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Chemical Profile, Antioxidant and Alpha-Amylase Inhibitory Activity of Leaves Extracts of *Annona muricata*: A Combined *In vitro* and *In silico* Study

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Abstract: Leaves of *Annona muricata* are commonly used for treating diabetes. This study was conducted to investigate the molecular mechanisms involved in the antidiabetic properties of leaves of *Annona muricata*. Leaves of Annona muricata were extracted separately with H_2O, hydromethanol (50% methanol), methanol, ethylacetate, and n-butanol. Chemical characterization of the extracts was performed by spectrophotometry and Gas chromatography-Mass Spectrometry (GC-MS) techniques. Biological activity was determined by α-amylase inhibition assays and molecular docking. The hydromethanol extract had a total phenolics concentration of 117.00±0.59 µg GAE/mg extract whereas; flavonoids were most abundant in the n-butanol extract accounting for 29.34±8.87 µg QE/mg extract. The n-butanol extract had the best FRAP value of 41.17±0.57 Vit C eqv mM, which was significantly higher than the value of the vitamin C reference. Estimated IC_{50} for all the extracts did not differ significantly but was significantly higher than the reference compound quercetin. All extracts inhibited α-amylase *in vitro* albeit significantly lower than acarbose. The hydromethanol extract had the highest inhibitory activity (53.31 ± 0.33%). Furthermore, chemical profiling of the hydromethanol extract revealed the presence of a variety of bioactive compounds. In silico analysis by molecular docking of the compounds identified by GC-MS on α-amylase revealed that the compounds had robust molecular interactions orchestrated by H-bonding and hydrophobic interactions. From the results, it can be concluded that extracts of *Annona muricata* possess antioxidant phytochemicals that inhibit α-amylase. Therefore, the results justify the use of the plant for the treatment of diabetes.

Keywords: diabetes mellitus; *Annona muricata*; alpha-amylase; phytochemical.

1. Introduction

Diabetes mellitus (DM) is non-communicable, characterized by a hyper-level of blood glucose which becomes known after a diagnosis [1, 2]. The World Health Organization (WHO) had opined that despite being a non-infectious disease, DM is a leading cause of death globally, with about 1.5 million deaths associated with the disease in 2019 [3, 4]. Several approaches had been adopted over the decades by researchers towards curbing or treating this disease. Based on the fact that two main types of DM exist [1, 5], type 1 DM (also known as Insulin-dependent diabetes mellitus) and type 2 DM (generally regarded as Non-insulin-dependent diabetes mellitus), therapeutic approaches used over the years have therefore focused on

different research niches [1, 6]. For type 1 DM, approaches such as insulin injection therapy, insulin receptor activation, insulin gene therapy had been tried amongst others, whereas for type 2 DM, the focus had been on the discovery of possible hypoglycemic agents targeted at reducing blood glucose levels to normal concentrations. Hence, orthodox drugs such as glibenclamide, acarbose, vildagliptin, voglibose, miglitol, metformin, and herbal extracts with bioactive compounds had been reported in the management of type 2 diabetes mellitus [7-11]. It is now common knowledge that the mechanism of action of some of these therapeutic agents used for the treatment of type 2 DM is by inhibition of one or more carbohydrate-hydrolyzing enzymes such as alpha-amylase, alpha-glucosidase, sucrase, etc., with the most commonly targeted enzymes being alpha-amylase and alpha-glucosidase [5, 12]. Alpha-amylases belong to the class of hydrolases, which hydrolyze α-1,4-glycosidic bonds in polysaccharides (such as starch, cellulose) to disaccharides and simple sugars [13, 14]. In type 2 DM, a sudden upsurge in blood glucose level in diabetic patients following a meal rich in carbohydrates (a condition referred to as postprandial hyperglycemia) is a major cause of untimely death. This is, however, prevented by the administration of drugs (e.g., acarbose, Glucobay, Precose) or herbal extracts that have inhibitory effects on α-amylase activity [8-11]. Inhibition of α-amylase helps suppress the rate of breakdown of carbohydrates in the meal, thereby slowing down the release of simple sugars into the bloodstream and preventing postprandial hyperglycemia [7, 13]. One of the side-effects of some orthodox drugs used in the past had been excessive inhibition of the carbohydrate-hydrolyzing enzymes, which in turn leads to abdominal discomfort and flatulence. Hence, since the last few decades, research is now being shifted to sourcing for enzyme inhibitors [7, 15] from natural sources (including plant extracts) for safer management of type 2 diabetes [7, 8].

Annona muricata (*A. muricata*), a plant species that belongs to the family of *Annonaceae*, is mostly found in the tropics and is well known for its various ethnomedicinal values [16]. The plant is traditionally used to treat insomnia, headache, malaria, diabetes, prostate enlargement, and as an antitumor [16, 17]. Most of these health benefits can be attributed to the plant's phytochemical composition [18-20]. There is, however, a limited volume of information associating the mechanism of action of *A. muricata* leaves (regarding its use in the management of type 2 DM) with alpha-amylase inhibition as well as the possible interaction of its phytochemical components with amino acid residues at the catalytic site of α-amylase, especially concerning bioactive components of plant extracts from species located in Nigeria, West Africa. Hence, in the present study, we comparatively investigated the inhibitory effects of various extracts (aqueous, hydromethanol, methanol, ethyl acetate, and n-butanol extracts) of *A. muricata* leaves on alpha-amylase activity and their relative antioxidant capacities. Further, we characterized the most bioactive extract of *A. muricata* leaves using Gas Chromatography-Mass Spectrometry (GC-MS) to identify the phytochemical compounds present that are responsible for its bioactivity. A detailed investigation of the potential interaction of the identified phyto-compounds with amino acid residues at the active site domain of pancreatic α-amylase was also carried out using molecular docking techniques.

2. Materials and Methods

2.1. Plant material: collection, processing, and extraction.

Leaves of *Annona muricata* were collected from Anyigba Town, Kogi State, Nigeria, and authenticated by the Department of Plant Science and Biotechnology, Kogi State

University, Anyigba. The leaves were washed, dried at room temperature, and grounded into fine powder. Five portions of 100 g of leaf powder of *Annona muricata* were weighed and suspended in 500 mL of the following solvents: water, 50% methanol (hydromethanol), methanol, ethylacetate, and n-butanol. The solvent extraction continued for 48 hr followed by filtration using Whatman No 1 filter paper. The filtrate was evaporated to give the solid extract.

2.2. Quantitative test for total phenolics.

The total phenol content of *Annona muricata* extracts was determined according to the method of Singleton *et al*. [21]. 2.5 ml of 10% Folin–Ciocalteau's reagent was added to dilutions of the plant extracts, mixed, and neutralized by adding 2.0 mL of 7.5% sodium carbonate. After incubating the mixture for 40 min at 8°C, the absorbance was measured spectrophotometrically at 765 nm. A calibration curve was plotted using gallic acid was plotted. The results were presented as gallic acid equivalent (mg GAE/g extract).

2.3. Quantitative test for total flavonoids.

The total flavonoid content of *Annona muricata* was determined by using the aluminum chloride's colorimetric assay method described by Bourhia *et al*. [22]. 1.5 mL of 2% $AlCl_3$ was added to dilutions of the plant extracts, mixed, and incubated for 60 min at room temperature. The absorbance was read using a spectrophotometer. The results were expressed as quercetin equivalent per gram of extract (mg QE/g).

2.4. Ferric ion reducing antioxidant power.

The methods of Benzie and Strain [23] were used to measure the Ferric reducing antioxidant power (FRAP) of the extracts. Briefly, 0.1 % of plant extract was mixed with 4 mL FRAP reagent (10:1:1 ratio of acetate buffer 300 mM pH 3.6 : TPTZ [2,4,6,-tripyridyl-s-triazine] 10 mM in 40 mM HCl : $FeCl_3.6H_2O$). The mixture was incubated in the water bath at 37°C for 5 minutes, and absorbance was taken at 593 nm. Ascorbic acid reference was prepared in the same manner. Given that FRAP value of Vit C = 2.0 (equivalent to 28.39 mM) [24], FRAP value of samples were calculated as follows:

FRAP value = (Change in sample absorbance / Change in Reference absorbance) x 2.0.

2.5. DPPH radical scavenging activity.

The Blois [25] method was using or the measurement of DPPH scavenging activity of the extracts. The assay is based on the ability of samples to donate hydrogen atoms and decolorize methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Antioxidants cause a change in the colors of DPPH from violet/purple to yellow. Briefly, 2.4 mL of 0.1 mM solution of DPPH in methanol was added to 1.6 mL of different concentrations of the plant extract or quercetin (6.25 -1000 µg/mL). The mixture was vortexed and left in the dark at room temperature for 30 min. Absorbance was after that measured spectrophotometrically at 517 nm. Percentage DPPH radical scavenging activity was calculated by the following equation:

$$\%\ \text{DPPH radical scavenging activity} = \frac{(\text{ABS}_{\text{reference}} - \text{ABS}_{\text{sample}})}{\text{ABS}_{\text{reference}}} \text{ x } 100$$

IC_{50} was derived from a plot of percentage inhibition against the concentration of the sample.

2.6. α-Amylase inhibition assay.

In vitro inhibition of α-amylase activity was accessed by the method described by Adefegha and Oboh [26] with slight modification. Briefly, 100 µL of 0.5% of plant extract was mixed with equal volume of alpha-amylase [EC 3.2.1.1, 1.0 mg/mL, prepared with 20 mM sodium phosphate buffer (pH 6.9) with 0.006 M sodium chloride] and incubated at 25°C for 10 min. At the expiration of the 10 min, 100 µL of 1% starch solution (prepared in 20 mM sodium phosphate buffer, pH 6.9) was added and incubated at 25°C for 30 min before adding 100 µL of dinitrosalicylic acid color reagent to stop the reaction. After that, the mixture was incubated in a boiling water bath for 5 min and cooled to room temperature. Absorbance was recorded at 540 nm against a distilled water blank. Acarbose was used as a reference and was assayed in a similar manner [26]. The alpha-amylase inhibitory activity was calculated using the formula:

$$\text{Percentage inhibition } (\%) = \frac{(\text{ABS}_{\text{reference}} - \text{ABS}_{\text{sample}})}{\text{ABS}_{\text{reference}}} \text{ x } 100$$

2.7. Gas chromatography-mass spectrometry analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out on the hydromethanolic extract of Annona muricata. The column temperature was set at 150-250°C at a flow rate of 79.5 mL/min. The eluates from GC were passed to the mass spectrometer to determine the mass spectral pattern. Identification of the eluted compounds was made by matching the Retention time, fragmentation pattern, and mass spectral data with the National Institute of Standard Technology (NIST) library database.

2.8. In silico analysis.

Investigation into the molecular interactions between α-amylase and compounds from *Annona muricata* was carried out using AutoDock Tool v1.5.6. The three-dimensional crystal structures of pancreatic α-amylase PBD ID: 5F0F were downloaded from the RCSB Protein Data Bank. Ligand structures were sketched and optimized by ChemDraw. The ligands were docked against α-amylase using the grid box size 80 X 74 X 68 Å in the x, y, and z dimensions with 0.375 Å spacing. PyMol was used to view the molecular interaction, while Discovery Studio software was used to analyze the protein-ligand interactions.

2.9. Statistical analysis.

GraphPad InStat version 3.05 was used for data analysis. Values are reported as mean ± standard deviation (SD) of three determinations. Data were analyzed by one-way analysis of variance (ANOVA) using the Tukey post hoc test. Values were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1. Results.

The total phenolic and flavonoid contents of solvent extracts of *Annona muricata* are presented in Table 1. The highest phenolic content was obtained from the hydromethanol extract 117.00±0.59 µg GAE/mg extract. Flavonoids were the best extract, with butanol

accounting for the total flavonoid content of 29.34±8.87 µg QE/mg extract. Results of in vitro antioxidant activity of the extracts measured using the FRAP and DPPH radical scavenging method is shown in Table 2. The FRAP value of the butanol extract (41.17±0.57 Vit C eqv mM) was the highest and significantly higher than the reference compound, Vitamin C (28.39±0.00 Vit C eqv mM). The DPPH radical scavenging capacity (IC_{50}) of all the extracts did not differ significantly (ranged between 51.92±1.42 - 54.29±3.13 µg/mL) but were significantly higher than the reference compound quercetin (35.67±0.29 µg/mL).

Table 1. Total Phenolics and flavonoid concentration of *Annona muricata* leave extract

Extract	Total Phenolic (µg GAE/mg extract)	Total Flavonoid (µg QE/mg extract)
Aqueous	67.71±8.00[a]	10.17±1.69[a]
Hydromethanol	117.00±0.59[b]	13.13±1.67[b]
Methanol	92.54±3.67[c]	12.04±2.82[c]
Ethylacetate	40.39±2.74[d]	20.35±1.80
n-Butanol	40.21±2.55[d]	29.34±8.87[a,b,c]

Values are expressed as mean±SD of three determinations. [a–d] Different superscript letters for a given value within a column are significantly different from each other, $p<0.05$.

Table 2. Antioxidant activity of extracts from leaves of *Annona muricata.*

Sample/Extract	FRAP Value (Vit C eqv in mM)	DPPH scavenging activity (IC_{50} µg/mL)
Reference	28.39±0.00[a]	Quercetin: 35.67±0.29
Aqueous	23.00±0.43 [b]	51.92±1.42[e]
Hydromethanol	27.40±2.13 [a]	54.29±3.13[e]
Methanol	36.77±0.43 [c]	51.96±4.39[e]
Ethylacetate	39.18±2.13 [c,d]	52.73±2.50[e]
n-Butanol	41.17±0.57 [d]	53.65±2.07[e]

Values are expressed as mean±SD of three determinations. [a–d] Different superscript letters for a given value within a column are significantly different from each other; [e]significantly different from Quercetin, $p<0.05$.

Antidiabetic activity measured by the ability of the extracts to inhibit α-amylase revealed that aqueous and hydromethanol extracts possessed the highest inhibitory activity; 48.84 ± 0.23% and 53.31 ± 0.33%, respectively. The inhibitory activity of the two extracts did not differ significantly but were both significantly lower than values obtained for the reference drug acarbose (87.24 ± 1.76%) (Table 3). The results of the GC-MS analysis shown in table 4 reveals that the hydromethanolic extract is rich in fatty acids, including n-Decanoic acid, Oleic Acid, n-Hexadecanoic acid, and other phytochemicals of medicinal value. These phytochemicals were found to interact with pancreatic α-amylase *in silico* with binding affinity ranging between 4.1 – 8.7 kcal/mol (Table 4 and Figure 1). The molecular interactions involved both H-bonding and hydrophobic interactions, as shown in Figure 1.

Table 3. Alpha-amylase inhibitory activity of extracts from leaves of *Annona muricata.*

Sample/Extract	Inhibition of α-amylase activity (%)
Acarbose	87.24 ± 1.76[a]
Aqueous	48.84 ± 0.23 [b]
Hydromethanol	53.31 ± 0.33[b]
Methanol	43.01 ± 0.87[c]
Ethylacetate	14.00 ± 3.35[d]
n-Butanol	25.49 ± 3.44[e]

Values are expressed as mean±SD of three determinations. a–e Different superscript letters for a given value within a column are significantly different from each other, $p<0.05$.

Table 4. Interaction of compounds identified by GC-MS analysis of hydromethanolic extract of *Annona muricata* assessed by molecular docking.

Compound	Retention time	Compound name	% Area	Chemical formula	Molecular weight (g/mol)	Binding affinity (kcal/mol)
1	14.929	n-Decanoic acid	0.32	$C_{10}H_{20}O_2$	172.26	-4.1
2	14.952	Oleic Acid	0.06	$C_{18}H_{34}O_2$	282.50	-4.5
3	14.998	1-Eicosene	0.09	$C_{20}H_{40}$	280.50	-4.2
4	15.100	n-Hexadecanoic acid	0.23	$C_{16}H_{32}O_2$	256.42	-3.5
5	15.177	Octadecanoic acid	0.10	$C_{18}H_{36}O_2$	284.50	-4.7
6	15.411	Tetradecanoic acid	0.26	$C_{14}H_{28}O_2$	228.37	-4.5
7	15.701	Heptadecanolide	0.22	$C_{17}H_{32}O_2$	268.40	-7.7
8	15.799	Cyclotetracosane	0.43	$C_{24}H_{48}$	336.60	-8.7
9	15.986	Tetracosanoic acid	1.22	$C_{24}H_{48}O_2$	368.60	-4.7
10	16.060	6-Octadecenoic acid, (Z)-	0.15	$C_{18}H_{34}O_2$	282.50	-4.2
11	16.598	Octadec-9-enoic acid	0.06	$C_{18}H_{34}O_2$	282.50	-4.2
12	17.994	Cyclopentadecanone, 2-hydroxy-	0.49	$C_{15}H_{28}O_2$	240.38	-7.9
13	18.028	1-Octadecene	0.53	$C_{18}H_{36}$	252.50	-5.1
14	18.487	3-Eicosene, (E)-	0.20	$C_{20}H_{40}$	280.50	-4.6
15	19.066	13-Tetradecen-1-ol acetate	0.41	$C_{16}H_{30}O_2$	254.41	-4.5
16	19.961	Erucic acid	1.32	$C_{22}H_{42}O_2$	338.60	-4.6
17	20.109	5-Eicosene, (E)-	0.64	$C_{20}H_{40}$	280.50	-4.8
Acarbose	-	-	-	-	-	-11.9

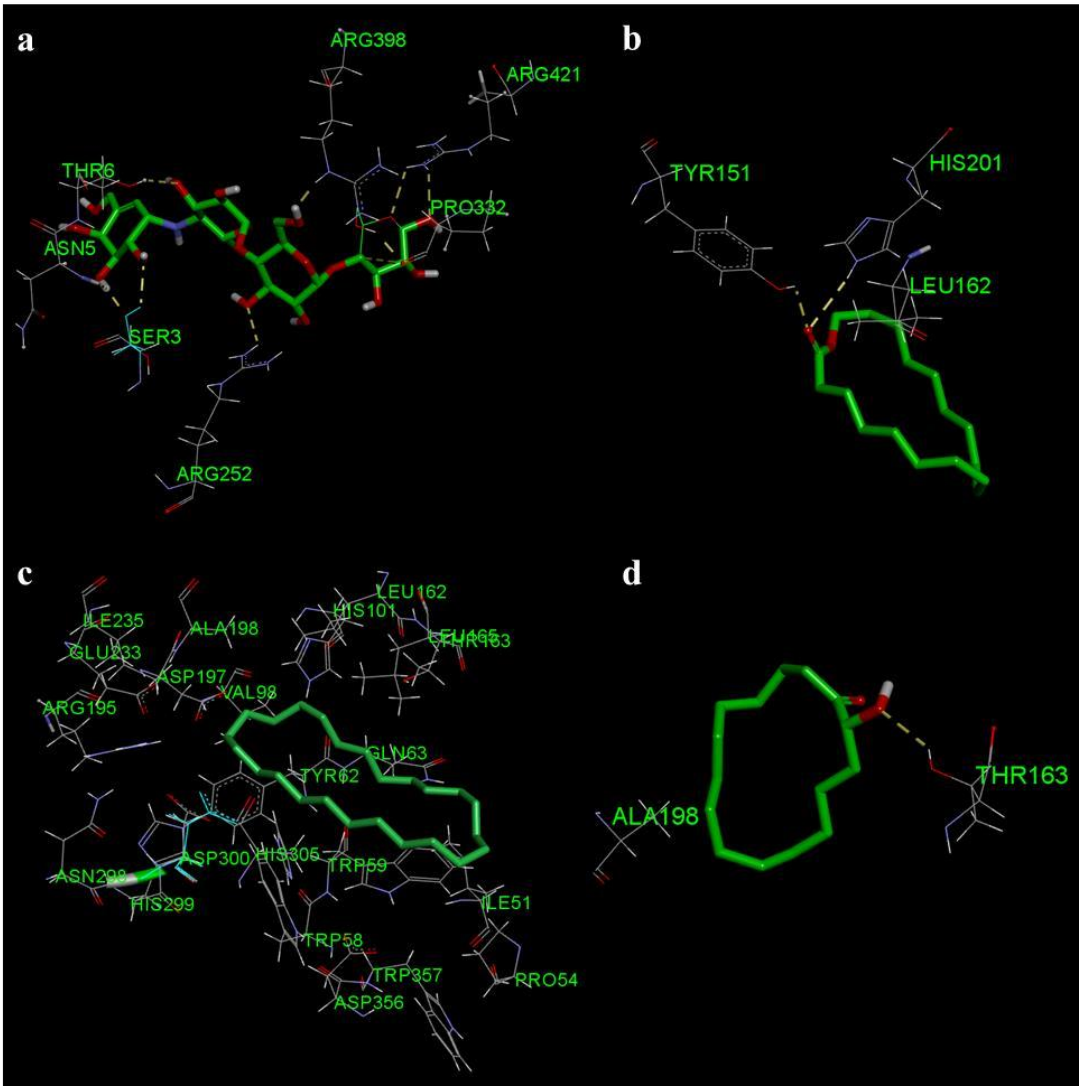

Figure 1. Molecular docking of selected compounds from *Annona muricata* into the binding site of α-amylase (PDB ID: 5E0F), acarbose (a), compound 7 (b), compound 8 (c), and compound 12 (d) represented as sticks. The interacting residues are represented as lines. Hydrogen bonds are shown as yellow dashes.

3.2. Discussion.

Diabetes is a disease affecting over 400 million adults, and therefore there is a need to discover a new drug to curb its scourge [27]. Most currently approved antidiabetic drugs have serious side effects and are costly [5, 13, 28]. In most traditional African and Asian settings, herbal formulations are used to manage diabetes and its complications [29, 30]. The use of herbal plants is gaining increased attention as they are deemed beneficial and atoxic. Researches that aim at drug discovery from plants fondly target specific pathways responsible for the pathogenesis of the disease [29]. In diabetes, inhibition of amylase is seen as one of the potent strategies; to minimize hydrolysis of carbohydrates, thereby managing glucose concentration in blood [30, 31].

In this present work, the phytochemical content, antioxidant activity, and α-amylase inhibitory activity of extracts from *Annona muricata* were investigated. Phytochemicals present in plants are responsible for the antioxidant activity they elicit *in vivo* or *in vitro* and, by extension, disease treatment [19, 20, 32, 33]. Our phytochemical analysis of the plant extracts revealed its richness in phenolic and flavonoid compounds. Different solvents extracted varying quantities of the phytochemicals, which could be attributed to the role of polarity in extraction efficiency. Other reports have shown that different plant parts of *Annona muricata* are rich in phytochemicals [17, 34]. However, this work presents a comparative approach and a guide for the choice of solvent where phenolic and flavonoid extract is desired. Oxidative stress has been implicated in most metabolic diseases that plague humans hence, the motivation to screen natural sources of antioxidants. Oxidative stress, resulting from increased levels of oxidants, decreased activity of antioxidant enzymes, and consequently increased levels of oxidatively damaged macromolecules, has also been implicated in the middle and advanced stages of diabetes mellitus [35]. From the results of this study, extracts of *A. muricata* possess antioxidant properties. In fact, antioxidant activity measured by the FRAP method revealed that the extracts were more potent antioxidants than Vitamin C. Similar results suggesting high antioxidant power have been reported in the literature [15, 36].

At the onset of diabetes, individuals present clinical symptoms such as sustained hyperglycemia [1, 8]. Amylases are responsible for the breakdown of complex carbohydrates to monosaccharides; therefore, inhibition of amylases is a viable route of tackling hyperglycemia [6, 7, 37]. Amylase inhibiting drugs such as acarbose are some of the most commonly prescribed for the management of diabetes [7, 38]. In this study, the inhibitory activity of the extracts was tested against α-amylase. All the extracts had α-amylase inhibitory activity, albeit less active than acarbose. This result complements other reports on the antidiabetic effects of crude extracts of *Annona muricata*. Purification of the active components of the extracts could yield pure compounds with improved inhibitory activity [7, 39]. More so, pursuing further studies on the crude extract is justified owing to the anticipated safety of plant-derived compounds.

To identify the active phytoconstituents in the extracts responsible for its antioxidant and α-amylase inhibitory activity, GC-MS analysis was performed. Among the compounds identified were n-Decanoic acid, Oleic acid, 1-Eicosene, n-Hexadecanoic acid, Octadecanoic acid, Tetradecanoic acid, Heptadecanolide etc. The compounds identified have diverse biological properties [40]. It could be concluded that these compounds act synergistically to affect biological activity. Further insight into the possible role of the identified compounds in regulating glucose levels via pathways involving α-amylase was given by *in silico* analysis [41,

42]. Molecular docking analysis of compounds identified by GC-MS with α-amylase showed significant interactions measured by binding energy. The compounds bound to the enzyme's active site forming stable interactions by hydrogen bonding and hydrophobic interactions with nearby amino acids [41-43]. Our results suggest that the extracts are rich in antioxidant phytochemicals that have amylase inhibitory activity.

4. Conclusions

The results of this study reveal that extracts of *Annona muricata* are promising sources of antioxidant compounds with antidiabetic properties. Based on the finding of the *in silico* analysis of GC-MS identified compounds, Heptadecanolide, Cyclotetracosane, Cyclopentadecanone 2-hydroxy- have the highest affinity for the active site of α-amylase. These compounds can therefore be purified, tested *in vivo*. This work provides direction for future research in drug discovery for diabetes therapy.

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Conflicts of Interest

The authors declare no conflict of interest.

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