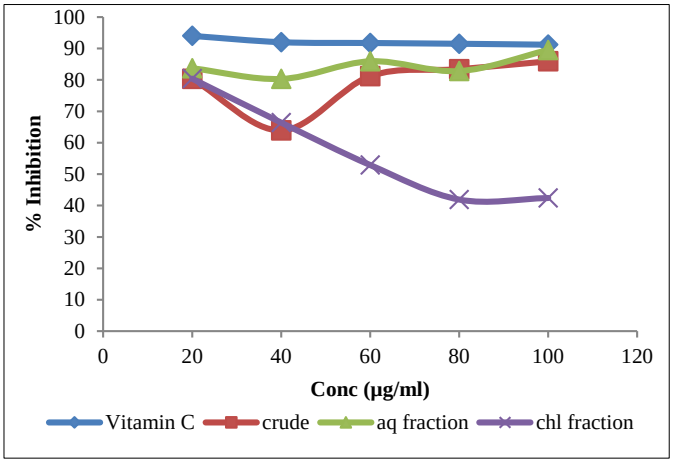

Fig 3.1: Effect of *A. muricata* crude and solvent fractions on DPPH radicals

3.3 Anti-lipid peroxidation study of *A. muricata* crude and solvent fractions sing *in vitro* model
The effect of crude and solvent fractions of *A. muricata* on lipid peroxidation is shown in Figure 3.2 below

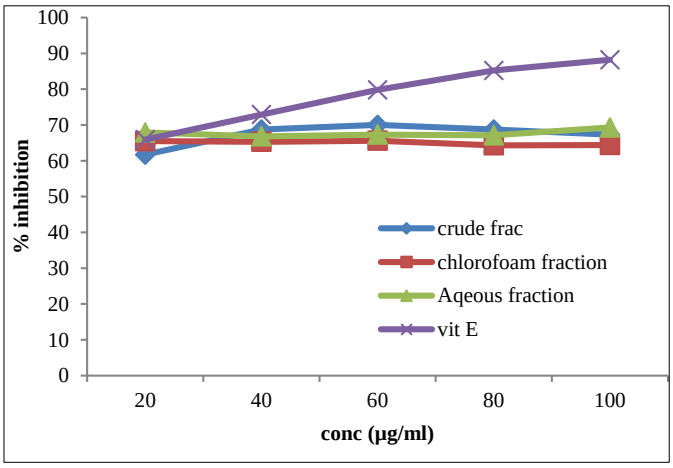

Fig 3.2: Antilipid peroxidation of *A. muricata* crude and solvent fractions

3.5 Effects of *A. muricata* leave crude and solvent fractions on alloxan induced Diabetes
The effects on the level of significant in which the various doses of treatment (250, 500, 1000 mg/kg) of *A. muricata* leaves extracts exhibited in the management of diabetes in animal study when compared with the results from the positive control (20 mg/kg) Glibenclamide and negative control are presented in Table 3.3

Table 3.3: Anti-hyperglycemic effect of *A. muricata* crude and fractions at various treatments across the groups

Group	Days				
	Before Induction	3 Days After Induction (T0)	3 Days After Treatment (T1)	6 Days After Treatment (T2)	9 Days After Treatment (T3)
Normal negative control	111.38 ±0.65	113.18±4.51	121.80±3.41	110.45±1.92	109.30±4.82
Diabetes negative control	101.53±1.55	216.21±0.52	265.72±0.89	318.61±1.59**	311.19±1.99
Positive control Glibenclamide 20 mg/kg	105.11±1.95	212.82±1.52	150.65±1.72	95.35±1.81	82.50±1.21**
500 mg/kg crude extract	107.79±1.57	251.99±2.51	103.52±4.32	92.12±2.52	81.11±4.81**
1000 mg/kg crude extract	109.82±2.32	219.7±3.21	100.18±4.11	90.31±3.21	80.51±3.41**
250 mg/kg chloroform fraction	105.95±1.89	279.18±2.65	215.12±1.98	218.92±2.98	210.52±1.89**
500 mg/kg chloroform fraction	111.32±1.52	265.00±2.88	205.65±2.98	198.12±1.77*	198.25±1.82*
250 mg/kg Aqueous fraction	112.52±0.79	259.62±1.00	148.19±2.15	110.53±2.51	92.95±1.87**
500 mg/kg Aqueous fraction	106.25±1.87	285.95±3.25	110.52±5.25	98.01±2.11	81.32±1.50**

The values above are mean ±SEM of six replicates. n = 6.Staitistical analysis was one across the column for each dose treatment relative to T0 at P< 0.0001 (**) and P< 0.0006 (*) using ANOVA (non-parametric).

4. Discussion

Medicinal plants has a lot of benefits which has motivated man to crave for them, in order to obtain a cure for any illness, to experience new sensations or just because of simple curiousity. Reasonable percentage of population in developing and underdeveloped nations in our present century depend on plants to cure themselves, most of which are so cheap and affordable by individuals who cannot afford modern medicines (Moghadamtousi *et al.*, 2015) [10].

However, the natural products which are derived from plants have been used to help mankind sustain its health since the dawn of medicine. Over the past century, the phytochemicals in plants have been a pivotal pipeline for the pharmaceutical discovery (Okhale *et al*., 2016) [13].

According to Table 3.0, the yield of *A. muricata* that was gotten is 24.9% using the Soxhlet method of extraction. The yield was found to be small as when compared to other high extraction techniques when used for such purposes such as the ultrasonic and percolation method of extraction.

Moreover, phytochemical screening of *A. muricata* as seen in Table 3.1 above showed the presence of various secondary metabolites such as flavonoids, alkaloids, steroids, saponins, tannins and reducing sugars (Table 3.1). These finding suggest that *A. muricata* is rich in different classes of phytochemicals, Hence their numerous role in therapeutic activities such as anti-diabetes, anti-inflammatory, anti-analgesic, anti-hyperlipidemic agent e.t.c.

The result of DPPH scavenging property of crude and solvent fractions of *A. muricata* is shown in Figure 3.1 above. Aqueous fraction showed the highest scavenging activity in a concentration non-dependent manner with the lowest and highest inhibition at 40 and 100 µg/ml. Although, scavenging property of Vitamin C (positive control) was higher than all samples examined. No significant difference in inhibition at 100 µg/ml between Vitamin C (positive control) and aqueous fraction. Chloroform fraction exhibited the least scavenging potential among the samples examined.

However, Figure 3.2 showed the effect of crude and solvent fractions of *A. muricata* in prevention of lipid peroxidation in goat liver. α – tocopherol (positive) exhibited the highest anti-lipid peroxidation potential. Crude extract and chloroform fractions exhibited the highest and lowest inhibition of lipid peroxidation as shown in Figure 3.2 above.

In addition, Table 3.2 showed the result of *in vivo* anti-diabetic effect of the crude extract, aqueous and chloroform leaves fractions of *A. muricata* in alloxan induced diabetic rats. The aqueous extract at 250 mg/kg showed very slight reduction in blood glucose level at 9 days after treatment and it reduced slightly with the number of days of treatment. The aqueous fraction at 500 mg/kg showed significant reduction in blood glucose level at 9 days after treatment which reduced further with the number of days of treatment. Chloroform fraction at 250 mg/kg there was no observable reduction in the blood sugar level after treatment, even at 500 mg/kg of chloroform extract there was no observable reduction in blood glucose level. Moreover, crude extract at 500 mg/kg showed a significant reduction in blood glucose level at 9 days after treatment while at 1000 mg/kg showed more significantly reduction in blood level than 500 mg/kg, the reduction in the glucose by the crude extract at 1000 mg/kg was comparable to that achieved with Glibenclamide (20 mg/kg).

5. Conclusion

Findings from this research showed that the plants is rich in phenolics and flavonoids, Hence their great antioxidant and anti-lipid peroxidation potentials which justify their usage in the ethno pharmacological claim of their uses in treatment of oxidative induced diseases, reduction of peroxidation of lipids in cell membrane and reduction of membrane lipid peroxidation associated with hyperglycemia. Moreover, their ethno-pharmacological claim in the treatment of diabetes was also justify with activity in crude extract and aqueous fraction. These results justify scientifically the local medicinal claims of A. *muricata* in the treatment of diabetes and other related oxidative stress induced diseases.

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