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Hematological and Hypoglycemic Effects of Ethanolic Extract of *Annona muricata* Ripe Fruits Pulp on Streptozotocin-induced Diabetes in Rats: *In vivo* and *In silico* Studies

O. S. Akinlolu [a*], T. H. Fatoki [b] and D. U. Momodu [a]

[a] Department of Chemistry, Faculty of Science, Federal University Oye-Ekiti, Ekiti State, Nigeria.
[b] Department of Biochemistry, Faculty of Science, Federal University Oye-Ekiti, Ekiti State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author OSA carried out sampling and hematological and hypoglycemic analyses. Author THF carried in vivo and in silico studies and interpreted the data. Author DUM did the editing. All authors read and approved the final manuscript.

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ABSTRACT

Diabetes mellitus has been a metabolic disorder characterized by interferences in the breakdown of carbohydrate, lipid, and protein as a result of insulin deficiency**.** Great efforts are ongoing in understanding and management of diabetes, and disease related complication. In this work, an attempt was made to study the hematological and hypoglycemic effects of *Annona muricata* ripe fruits pulp in Streptozotocin (STZ)-induced diabetic rats and validates its traditional claim. The forty-eight (48) Albino rat were divided into six groups which include normal, tests and controls. The

**Corresponding author: E-mail: olapade.akinlolu@fuoye.edu.ng, daniel.momodu@fuoye.edu.ng;*

diabetes–induced rats were fed orally with *A. muricata* ripe fruits pulp extract in concentrations of 750 mg, 1000 mg, and 2000 mg respectively. The results showed that the extract caused fasting blood sugar glucose levels to remarkably reduced to near normal. Hematological studies revealed that there were improvements in the hematological indices tested groups when compare with diabetes normal group. The molecular docking results indicate that the phytochemicals in *A. muricata* ripe fruit pulp have more affinity for aldose reductase followed by alpha-amylase and then alpha-glucosidase, and that montecristin, epoxymurin-A, dicaffeoylquinic acid, and kaempferol 3-O-rutinoside show higher affinity to the three targets than acarbose. These results indicate that ripe fruits pulp of *A. muricata* possesses a strong hypoglycemic effect in STZ–induced diabetic rats, thus supporting its traditional use in the management of diabetes mellitus.

Keywords: Phytochemical; hematological; hypoglycemic; streptozotocin; metabolic disorder.

1. INTRODUCTION

"Chronic disorder of diabetes has appeared to be the major causes of adult mortality and morbidity over the last three decades all over the world" [1]. "It is a metabolic condition characterized by disruption in the breaking down of carbohydrates, proteins and lipids as a result of insulin deficiency" [2]. It is also a complex disease that has high blood glucose level feature due to imbalanced insulin production [3].

Traditional medicine usage in the treatment of different ailments has immensely expanded in both developed and developing countries of the world due to their cost effectiveness, availability and efficacy [4]. Some medicinal plants have medicinal value believed to promote good health and stimulate resistance against infection by restoring body equilibrium and conditioning body tissue [5]. Various parts of plants are used for different purposes all over the world.

"Annona muricata (L.) referred also as graviola and soursop belongs to a family called *Annonaceae*. It is grown across the world in the tropical regions" [6]. "The plant produces an edible fruit green in colour, heart-shaped, large and 15–20 cm in diameter. It has white fleshy mesocarp. Soursop has been indigenous plant; historical used as herbal medicine for decade. The fruit juice is generally used to fight parasitic organisms and worms, to treat fevers, improve breast milk production, and to halt diarrhea and dysentery" [7]. "Tropical *A. muricata* tree parts are used in natural medicine including the bark, root, leaves, nand fruit-seeds. It bark are considered sedative, smooth muscle relaxant, hypoglycemic, hypotensive, and a tea from it is used for various disorders" [8]. In this work, the hematological and hypoglycemic effects of *A. muricata* ripe fruits pulp was investigated in Streptozotocin (STZ)-induced diabetic rats and the binding affinity was predicted computationally.

2. MATERIALS AND METHODS

2.1 Plant Collection, Identification and Extraction

Anona muricata ripe fruits were purchased from the market area of Ikeji-Arakeji town in Osun state, Nigeria and were authenticated and identified at the herbarium of the Crop Science Department, Joseph Ayo Babalola University (JABU), Osun state, Nigeria. The fruits were washed with distilled water, then the peels and seeds of the fruits were cut into pieces after separation from the pulp. The pulp pieces were oven dried at 50°C, then powdered using a blender, and kept in an airtight container to avoid moisture. The extraction was according to the method described by Jothy et al. [9] and Nweke et al. [10]. Concisely, 300 g of the powdered sample was soaked in 2500 mL of ethanol to extract the compound constituents. It was left for 72 hours (3 days) in the labeled container after which it was sieved using muslin cloth and then filtered with 0.45 µm micropore filter. The filtrates were vaporized using rotary evaporator to powder by removal of ethanol. The extract was preserved in a sterile bottle at 4°C until use.

2.2 Experimental Animals

Forty-eight albino rats (100-150 g) were obtained from Department of Animal Production and Health, Federal University of Technology, Akure, Ondo State, Nigeria. They were fed with standard rat pellets (Livestock Feeds, Ikeja, and Lagos State) and water ad libitum. They were housed under standard laboratory conditions acclimatized for 15days before the treatment. The experimental procedures were conducted in

conformity with international, national and institutional guidelines [8].

2.3 Induction of Experimental Diabetes

The induction of diabetes mellitus was done by single intraperitoneal injection of STZ (75 mg/kg) freshly dissolved in 0.1mol/l citrate buffer. Normal control rats were injected with only citrate buffer solution (pH 6.3) intraperitoneally. The 'test' animals in groups 2 to 6 became diabetic within 48 hours after STZ administration. Diabetic state was confirmed by measuring blood glucose concentration 48 hours after STZ injection. Diabetes was allowed to develop and stabilize in these STZ-treated rats over a period of 3-5 days. Before the commencement of our experiments, both the control normal (normoglycemic) and STZ-treated, diabetic (hyperglycemic) test rats were fasted for 16h, but still allowed free access to water throughout. Fasted STZ-treated rats with blood glucose concentration ≥18 mmol/L were considered to be diabetic, and used in this study.

2.4 Experimental Design

Forty-eight albino rats (150-250 g) were divided into 6 groups of 8 rats each, according to their average weight, daily fed with pellet feed and water, and test groups also received daily oral dose of extract as follow:

Group 1: Normal rat (positive control)
Group 2: Diabetic rat control not treated
Group 3: Diabetic rat control treated with insulin standard drug
Group 4: Diabetic rat treated with ethanolic extract of 750 mg/kg body weight daily
Group 5: Diabetic rat treated with ethanolic extract of 1000 mg/kg body weight daily
Group 6: Diabetic rat treated with ethanolic extract of 2000 mg/kg body weight daily

Blood through the ocular puncture was taken after the 6th week of administration. Two ml of the blood samples from each group were collected in test tubes and put into centrifuge tubes, spun at 3000 rpm for 10 min and the serum collected for hormonal assays. Whole blood (2 ml) for hematological studies were placed in EDTA tubes and assayed for full blood count. The rats were sacrificed under chloroform anesthesia after collection of blood samples.

2.5 Preparation of Tissue Homogenates

The rats were sacrificed and pancreas and kidney were excised by cervical dislocation and, rinsed with ice-cold physiological saline, and homogenized with Potter Elvehjem homogenizer. 10% homogenates were prepared in 6.7 mM phosphate buffer (pH 7.4), and centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was used for antioxidant enzyme assays.

2.6 Blood Parameter Analyses

"Blood was collected pre-feeding in triplicate per treatment on day 0, 7 and 14 using a syringe and a needle from the wing vein" [11]. "Heparinized tubes were used for the collection of blood for FBC while non-heparinized tubes were used to collect blood for serum biochemical assay (AST and ALT). The hematological and serum biochemical parameters were determined. Hematological parameters assayed for, were red cell distribution width, red blood cells (RBC), platelets, hemoglobin estimation, hematocrit, MCV, MCH and MCHC. Aspartate transaminase and ALT concentrations were also assayed from the serum" [12].

2.7 Hematological Studies

"Determination of hematological parameters such as hemoglobin concentration (Hb), packed cell volume (PCV), platelet count, total white blood cell count (TWBC neutrophils and lymphocytes) were done using standard operative procedures" [13].

2.8 Phytochemical Analysis

"Mayer, Dragendoff, Wagner and picric reagents test were used to test for alkaloid. Frothing test for saponin, ferric chloride test for tannin while Salkowski test for cardiac glycosides" [14].

2.9 Biochemical Analysis

"Serum blood samples were analyzed for alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST)" [15].

2.10 Statistical Analysis

"All the results were expressed as mean values ± SEM. One-way analysis of variance (ANOVA)

was performed to compare the differences between two or more means followed by Dennett's post tests using SPSS version 20.0. A mean difference was considered significant when p< 0.05" [15].

2.11 Molecular Docking Simulation

The phytochemical constituents of *A. muricata* fruit was obtained from the literature [16], the chemical structures of this phytochemicals and a standard drug (acarbose) were obtained from NCBI PubChem Compound database (http://www.pubchem.ncbi.nlm.nih.gov/) in SDF formats. The diabetic protein targets used were according to the previous report [17]. Briefly, the AlphaFold modelled structures of three carbohydrate metabolizing enzymes (targets); alpha-amylase (UniProt UD: P04746), alpha-glucosidase (UniProt ID: O43451), and aldose reductase (Uniprot ID: P15121) were obtained from UniProt database. The ligands (phytochemicals and standard drug) were prepared for molecular docking by using AutoDock Tools (ADT) v1.5.7 [18], and the docking parameters used were: alpha-glucosidase (center grid box: 5.978 × 5.605 × -1.129; Size: 126 × 126 × 126; Spacing: 1.000); alpha-amylase (center grid box: -7.279 × 1.038 × -2.578; Size: 126 × 126 × 126; Spacing: 0.500); and aldose reductase (center grid box: 1.346 × 2.385 × 0.255; Size: 126 × 126 × 126; Spacing: 0.400). Molecular docking simulation was instantiated using AutoDock Vina v1.1.2 [19], from the command line. After docking, close interactions of binding of the targets with the ligands were analyzed and visualized using PyMol v2.0.7.

3. RESULTS

The results in Table 1 revealed that high dose concentration of ethanoic extract of Anona muricata ripe fruit significantly reduced blood glucose level. The results in Table 2 showed the hematological parameter (PCV, Hemoglobin, White blood cell count, Neutrophils, Platelets} which increased compare to the normal. Hematological parameters are usually associated with health status and are of diagnostic importance in clinical assessment of the state of health of a patient. Blood parameter is good indicators of physiological, pathological and nutritional status of an animal and changes in hematological parameters have the potential to explicate the impact of therapeutic drug testing. The results of the hematological studies show that *A. muricata* has proportionally increased effect on PCV, Hb, platelets number, WBC, and neutrophil.

Table 3 showed the weight of rat used for the experiment before the experiment and after the treatment. The results showed that *A. muricata* ripe fruit extract increases the body weight of the rat. Group A (+control) showed a 5.69% increase in body weight during the experimental period while a 25.88% body weight reduction was recorded in group B which is diabetic rat without treatment. This was highly significant ($p<0.05$) when compare with other groups of diabetic rats treated with *A. muricata*. Interestingly, group F which received the highest dose (2000 mg/kg) of ethanolic extract of *A. muricata* fruit pulp, showed as significant body weight improvement of 3.12% when compare with group B and this is closer to the normal weight seen in group A.

The enzyme activities in the serum of the rats administered with ethanolic extract of *A. muricata* ripe fruits pulp, showed that the control group has the lowest level of enzyme activity in the serum with values of 62.17 UL, 58.71UL, and 10.87UL for ALP, AST and ALT activities respectively, while group D (the group administered with lowest concentration of the extract (750mg/kg) have highest level of enzyme activity. Table 5 showed that phytochemicals that were present or absent in ethanolic extract of *A. muricata* ripe fruit pulp. Cardiac Glycosides, Steroids, Saponins, and Phenol were present while flavonoid, tannins, terpenoids and quinine were absent.

The results of the molecular docking show that the phytochemicals in *A. muricata* ripe fruit pulp have more affinity for aldose reductase followed by alpha-amylase and then alpha-glucosidase, at a binding energy cut-off score of -10.0 kcal.mol^{-1} (bolded). It was observed that montecristin, epoxymurin-A, dicaffeoylquinic acid, and kaempferol 3-O-rutinoside show better affinity to the three targets than acarbose.

Table 1. Blood Glucose Level

Group	Initial level (mg/dl)	Final level (mg/dl)	Change (mg/dl)	% Change
A (+ control)	117.02±1.41	117.18±0.16	0.16	0.13
B (- control)	126.02±1.40	237.17±0.53	111.15	88.20
C (standard drug)	140.30±1.20	122.93±0.55	-17.37	-12.40
D (750mg/kg)	126.50±1.00	315.33±1.11	188.83	149.20
E (1000mg/kg)	138.10±0.70	131.02±1.39	-6.98	-5.05
F (2000mg/kg)	126.16±0.54	124.02±1.41	-2.14	-1.72

The results are mean of 8 determinants ±SD. Significant (p<0.05)

Table 2. Hematological Parameter of PCV, Hemoglobin, White Blood Cell Count, Neutrophils, Platelets

Group	PCV (%)	Haemoglobin (g/l)	WBC Count (mm^3)	Neutrophils	Platelets
A (+ control)	57.25±0.96	18.75±0.50	38.02±5.00	42.16±0.08	988.74±0.15
B (- control)	40.25±0.50	12.63±0.48	27.04±0.50	30.43±4.55	395.67±0.01
C (standard drug)	56.76±0.05	18.77±0.50	54.03±4.79	32.66±0.06	694.65±0.01
D (750mg/kg)	51.75±0.50	17.31±0.01	56.00±0.96	35.98±0.01	533.68±0.01
E (1000mg/kg)	51.38±0.48	16.01±0.01	44.00±1.00	36.98±0.01	646.87±0.01
F (2000mg/kg)	65.01±0.01	21.71±0.01	83.04±4.79	40.57±0.01	790.55±0.01

The results are mean of 4 determinants ±SD. Significant (p<0.05)

Table 3. Result of Body Weight

Group	Initial weight (mg)	Final weight (mg)	Change (mg)	% Change
A (+ control)	208.22±1.18	220.22±1.07	11.80	5.69
B (- control)	193.61±1.23	143.48±1.20	-50.12	-25.88
C (standard drug)	171.15±1.53	178.64±1.07	7.49	4.37
D (750mg/kg)	134.70±1.22	107.05±1.23	-27.65	-20.53
E (1000mg/kg)	149.90±1.24	137.77±1.08	-10.13	-4.75
F (2000mg/kg)	141.03±4.91	136.63±1.24	-4.4	-3.12

The results are mean of 8 determinants ±SD. Significant (p<0.05)

Table 4. Enzyme Activities

Group	ALP (U/L)	AST (U/L)	ALT (U/L)
A (+ control)	62.17±2.83	58.71±4.15	10.87±2.04
B (- control)	276.16±0.53	87.31±1.47	20.23±1.04
C (standard drug)	64.17±1.63	60.73±2.95	12.85±0.84
D (750mg/kg)	290.50±0.93	100.05±1.06	38.18±0.73
E (1000mg/kg)	152.22±2.18	67.55±1.06	16.81±1.34
F (2000mg/kg)	90.54±0.90	65.12±1.23	13.92±0.31

The results are mean of 3 determinants ±SD. Significant (p<0.05)

Table 5. Phytochemical Analysis of Ripe *A. muricata* Ethanolic Extract

Phytochemicals	Result
Flavonoid	-
Cardiac Glycosides	+
Steroids	+
Saponins	+
Tannins	-
Phenolics	+
Terpenoids	-
Quinine	-

(-) Absent. (+) Present. The results are mean of 3 determinants ±SD. Significant (p<0.05)

Table 6. Binding affinity of phytochemicals in *A. muricata* fruit pulp with three carbohydrate metabolizing enzymes

S.N	Phytochemicals	PubChem CID	Binding Energy ΔG (kcal.mol^{-1})		
			Alpha-amylase (AF-P04746-F1)	Alpha-glucosidase (AF-O43451-F1)	Aldose Reductase (AF-P15121-F1)
0	Acarbose	444254	-10.3	-10.8	-10.1
1	Annonacin	354398	-10.3	-9.7	-13.7
2	Annonacin-10-one	180161	-9.9	-9.8	-13.4
3	Annonaine	160597	-9.2	-9.0	-7.7
4	Cis-Annoreticuin	72778911	-10.2	-9.9	-13.7
5	Asimilobine	160875	-8.5	-8.9	-8.8
6	Cinnamic acid	444539	-7.2	-8.1	-8.3
7	Corossolone	4366126	-10.5	-9.6	-12.3
8	Coumaric acid	637542	-7.1	-8.2	-8.1
9	Dicaffeoylquinic acid	12358846	-11.3	-11.2	-14.2
10	Dihydrokaempferol-hexoside	10478918	-10.1	-10.5	-9.0
11	Epoxymurin-A	5281161	-11.2	-10.3	-14.9
12	Epoxymurin-B	131752983	-8.0	-9.1	-13.2
13	Epomusenin-A	10507050	-6.9	-8.4	-11.6
14	Epomusenin-B	10698082	-9.1	-8.5	-13.5
15	Fisetin	5281614	-9.6	-9.0	-11.7
16	Kaempferol	5280863	-9.5	-10.2	-7.3
17	Kaempferol 3-O-rutinoside	5318767	-11.2	-12.3	-11.4
18	Luteolin 3´7-di-O-glucoside	133611799	-10.8	-10.2	-10.4
19	Montecristin	102083640	-11.2	-10.4	-14.4
20	Morin	5281670	-9.4	-9.3	-7.6
21	Muricatocin A	133072	-9.3	-8.5	-13.6
22	Myricetin	5281672	-9.9	-9.9	-11.9
23	N-methylcoclaurine	40595	-10.2	-8.6	-11.8
24	Nornuciferine	41169	-9.3	-9.7	-9.7
25	Reticuline	439653	-8.4	-9.8	-8.2
26	Sabadelin	101006011	-8.1	-6.8	-12.3
27	Xylomatenin	10484035	-9.6	-9.8	-13.4

S.N 0: Standard drug. S.N 1-27: phytochemicals in A. muricata fruits pulp

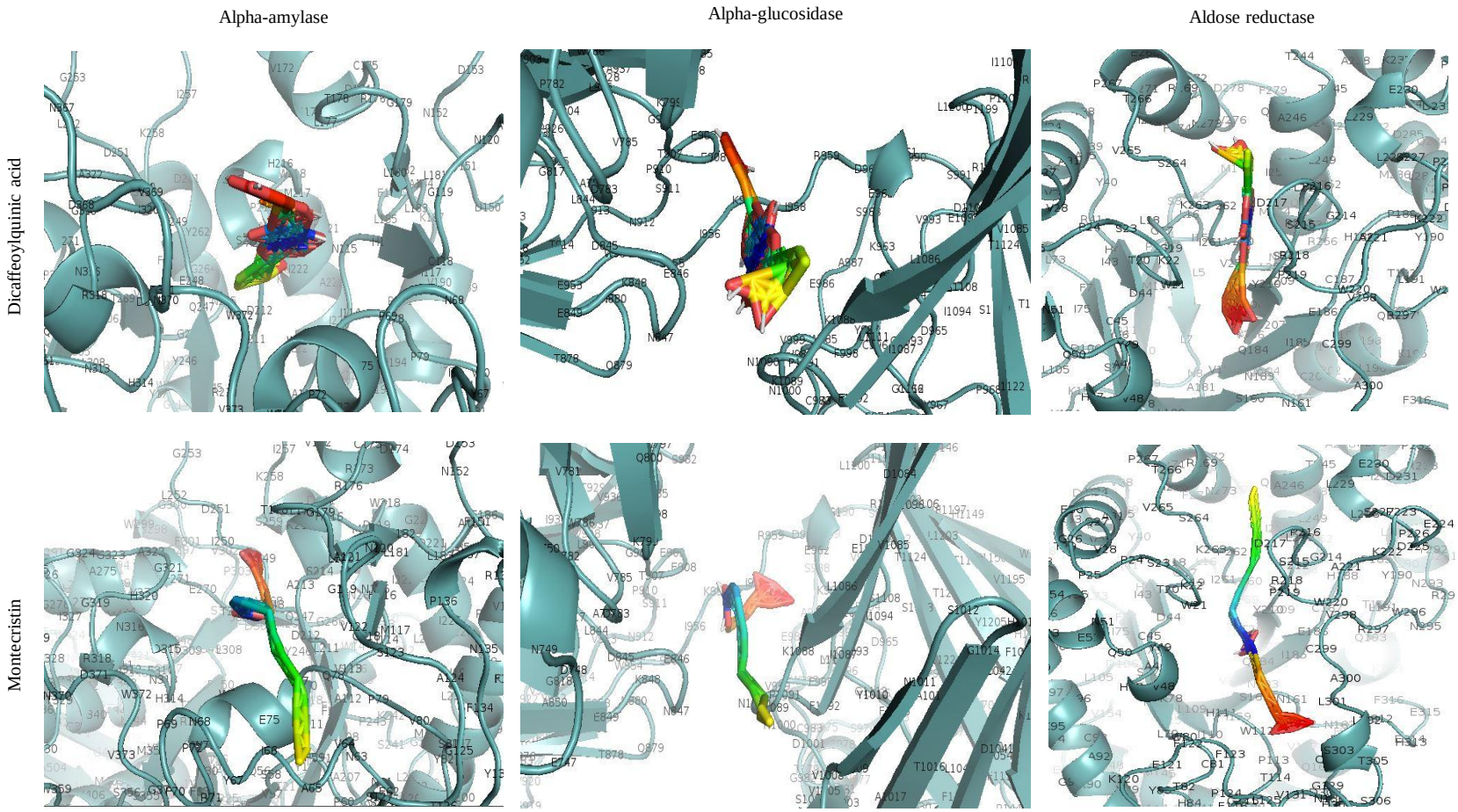

Fig. 1. Binding pose of dicaffeoylquinic acid and montecristin from *A. muricata* fruit pulp on alpha-amylase, alpha-glucosidase and aldose reductas

4. DISCUSSION

"A. muricata is widely used in traditional medicine to treat a variety of ailments, such as hypertension, diabetes, and cancer. Research also stated that these plants contain various types of bioactive compounds from certain classes, such as acetogenins, flavonoids, phenols, alkaloids, and megastigmane. In vivo and in vitro research showed that it has potential to treat various conditions, such as wound healing, ulcer, inflammation, cancer, diabetes, and hypertension" [20].

"Results of this study indicate that high dose concentration of ethanoic extract of *Annona muricata* ripe fruit significantly reduced blood glucose level. Several studies have shown that *A. muricata* possesses antihyperglycemic activities" [21,22] "Also, it has been reported that treatment of diabetic rats with *A. muricata* extracts caused a marked amelioration of hyperglycemia with pronounced increase in serum insulin levels" [23]. These reports corroborate with findings of this study, that *A. muricata* ripe fruits pulp extract lowers blood glucose levels.

This study shows that hematological parameter (PCV, Hemoglobin, White blood cell count, Neutrophils, Platelets} increased compare to the normal. "Hematological parameters are usually associated with health status and are of diagnostic importance in clinical assessment of the state of health of a patient. Blood parameter is good indicators of physiological, pathological and nutritional status of an animal and changes in hematological parameters have the potential to explicate the impact of therapeutical drug testing. The results of the hematological studies show that *A. muricata* has proportionally increased effect on PCV, Hb, Platelets number, WBC, Neutrophil" [22].

This present study shows that *A. muricata* ripe fruit extract increases the body weight of the rat. Group A (+control) showed a 5.69% increase in body weight during the experimental period while a 25.88% body weight reduction was recorded in group B which is diabetic rat without treatment. This was highly significant ($p<0.05$) when compare with other groups of diabetic rats treated with *Anona muricata.* In fact, group F which received the highest dose of the extract (2000mg/kg of ethanolic extract of *A. muricata* ripe fruit pulp) showed a significant body weight improvement of 3.12% when compare with group B and this is closer to the normal weight seen in group A. The reduction in the body weight noticed may be as a result of damage that has occurred to the ß-cell in the pancreas which leads to the inability of the organ to produce insulin hormone responsible for the conversion of excess glucose to glycogen.

"In this study, the liver enzymes were determined to evaluate the effect of the extract on Serum ALP, AST, and ALT after STZ-induced diabetic mellitus. The result of this study showed that serum ALP, AST, and ALT were significantly increased in untreated diabetic rat (Group B). This increase could possibly as a result from the cell membrane leaking and even completely ruptured" [24]. However, the extract reduced the liver enzymes in a dose-dependent fashion. Control group having the lowest level of enzyme activity in the serum with the values of 62.17U/L, 58.71U/L and 10.87U/L respectively while group B (negative control) and group D (the group administered with the lowest concentration of the extract (750mg/kg) have highest level of enzymes activity with the values of 276.16U/L, 87.31/L, 20.23U/L in the serum.

Study has shown that daily treatment of STZ-induced diabetic rats with *A. muricata* extract for 4 weeks, could prevent the deleterious effect of STZ, based on its antioxidant and protective effect of pancreatic β-cells [25]. "Also, daily intraperitoneal injection of STZ-induced diabetic Wistar rats with the methanol extract of *A. muricata* leaves (100 mg/kg) for two weeks significantly reduced their blood glucose concentration from 21.64 to 4.22 mmol/L, and significantly decreased the serum total cholesterol, low-density lipoprotein, triglyceride and very low-density lipoprotein cholesterol" [26]. "Additionally, it has been reported that the glycemic index (GI) and glycemic load (GL) are considered low for *A. muricata*, which agrees with its hypoglycemic potential" [27]. "GI indicates the effect of the content and type of carbohydrates of a food on blood glucose content, while GL estimates how much the food will raise blood glucose level after eating it. GI and GL" [28].

The screening of *A. muricata* in this study show that it contained saponin, cardiac glycosides, phenolic compounds and steroids. The phytochemicals present in *A. muricata* are classified as alkaloids, phenolics, and acetogenin [28]. "It has been well documented that tannin and other polyphenolic compounds, flavonoids,

triterpenoid saponins, and a host of other plant secondary metabolites possess hypoglycemic, hypolipidemic, anti-inflammatory, and other pharmacological and biochemical properties in various experimental animal models" [29].

The key concept of molecular docking is to develop an appropriate solution to elucidate the minimum binding affinity interaction per mole of ligand [30]. The molecular docking results of this study showed that that montecristin, epoxymurin-A, dicaffeoylquinic acid, and kaempferol 3-O-rutinoside have good binding affinity to the three carbohydrate metabolizing enzymes than acarbose. This study confirms the antidiabetic activity of these phytochemicals in the fruits pulp of *A. muricata*. The antioxidant activity of kaempferol, kaempferol 3-o-rutinoside, luteolin 3´7-di-o-glucoside, montecristin, and morin has been reported, while annonacin, corossolone, cis-annoreticuin, and annonacin-10-one were found to be cytotoxic [16,31,32].

Studies have shown the effect of selected *A. muricata* active compounds (rutin, muricatocin A, anonaine, isolaureline, xylopine, and kaempferol 3-O-rutinoside) on inhibition of the Forkhead Box O1 (FOXO1) protein, and the results revealed that FOXO1 protein plays a crucial role in the proliferation process of pancreatic β-cells and inhibition of the FOXO1 protein activity in the nucleus will enhance insulin sensitivity [33,34]. Furthermore, a study has reported that an aqueous extract of *A. muricata* peel (AEAMP) inhibited α-amylase and α-glucosidase enzymes more effectively than acarbose, and that it improve β-cell dysfunction by upregulating liver AKT and pancreatic PI3K and AKT genes, and preventing apoptosis by upregulating liver and pancreatic Bcl2 [35].

5. CONCLUSION

A. muricata dried ripe fruit pulp contains many important phytochemicals such as saponin, cardiac glycosides, phenolic compounds and steroids, which have antioxidant properties that may prevent cellular injuries. This study confirmed that Streptozotocin (STZ) is capable of inducing diabetes and that effect causes hyperglycemia in albino rat. Moreover, we showed that the *A. muricata* ripe fruit pulp has potential to reduce the blood sugar level in albino rat. This study showed that *A. muricata* ripe fruit pulp has proportional increasing effect on PCV, Hb, platelets number, TWBC, neutrophil, eosinophil, basophil, monocytes and lymphocyte. Finally, we showed that increase in dose of ethanolic extracts of *A muricata* ripe fruit pulp restored liver function by significant increase in serum ALP, AST, and ALT in STZ-induced diabetic rat with reduction in the sugar level and increase in hematological parameters.

CONSENT

It is not applicable.

ETHICS APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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