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Exploring antioxidant and antidiabetic potential of *Mutingia calabura* (Kerukupsiam) leaf extract: *In vitro* analysis and molecular docking study

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ABSTRACT

Antioxidant activity and antihyperglycemic constituents and of traditional medicinal plants are currently the preferred therapeutic means of treatment and management of diabetes because of the undesired adverse effect of synthetic drugs. *Muntingia calabura* (Kerukupsiam) leaves and other parts are considered as alternative natural sources of treatment for diabetes. Ultrasonic assisted extraction is a novel approach for extraction of phyto-constituents which gives high extraction yield of bioactive compounds. However, there has been no published information presently on the use of ethanol ultrasonic assisted extraction method for assessment of antioxidant and antidiabetic activities of *M. calabura leaves.* Hence, the current study aims to evaluate the *in vitro* antioxidant and antidiabetic activities of *M. calabura* leave extract. IC_{50} analysis was done to determine theinhibitory concentration and the results obtained from 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay showed IC_{50} of gallic acid to be 1.0 μg/ml, which is lower than *M. calabura* leaves extract at 2.54 μg/ml, indicating that only small concentration of gallic acid was required to inhibit the free radicals at 50 %. However, IC_{50} analysis for amylase inhibition showed that *M. calabura* extract had 44.39 μg/ml antidiabetic activity compared to acarbose with 57.1 μg/ml activity. This indicates that *M. calabura* leaves extract has a better inhibition on amylase activity compared to the acarbose which is a synthetic drug. Further still, *in silico* study was carried out and the molecular docking result of eight ligands against amylase indicates quercetin had the least binding free energy of −9.1 kcal/mol, indicating the strongest interaction. Using Lineweaver-Burk plot, the results showed a competitive inhibition, hence, it was justified that *M. calabura* has the potential to manage diabetes and other diseases related to free radicals.

Introduction

Diabetes is a chronic disease that has become a global threat to most people around the world and affects a large percentage of world proportion due to the lack of insulin production, intolerance of glucose during pregnancy, and also other factors like depletion of protein in tissues and lack usage of glucose by the body cells [1]. Diabetes mellitus is classified as a metabolic disorder that is characterized by hyperglycaemia, which is caused by low level of insulin production by the pancreas or the inability of insulin generated to control the blood glucose levels [2]. Type 2 diabetes also known as insulin-independent diabetes mellitus is the most common and prevalent form of diabetes, which accounts for 90–95 % of diagnosed cases wherein enough insulin is produced by the pancreas but the body unable to effectively use insulin during glucose metabolism [3]. According to World Health Organization forecast, individuals with diabetes is projected to increase to

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700 million by 2045 [4].

There are currently some therapeutic agents available in the market, ranging from oral hypoglycaemic agents such as; biguanidine, sulfonylurea, thiazolidinediones, meglitinides and α-glucosidase inhibitors to insulin therapy for management and treatment of diabetes. However these antidiabetic drugs have undesired adverse effects such as diarrhoea, ketoacidosis, hypothyroidism, tachycardia weight gain and others [5]. Plants are generally known as good source of therapeutic agents for treatment of diseases, and several of them namely; *Morus alba*, *Muntingia calabura*, *Allium sativum*, *Zingiber officinale*, *Panax ginseng*, *Moringa oleifera* and so on have been reported [6,7] to possess anti-hyperglycemic properties. Bioactive compounds from these plants with antidiabetic properties have gain popularity as reliable and alternative approach for management of diabetes because they are relatively safer inexpensive and do not cause undesired adverse effect unlike the synthetic drugs [8]. The bioactive components of these traditional medicinal plants with antidiabetic activities include; saponins, flavonoids, phenolics, glycosides, carotenoids, terpenoids, alkaloids. Generally, six notable mechanisms including inhibition of glucose metabolism, improved insulin secretion, decreased lipid peroxidation, inhibition of pro-inflammatory pathway, enhancement of plasma anti-oxidant capacity, and insulin-like effect has been considered as mechanism of action of antidiabetic compounds [6].

Muntingia calabura (Kerukupsiam) belongs to the family *Muntingiaceae* and a native plant of Central America, but widely found in countries with tropical climates like Malaysia, Indonesia, Philippines, India, China, and Brazil [9]. It is the most commonly known roadside plant in Malaysia [8]. The plant has been used over time as a traditional medicinal herb against illnesses like flu, cough, and fever. Different parts of the plant possess high therapeutic value, and their antiseptic, antioxidant, antidiabetic, anti-inflammatory, antihypertensive, laxative, gastroprotective and anti-gout properties have been reported [10].

Molecular docking is one of the most used techniques in drug design because of its ability to predict with a considerable amount of accuracy, the conformation of ligands within the specific target binding site. Predicting a ligand's primary binding mode with a protein having a known three-dimensional structure is the goal of ligand–protein docking. For the purpose of lead chemical optimization, docking can be used to do virtual screening on a large number of compounds, rank the results, and indicate how the ligands block the target. Based on earlier study [11] on the antioxidant activity of *M. calabura* using water extraction method, the major constituents detected by Gas chromatography–mass spectrometry (GC–MS) were alpha-terpineol, geraniol, citronellol and eugenol. Meanwhile, gallic acid, fructose, catechin and quercetin were detected by LC-MS. Since water and ethanol are polar, the major compounds extracted [11]were used as ligands in molecular docking of the current study.

Although some studies on the antidiabetic activity of *M. calabura* leaf extract have been recently reported [8,12], experimental evidence on the antidiabetic and antioxidant properties of the plant using molecular docking, and study on the mechanism of antidiabetic action of the plant extract has not been investigated. Moreover, this is the first scientific report utilizing ethanol ultrasonic assisted extraction of *M. calabura* leaves for in-vitro antidiabetic and antioxidant study. Ultrasonic assisted extraction was utilized in the extraction process of the *M. calabura* leaves extract because this novel approach gives higher extraction yield of bioactive constituents and also have higher recovery rate of other bioactives like phenolic compounds than using the conventional water/ethanol extraction approach [11]. Thus, the present study was conducted to assess the *in vitro* antioxidant and antidiabetic activities of *M. calabura* leaves using ultrasonic assisted extraction approach.

Materials and methods

Plant material preparation

Mutingia calabura leaves were obtained from Institute of Sustainable Agro Technology, Perlis and conveyed to the lab within 8 h for the experiment. The plant material was washed with distilled water. The leaves of *M. calabura* were dried at 50℃ using a vacuum oven [13]. The leaves were then ground to powder and kept under storage away from light and humidity [2].

Extraction process

One gram of powdered leaves were weighed and placed in a conical flask. 100 mL of 90 % ethanol was measured and poured into the conical flask. The sonicator was set to 450 rpm and 25℃ along with a frequency of 20 kHz. The samples were extracted using an ultrasonic assisted extraction according to the procedure described [14]. Rotary vacuum evaporator was used to remove excess solvent and concentrate the leave extract [1]. The process of centrifugation was carried out at 3000 rpm for about 10 min. A micropipette was later used to collect the supernatant.

1,1-diphenyl-2-picrylhydrazyl (DPPH) solution effect on the extract

A 0.2 mM concentration of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was prepared in ethanol. The DPPH solution (2 mL) was added into the 200 μL of extract and 800 μL of ethanol [15]. The blank solution was ethanol and 2 mL of DPPH solution together with 800 μL of ethanol was the negative control. Gallic acid was used as a standard positive control [16]. The mixture was left in the dark for 30 min and a spectrophotometer was used to determine the absorbance at 517 nm. The percentage of inhibition was calculated using Equation 1:

% inhibition = (absorbance of control (As)/ absorbance of sample (Ac)) x 100 % (1).

Inhibitory effect of extract on amylase activity in starch solution

Starch solution of 1 g/10 mL was prepared in test tubes at the concentrations of 20, 40, 60, 80, and 100 μg/mL of the extract by adding into 0.05 M of sodium phosphate buffer solution, pH 7.0. About 200 μL of the extract prepared previously was mixed with 200 μL of amylase solution and was left to incubate at 30℃ for 10 min. Then, 200 μL of starch were added and incubated for 3 min. Then a fixed time was set for the reaction to occur, and the samples were taken to record its absorbance value. The negative control for this assay was DMSO while the positive control was prepared in the similar way along with the same concentration using acarbose. A sodium phosphate buffer was used as the blank solution and the absorbance was set at 517 nm. The percentage of inhibition was calculated using Equation 1.

Enzyme kinematic study

The Lineweaver-Burk plot was used to show the kinetics of the extract. This assay was carried out with varying substrate (starch) concentrations at 20, 40, 60, 80 and 100 μM with or without the extracts. With the aid of UV–vis spectrophotometer the solution either with or without the samples was tested for inhibitory effects of the extract towards the enzyme at 540 nm.

Inhibitory concentration at 50 % inhibition (IC_{50}) analysis

The IC_{50} method for amylase inhibitory activity was done by preparing different concentrations (20, 40, 60, 80, and 100 μg/mL) of the plant leave extract and acarbose in sodium phosphate buffer (pH 7.0). The values of IC_{50} of acarbose and the plant leaves extract were

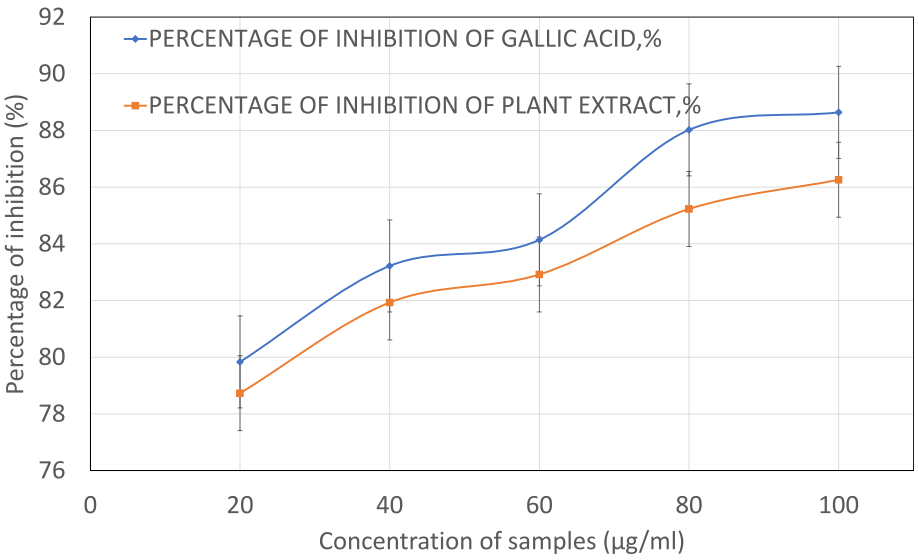


Fig. 1. Percentage inhibition of DPPH by leaves extract of *Mutingia calabura* and gallic acid.

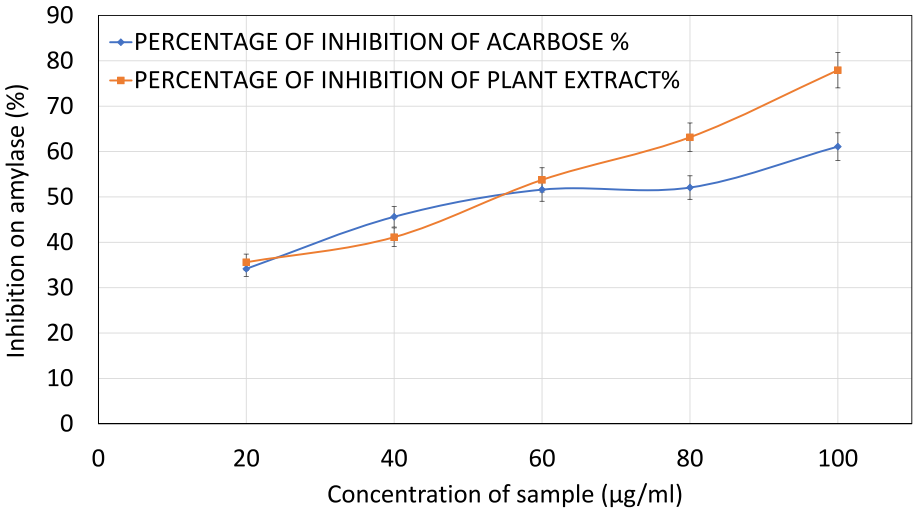


Fig. 2. Percentage amylase inhibition assays by leaves extract of *Mutingia calabura* and acarbose.

determined based on the regression method through Microsoft Excel and a graph was obtained by probit analysis. A similar method was performed for antioxidant activity by preparing different concentrations of extract and gallic acid, which were dissolved in 90 % ethanol to give 20, 40, 60, 80, and 100 µg/mL. The same method was used for amylase inhibitory assay.

Correlation of antioxidant and amylase inhibitory activities

The correlation between the antioxidant activity and the amylase inhibitory activity was carried out by plotting a graph with different concentrations of *M. calabura* leaves extract for the antioxidant and amylase inhibitory activities.

Determination of enzyme inhibition type

The type of enzyme inhibition was based on the Lineweaver-Burk plot where 1/V against 1/ [amylase] and 1/ [starch] and the pattern of the plot. The values of Km and Vmax were obtained from Michaelis-Menten equation and also from Microsoft Excel using solver function. The reason for using line weaver plot was to investigate the enzyme kinetics, that is, inhibition of amylase. The type of enzyme inhibition could be competitive, non-competitive or uncompetitive at the end of the experiment.

Molecular docking experiments

The protein crystal structure of Human Salivary Amylase (1C8Q) was obtained from protein data bank. The hetero atoms and the water molecules of the structure were removed using AutoDock tools, and the input files were prepared using the same tool. The polar hydrogen atoms and Kollman charges were added to the protein macromolecule. The ligands were prepared using Pymol and these compounds were docked with alpha-amylase enzyme using AutoDock Vina program, version 1.2 [17,18]. The receptor protein was kept rigid in each docking experiment. Docking was done in a grid box of 60 x 60 x 60 Å dimensions based on active site of the protein [19]. Starting at random positions, a total of 20 independent docking runs were done for each compound. Best binding free energies among the average 20 runs were reported here. The interaction of the ligand and protein was analysed in 2-D with Discovery Studio [20].

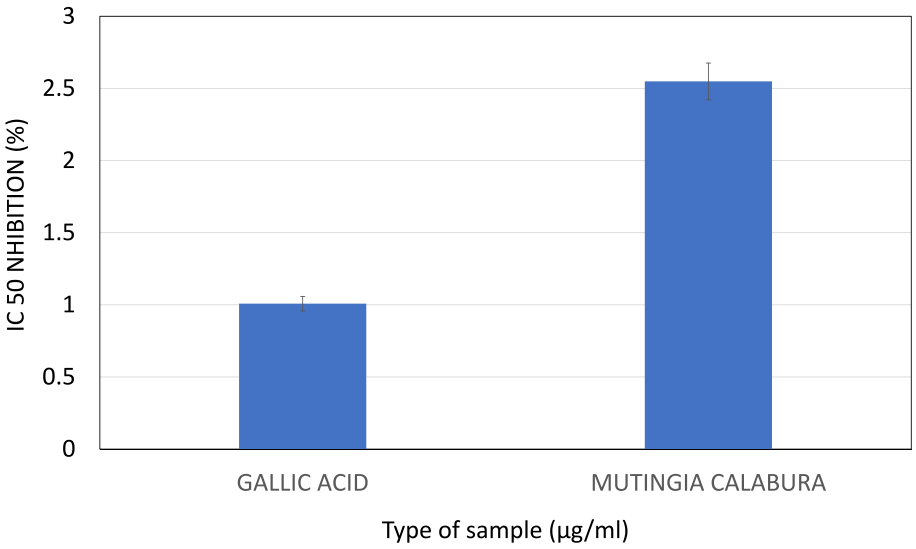


Fig. 3. IC_{50} inhibition on DPPH scavenging assay of *Mutingia calabura* leaves extract and gallic acid.

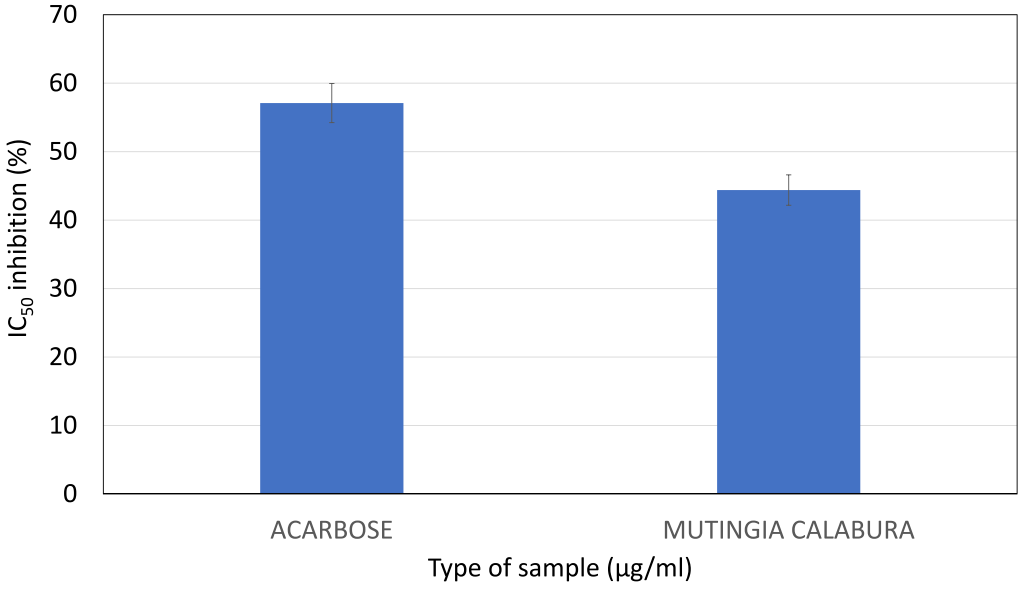


Fig. 4. IC_{50} amylase inhibition of *Mutingia calabura* leaves extract and acarbose.

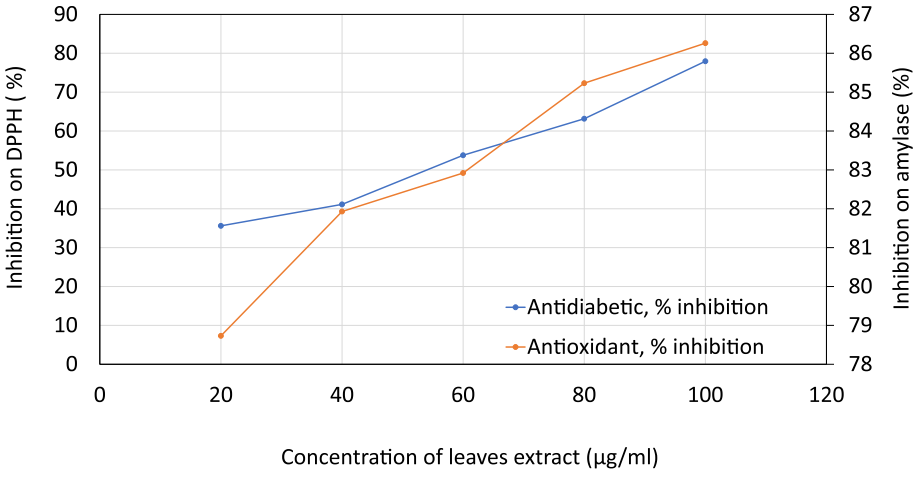


Fig. 5. Correlation graph of DPPH radical scavenging assay and amylase inhibition assay.

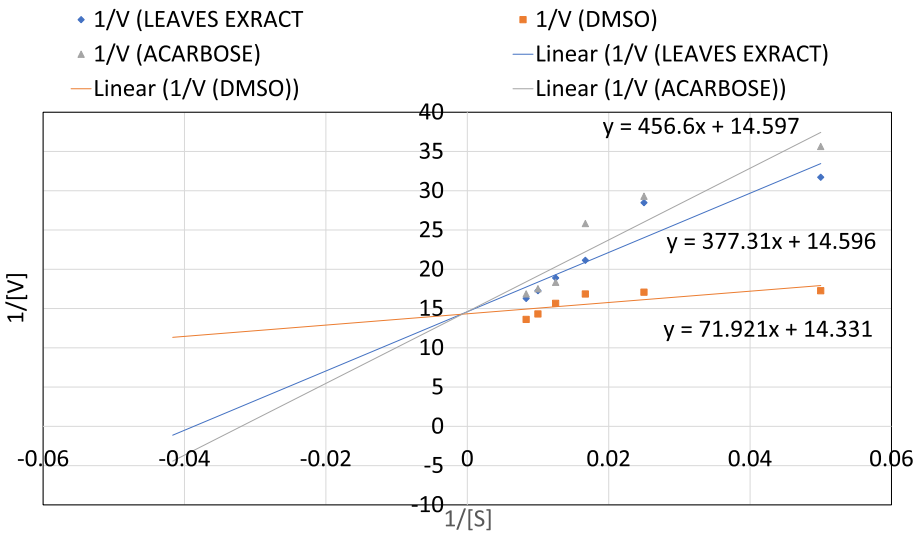


Fig. 6. Line weaver-Burk Plot of amylase inhibition by *Mutingia calabura* leaves extract.

Table 1
Enzyme Kinetic Parameters.

Sample	V_{max} (abs/min)	K_m (μM)
Mutingia calabura leaves extract	0.068512	25.8495
Acarbose (positive control)	0.068510	31.2804
Dimethyl sulfoxide (negative control)	0.06978	5.0186

Results and discussions

1, 1-Diphenyl-2-Picryl-hydrazyl (DPPH) radical scavenging assay

Fig. 1 shows the comparison between the percentage of inhibition on DPPH of leaves extract of *M. calabura* and gallic acid which is the positive control. Gallic acid showed the highest percentage of DPPH inhibition (88.64 %) while *M. calabura* leaves extract showed 86.26 % inhibition at the concentration level of 100 μg/mL for both samples. The antioxidant activity of the *M. calabura* leaves extract increased with increasing concentration probably due to the high concentration of phenolic compounds in the higher amount of the extracts as reported [21].

Amylase inhibition by Muntingia calabura leaves extract

Fig. 2 depicts the comparison between amylase inhibition percentage by *M. calabura* leaves extract and acarbose in starch solution. Acarbose is a synthetic medicine used to treat diabetes mellitus whose mechanism of action is based on amylase activity inhibition, thus hindering starch conversion to glucose. The result in Fig. 2 shows that the percentage amylase inhibition for both *M. calabura* leaves extract and acarbose increased gradually as the sample concentration increases. Acarbose usually shows a higher inhibition percentage when compared to plant leaves extract at the same concentration. However, based on the experimental data collected, *M. calabura* leaves extract has shown the highest percentage of inhibition at 77.94 % while acarbose shows at 61.1 % at100 μg/mL. The plant extract showed potent inhibition of α-amylase activity.

IC_{50} analysis on 1, 1-Diphenyl-2-Picryl-hydrazyl (DPPH) radical scavenging assay

Fig. 3 shows IC_{50} inhibition on DPPH scavenging assay of *M. calabura* leaves extract and gallic acid. From the result depicted in Fig. 3, it is observed that IC_{50} of gallic acid is 1.0 μg/mL, which is lower than that of the plant leave extract at 2.5 (μg/mL). This result implies that only small concentration of gallic acid was required to inhibit the free radicals at 50 %. Gallic acid is known to be a synthetic pure antioxidant which shows low IC_{50} values compared to other sample extracts. The IC_{50} value of 2.5 (μg/mL) obtained by *M. calabura* leave extract proves that it has antioxidant property.

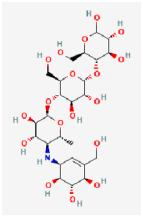
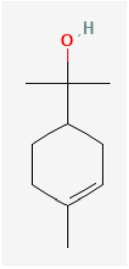
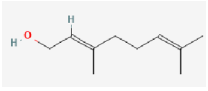
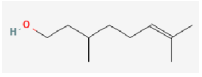
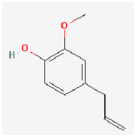
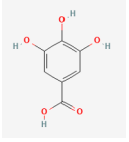
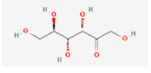
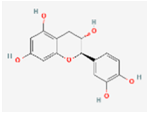
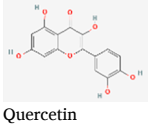
IC_{50} analysis on amylase inhibition assay

Fig. 4 shows IC_{50} amylase inhibition by *M. calabura* leave extract and acarbose. It is observed in the figure that the concentration of acarbose that inhibit 50 % of amylase is 57.1 μg/mL, while that of *M. calabura* leaves extract was only 44.39 μg/mL. This indicates that acarbose requires a higher dose than *M. calabura* leaves extract to inhibit the amylase activity at 50 % inhibition response. It is known that the lesser the IC_{50} values of a sample, less amount or concentration is sufficient to show 50 % inhibition, Hence, if lesser is the IC_{50} value then the better the is the result. Based on the result of IC_{50}, it is found that *M. calabura* leave extract has a better inhibition capability on amylase activity compared to acarbose which is a synthetic therapeutic agent.

Correlation study of antioxidant and amylase inhibitory activities

The correlation study of *M. calabura* leaves extract was between antioxidant activity and amylase inhibitory properties. The relation is done based on the response of inhibition activity on free radicals and amylase enzyme. The potentials of *M. calabura* leaves extract were tested in two assays, which are amylase inhibition assay and DPPH radical scavenging assay. Hence, the correlation study obtained was determined from these assays. Fig. 5 shows the correlation graph of *M. calabura* leave extract for DPPH radical scavenging assay and amylase inhibition assay. The graph shows that the inhibition percentage of DPPH radical scavenging assay and percentage of amylase inhibition assay increases gradually with increase in leaves extract concentration. The antidiabetic potential depicted by *M. calabura* explains that the presence of phytochemicals like flavonoids are working in synergy. These flavonoids are known antidiabetic compounds found commonly in medicinal plants [22]. This working synergy indicates that phytochemical components such as flavonoids are also responsible for antioxidant properties of *M. calabura* leaves extract. Moreover, the coefficient of correlation is 0.9809, indicating that the graph showed a strong correlation between anti-oxidative properties and anti-diabetic properties of the *M. calabura* leaves extract.

Table 2
Binding free energies calculated by AutoDock vina for ligand binding to α-amylase.

Ligand 2D Structure	AutoDock vina binding free energy (kcal/mol)
Acarbose (positive control)	−8.2
 Alpha-Terpineol	−5.9
 Geraniol	−5.1
 Citronellol	−4.9 −5.9 −6.1
 Eugenol	−5.1
 Gallic acid	−8.8 −9.1
 Fructose	
 Catechin	
	
 Quercetin	

Kinetic study of amylase enzyme

The mode of inhibition of the aqueous extract of M. calabura leaves extract on amylase activity was determined using the Lineweaver-Burk plot as shown in Fig. 6 which revealed that the extract displayed a competitive inhibition of the enzyme activity. The y-intercept (1/Vmax) remains unaffected, and slope (Km/Vmax) increased with the concentration of the inhibitor (sample extracts). This explains the active components in the leaves extract compete with the substrate to bind to the active site of the free enzyme and altering the structure of the enzyme and affect the performance of the enzyme which in turn reducing the breakdown of starch to simple sugar [23].

Based on the graph plotted and Table 1, the Vmax of *M. calabura* leave extract is 0.068512 abs/min while the Km value 25.8495 μM and the Vmax of DMSO which is the negative control in this assay is 0.06978 abs/min and the Km value obtained is 5.0186 μM. On the other hand, the Vmax of Acarbose which is the positive control in this assay is 0.068510 abs/min while the Km value obtained is 31.2804 μM. Based on the values obtained the Vmax of DMSO is highest compared to Vmax of *M. calabura* leave extract and also the Vmax of Acarbose. The Vmax of Acarbose is the lowest in this assay. This shows that there is highest maximum reaction rate in the DMSO compared to acarbose which showed lowest reaction rate. Further still, *M. calabura* leaves extract comparably has a slightly higher Vmax value than acarbose but with lower Vmax than DMSO. This indicates that there are inhibitors present in *M. calabura* leaves extract. The Km values of DMSO, acarbose and *M. calabura* leaves extract are 5.0186 μM, 25.8495 μM and 31.2804 μM, respectively. The varying Km value indicates that the three substrates (samples) have different affinities for the amylase enzyme; *M. calabura* leaves extract have higher Km value than acarbose, which implies that its inhibitors have weak binding, that is, low affinity with the enzyme than acarbose [24].

Molecular docking

As shown in the results of correlation coefficient study, antioxidant compounds regulate the antidiabetic activity of *M. calabura* leaves extract, hence, molecular docking studies were performed to investigate and further consolidate the result of antioxidant and antidiabetic activity of the reported chemical constituents (see Table 2). Result for the binding free energy of nine ligands including one positive control are represented in Table 2. Quercetin and catechin showed the potent binding affinity of −9.1and −8.8 kcal/mol, respectively. Quercetin present in various natural sources as a flavonoid has demonstrated *in vitro* and *in vivo* antidiabetic properties [25]. As expected from the binding free energy results, quercetin showed the highest number of conventional hydrogen bond, a total of six with α-amylase compared to other ligands tested. The active site amino acid residues of α-amylase are Asp 197, Glu 233 and Asp 300. These three amino acids are interacting with quercetin with two of them forming conventional hydrogen bond as depicted in Fig. 7.

Conclusion

Mutingia calabura leaves extract was discovered to have a capacity to act as an amylase inhibitor and obtaining a higher value of antioxidant in the presence of flavonoids. Based on this research study *M. calabura* leaves extract obtained a value of 78.73 % in DPPH radical scavenging assay, which is slightly lesser than the value obtained by gallic acid, which is 79.33 %. On the other hand, IC_{50} analysis for DPPH radical scavenging assay was also done where *M. calabura* leaves extract obtained a value of 2.5 μg/ml, while gallic acid obtained a value of 1.0 μg/ml. In the amylase inhibitory assay, the *M. calabura* leaves extract obtained an inhibition percentage value of 77.94 %, while the acarbose obtained is about 61.1 %. In addition, IC_{50} analysis for this anti-diabetic assay was also done where the *M. calabura* leaves extract obtained a

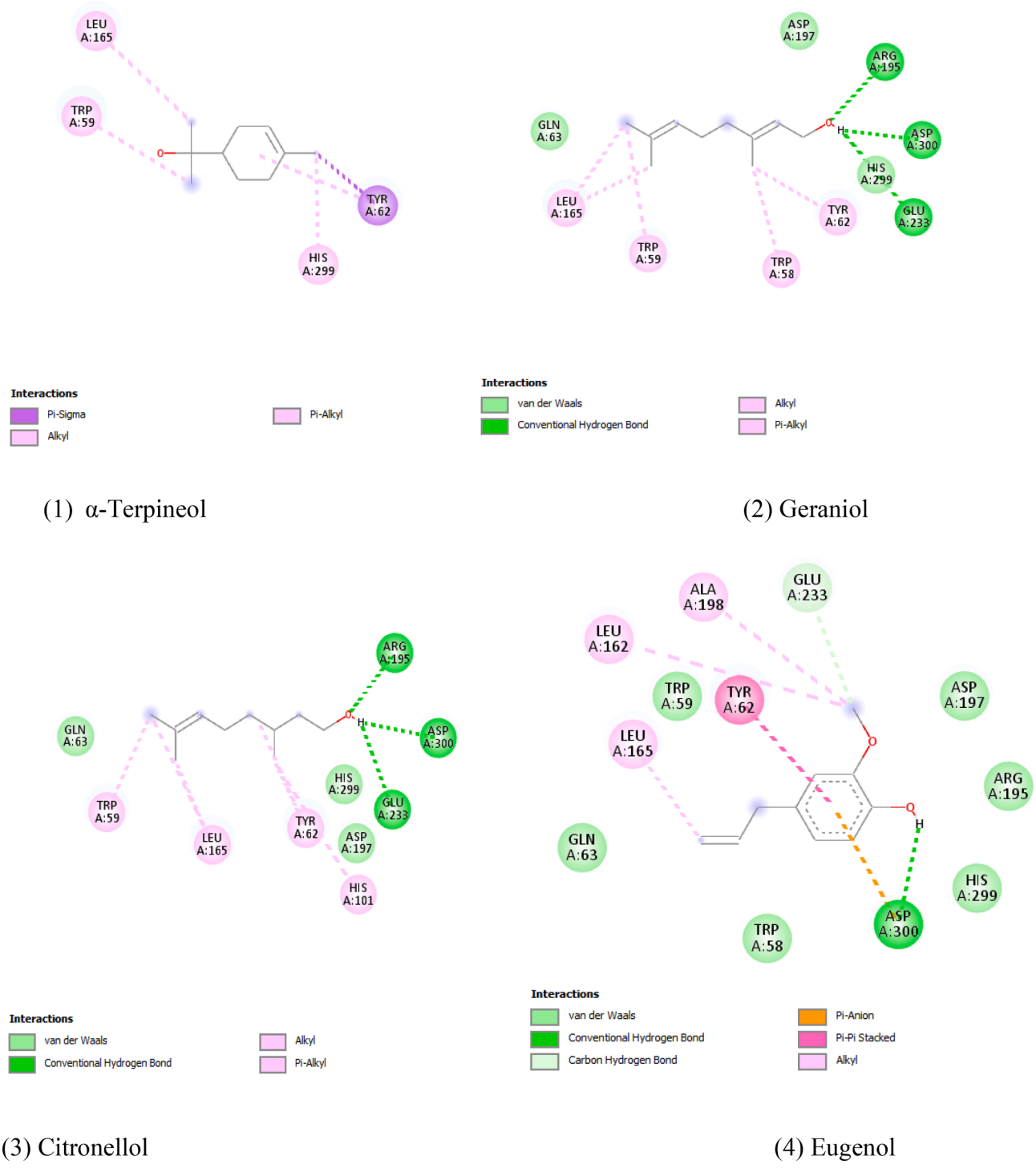


Fig. 7. Binding interactions between α-amylase and probable inhibitors: (1) α-Terpineol, (2) Geraniol, (3) Citronellol, (4) Eugenol, (5) Gallic acid, (6) Fructose, (7) Catechin, (8) Quercetin and (9) Acarbose. Various types of enzyme-ligand interactions are depicted in different colours.

value of 44.39 μg/ml, while the acarbose obtained a value of 57.1 μg/ml. Furthermore, in the correlation study showed that coefficient correlation is 0.9809. This displays the graph portrays a strong coefficient between anti-oxidative and anti-diabetic properties of *M. calabura* leaves extract. From these obtained experimental data showcases that *M. calabura* leaves extract has the potential to act as natural antioxidant and also a natural amylase inhibitor. The molecular docking results suggests the ligand, quercetin is the most potent inhibitor of alpha

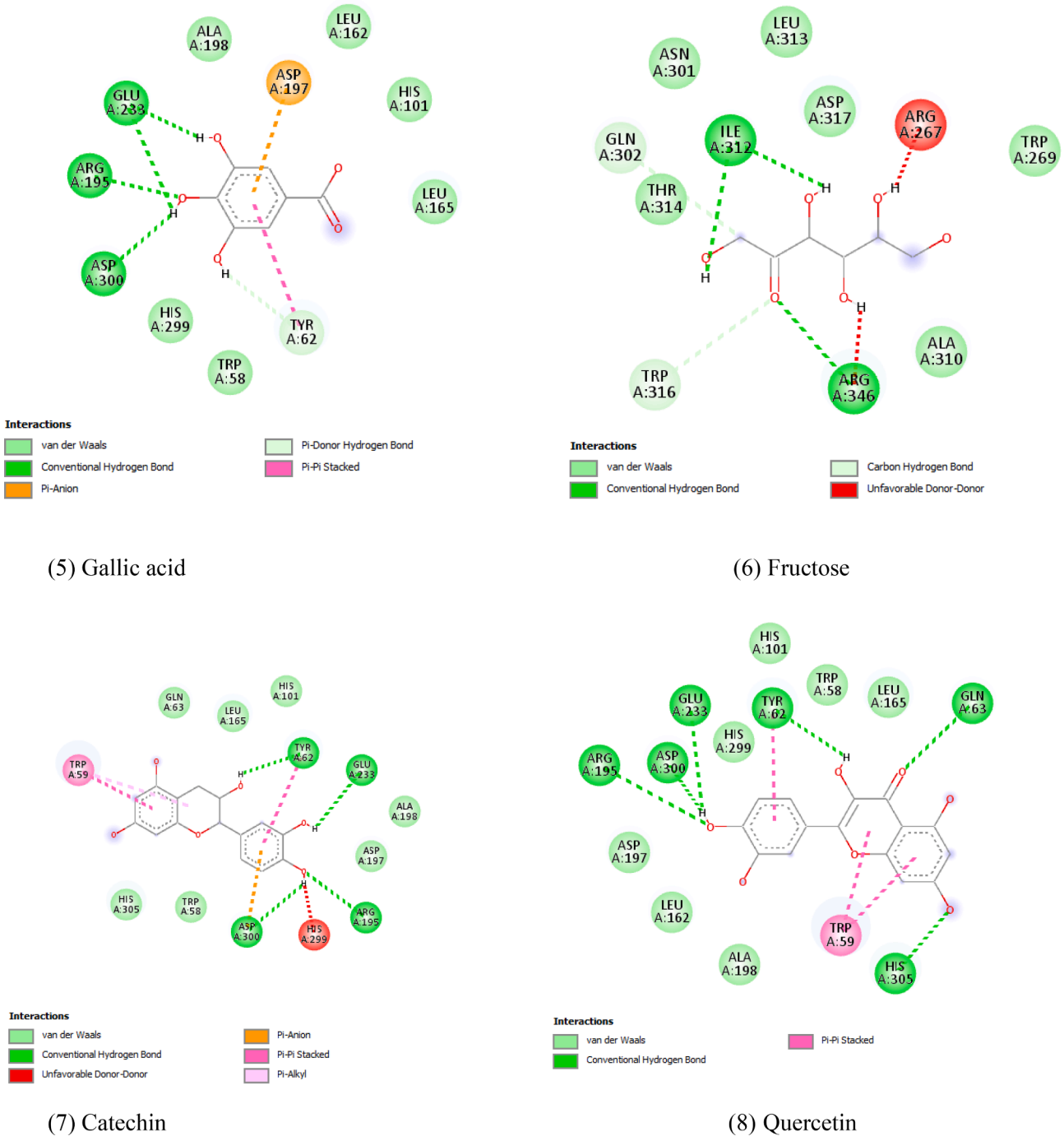


Fig. 7. (*continued*).

amylase with the least binding free energy of −9.1 kcal/mole among all the ligands computed. These findings provide intense motivation for further *in vivo* study and drug identification from *M. calabura* leaves extract.

CRediT authorship contribution statement

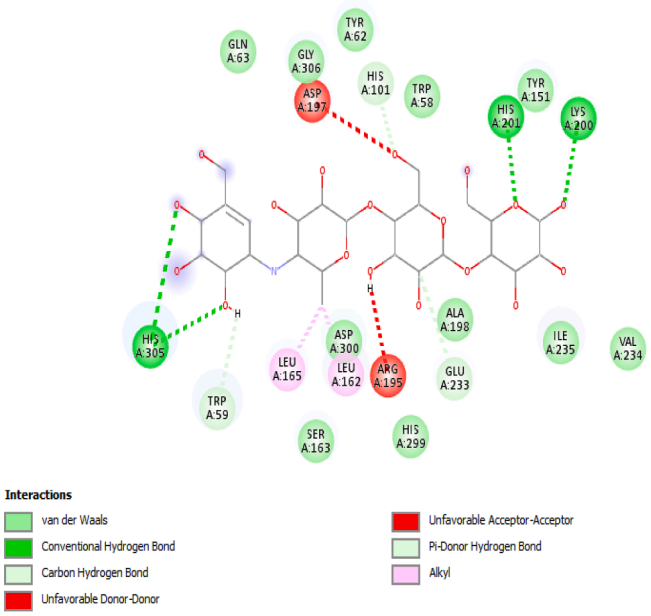
Ahmad Anas Nagoor Gunny: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Nimaal K. Prammakumar:** Formal analysis, Validation, Writing – original draft. **Abdul Aziz Ahmad:** Resources, Validation, Writing – review & editing. **Subash C.B. Gopinath:** Validation, Writing – review & editing. **Amirul Ridzuan Abu Bakar:** Validation, Writing – review & editing. **Haliru Musa:** Resources, Writing – review & editing. **Mohd Hishamuddin Che Mat:** Resources, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.



(9) Acarbose (Positive control)

Fig. 7. (*continued*).

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