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Phytochemical Constituents, Anti-Diabetic and Antioxidant Activities of Methanol Extracts of *Diospyros malabarica* (Desr.) Kostel Leaves and Stem Bark

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ABSTRACT

Diospyros malabarica (Desr.) Kostel is widely used in traditional medicine as an antioxidant and anti-diabetic agent. This study aims to evaluate the phytochemical constituents, the antioxidant and anti-diabetic activities of the methanol leaf extract (MLE) and methanol stem bark extract (MBE) of *Diospyros malabarica*. The extracts were obtained by Ultrasound-Assisted Extraction (UAE). Phytochemical screening was done using standard methods. The total phenolic content (TPC) and total flavonoid content (TFC) were determined using the Folin-Ciocalteau and Aluminium chloride colorimetric methods, respectively. The antidiabetic activity was assessed using α-glucosidase and dipeptidyl peptidase-4 (DPP-4) inhibitory assays. The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, and Ferric Reducing Antioxidant Power (FRAP) assays. The results showed that the TPC (621.31 ± 0.74 mgGAE/g) and TFC (32.86 ± 0.31 mgQE/g) of MBE are 14 times higher than that of MLE with TPC and TFC of 42.83 ± 0.15 mgGAE/g, and 2.37 ± 0.02 mgQE/g, respectively. MBE also showed higher antioxidant and anti-diabetic activities than MLE. The IC_{50} values of MBE for DPPH radical scavenging, ABTS radical scavenging, and FRAP activities were 8.04 ± 0.05 µg/mL, 2.61 ± 0.03 µg/mL, and 6803.86 µMFSE/g, respectively. For the anti-diabetic activity, MBE had IC_{50} values of 14.36 ± 0.21 µg/mL, and 205.39 ± 2.94 µg/mL for α-glucosidase, and DPP-4 inhibitory activities, respectively. Therefore, the stem bark of *D. malabarica* has better anti-diabetic and antioxidant activities than the leaves, and thus has a potential for use as an antioxidant and anti-diabetic agent.

***Keywords*:** Anti-diabetic, Alpha-glucosidase, DPP-4, Antioxidant, *Diospyros malabarica* (Desr.) Kostel

Introduction

Diabetes is a complex and chronic disease caused by increased blood glucose levels. People with diabetes generally experience a decreased ability to respond to insulin or a decreased level of insulin produced by the pancreatic *β*-cells, which results in abnormalities in the metabolism of carbohydrates, proteins, and fats.[1] One type of diabetes is non-insulin-dependent diabetes mellitus or type 2 diabetes mellitus (T2DM), which is caused by insulin resistance or insulin secretion deficiency. Adequate management of post-prandial hyperglycemia has the potential to reduce chronic complications because increased blood sugar levels will increase the number of free radicals in the body, which can trigger oxidative stress.

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Therefore, there is the need for a therapeutic agent that will reduce glucose absorption by inhibiting the action of carbohydrate hydrolyzing enzymes such as α-glucosidase,[2,3] inhibiting the action of the DPP-4 enzyme which plays a role in reducing glucagon secretion and stimulating insulin secretion from the pancreas,[2] and scavenging free radicals.

Diabetes mellitus and its complications are associated with increased oxidative stress caused by free radicals.[3] Free radicals are compounds with unpaired electrons which make them unstable and very reactive. In order to attain a paired electron state and become stable, free radicals oxidizes biomolecules and cell organelles, thus inducing cell damage.[4] Most free radicals in the body are reactive oxygen species (ROS) consisting of superoxide anion (O_2•–), alkoxyl radical (RO•) radical, hydroxyl radical (OH•), peroxyl radical (ROO•), and hydroperoxyl radical (HO_2•), while nitric oxide (NO•), nitrite (NO_2•), and nitrate radicals (NO_3•) are termed the reactive nitrogen species (RNS).[5] Free radical activity causes physiological disturbances in cells; therefore, antioxidants are needed to neutralize or mitigate free radical levels to maintain cell physiological functions and contribute to disease prevention. Antioxidant activity protects the body against the damaging effects of oxidative stress due to hyperglycemia and can improve carbohydrate, protein and lipid metabolism and glucose absorption in people with diabetes mellitus.[6]

Currently, several types of drugs are available for the treatment of diabetes including non-insulin anti-diabetic drugs such as sulfonylureas, biguanides, thiazolidinediones, α-glucosidase inhibitors, *Dipeptidyl peptidase 4* (DPP-4) inhibitors, and Sodium-glucose co-transporter-2 (SGLT2) inhibitors.[7] However, the use of anti-diabetic

drugs is still limited by side effects. Therefore, the search for medicinal compounds for the treatment of diabetes continues to grow, especially drugs derived from natural ingredients such as plants reported as diabetes agents with low cost and fewer side effects.[8]

Diospyros malabarica (Desr.) Kostel, which is in the Ebenaceae family, is one of the plants that are widely used in traditional Indian medicine because it is known to have hepatoprotective, hypoglycemic,[9] analgesic,[10] antibacterial,[10,11] anthelmintic,[12] antidiarrheal, and strong antioxidant activities.[10,13] *D. malabarica* is also known to have antihyperglycemic activity, which is listed in Ayurveda as a medicine for diabetes mellitus. Generally, the woody parts of this plant is mostly used in traditional medicine, but Ogata *et al.* (1995)[14] stated that the fruit of *D. malabarica* is also used in traditional medicine. The methanol extract of the leaves and stem bark of *D. malabarica* obtained by maceration have previously shown anti-diabetic activity by inhibiting α-amylase.[15] In addition, *in vivo* studies on the methanol extract of the leaves and stem bark of *D. malabarica* have shown a significant hypoglycaemic effect,[16,17] and good antioxidant activity.[10]

Based on a review of previous studies, extraction using ultrasound-assisted extraction (UAE) of *D. malabarica* has not been reported. In addition, although information related to antioxidant activity has been reported previously, no data have been found regarding its activity against α-glucosidase and DPP-4 enzyme inhibition. Therefore, further research is needed to explore the potential anti-diabetic and antioxidant drug candidates from *Diospyros malabarica*, especially the leaves and stem bark, considering its wide use in traditional medicine as an anti-diabetic agent.

Materials and Methods

Chemicals and Reagents

Chemicals used in this study include analytical grade solvents [n-hexane (Brataco Indonesia), ethyl acetate (Brataco Indonesia), methanol (Brataco Indonesia), ethanol pro analysis (Merck), methanol pro analysis (Merck, Germany)], sulfuric acid (Merck, Germany), gallic acid (Sigma-Aldrich, USA), Folin Ciocalteau (Sigma-Aldrich, F9252/F47641, USA), aluminium chloride, sodium acetate, DPPH (Sigma-Aldrich, USA), TPTZ, ABTS, quercetin (Sigma-Aldrich, USA), sterile water for injection (API), phosphate buffer pH 6.8, baker's yeast α-glucosidase enzyme (Sigma-Aldrich, USA), acarbose (Sigma-Aldrich, USA), p-nitrophenyl-α-D-glucopyranoside (p-NPG) (Sigma-Aldrich, USA), sodium carbonate (Merck cat. 1.09940 Germany), substrate glycyl-prolyl-para-nitroanilide (GPPN) (Sigma-Aldrich, USA), human DPP-4 enzyme (Sigma-Aldrich, USA), Trizma base (Sigma-Aldrich, USA), and Sitagliptin.

Plant Materials

The leaves and stem bark of *Diospyros malabarica* were obtained from the Bogor Botanical Garden in November, 2022. The plant materials were identified and authenticated in the Research Center for Plant Conservation and Botanic Gardens, the National Research and Innovation Agency, Bogor, West Java, with a herbarium number B-3990/IPH.3/KS/XI/2019. An image of the samples of *D. malabarica* is shown in Figure 1.

Sample Preparation

The plant material was dried and ground into powder form. Approximately 25 g of powdered leaves and stem bark was extracted with methanol using the Ultrasound-Assisted Extraction (UAE) method (QSONICA Sonicator – Q2000, USA) with a sample-solvent ratio of 1:10 (g/mL), at a frequency of 20 kHz, and amplitude of 30% at room temperature for 15 minutes.[18] The extraction process was repeated three times, with a sample-solvent ratio of 1:5 (g/mL) in the subsequent steps. The extract was filtered using Whatman No. 1 filter paper and evaporated at 50ºC using a rotary evaporator at a speed of 70 rpm to obtain a crude methanol extract.[19] Extraction with UAE was chosen because it reduces extraction time and solvent consumption. In addition, it can be performed at low temperatures to prevent extract degradation and loss of volatile compounds.[20] The crude extract was then dried in an oven at 50ºC, and the percentage yeild was calculated.[15]

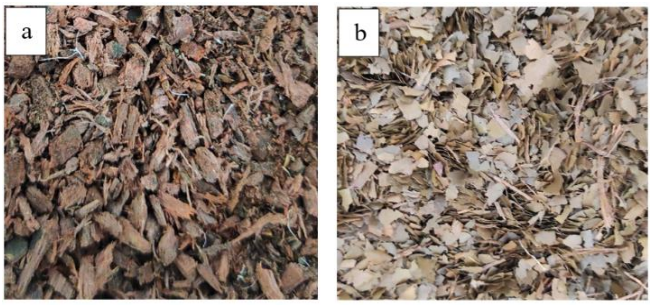

Figure 1: (a) Dried stem bark **(b)** Dried leaves of *Diospyros malabarica*

Phytochemical Screening

Phytochemical screening aims to determine the group of compounds as an initial information on the compounds present in the sample. Qualitative tests for the presence of alkaloids, anthraquinones, flavonoids, glycosides, saponins, tannins, and terpenoids in the leaf and stem bark extracts of *D. malabarica* were carried out. The presence of alkaloids was determined using Bouchardat, Mayer, and Dragendroff reagents, anthraquinone group was identified by mixing 3 mL of the concentrated extract with 2 N sulfuric acid (H_2SO_4) in a ratio of 1:1, while flavonoid group was detected using Shinoda and with 5% $AlCl_3$ reagent. The class of glycoside was identified by adding five drops of Molisch LP reagent added and 2 mL of sulfuric acid (H_2SO_4). The presence of saponin groups was identified with foam index, terpenoids group was identified with the Lieberman-Burchard reagent, and tannin group identified with gelatin and $FeCl_3$ reagents.[21]

Determination of Total Phenolic Content (TPC)

As much as 75 mL of the crude methanol extract of the stem bark and 750 mL of the crude methanol extract of the leaves at a concentration of 1000 µg/mL were added to 0.5 mL of Folin-Ciocalteau reagent (1:10) in a vial. The mixture was shaken for 1 min and allowed to stand for 3 min. Then, 1 mL of 7.5% sodium carbonate solution was added followed by the addition of distilled water to make up to 5 mL.[22] The mixture was vortexed until it became homogeneous and then incubated in a dark room at room temperature for 30 min. The absorbance of the resulting mixture was measured at 671 nm using a UV-Vis spectrophotometer. A standard curve was constructed with gallic acid (2 - 6 µg/mL). the experiment was done in triplicates and the TPC was expressed as mg gallic acid equivalent (mg GAE)/g extract.[23]

Determination of Total Flavonoid Content (TFC)

A total of 500 mL of crude methanol extract of the stem bark and 3750 mL of crude methanol extract of the leaves at a concentration of 1000 µg/mL were added to 0.1 mL of 10% aluminium chloride, and allowed to stand for 3 min. Thereafter, 0.1 mL of 1 M sodium acetate solution and distilled water were added until a 5 mL volume was reached. The mixture was vortexed until it became homogeneous and then incubated in a dark room at room temperature for 30 min. Then, a UV-Vis spectrophotometer was used to measure the absorbance at 441 nm. A blank solution was prepared by mixing the sample and distilled water up to a 5 mL volume. A standard curve was constructed with quercetin at concentrations ranging from 1 - 5 µg/mL. The test was performed in triplicates. TFC was expressed as mg quercetin equivalent (mg QE)/g extract.[22]

Determination of Anti-diabetic Activity

Alpha-Glucosidase Inhibitory Assay

The α-glucosidase enzyme inhibition assay was done following the method previously described by Elya *et al.* (2015),[24] with slight modification. The crude extract solutions were diluted in 5% DMSO. The solution was sonicated with an ultrasonic water bath. To 30 mL of the sample solution at different concentrations (300, 375, 450, 525, and

600 µg/mL for MLE; 12, 15, 18, 21, and 24 µg/mL for MBE) were added 36 µL phosphate buffer (pH 6.8) and 17 µL p-Nitrophenyl-*β*-D-glucopyranoside (p-NPG) substrate, then incubated for 5 min at 37ºC. After the incubation period, 17 µL of 0.12 U/mL α-glucosidase enzyme solution was added and incubated again for 15 min at 37ºC, after which 100 µL of 200 mM sodium carbonate was added. The absorbance of the resulting solution was measured at 405 nm using a microplate reader. Acarbose was used as the positive control at concentrations of 45, 60, 75, 90, and 105 µg/mL. The same procedure was used for the control test. However, after incubating for 5 min at 37ºC, 100 µL of 200 mM sodium carbonate was added, followed by 17 µL of 0.15 U/mL α-glucosidase enzyme solution, then solution was incubated again for 15 minutes at 37ºC. The IC_{50} values were calculated from a linear regression equation obtained from the plot the absorbance readings versus the concentrations of acarbose and the samples.

Dipeptidyl peptidase 4 (*DPP-4) Inhibitory Assay*

The DPP-4 inhibitory assay was carried out following the procedure prescribed by Arabiyat *et al.* (2019)[25] with slight modifications. To 35 µL of extract solutions at different concentrations (560, 1120, 1680, 2240, and 2800 µg/mL for MLE; 168, 224, 280, 336, and 392 µg/mL for MBE) was added 15 µL of 0.1 U/mL DPP-4 enzyme, and then incubated for 10 min at 37ºC. After the incubation period, 50 µL of 1.25 mM Glycyl-prolyl-para-nitroanilide (GPPN) substrate solution was added and re-incubated for 30 min at 37ºC. Thereafter, 25 µL of 30% glacial acetic acid was added to stop the reaction. Then, the absorbance was measured at 405 nm using a microplate reader. The same procedure was carried out for the control assay, but 15 µL of Tris-HCl solution was added after the sample solution was added. The control sample was incubated for 10 min at 37ºC, then 50 µL of 1.25 mM GPPN substrate solution and 25 µL of 30% glacial acetic acid were added, incubated for 30 min, and the absorbance of the solution was measured using a microplate reader at 405 nm. Sitagliptin at concentrations of 0.035, 0.07, 0.105, 0.14, and 0.175 µg/mL was used as the positive control. A calibration curve was created from the inhibition percentages obtained for the different concentrations. A linear regression equation was generated from the calibration curve and was used to calculate the IC_{50} values for the extracts and positive control.

Determination of Antioxidant Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The antioxidant activity of MLE and MBE was assessed using the DPPH radical scavenging assay. Briefly, different concentrations of the test samples (MLE at 50, 75, 100, 125, and 150 µg/mL, MBE at 2, 4, 6, 8, and 10 µg/mL) and standard (ascorbic acid and quercetin at 1, 1.5, 2, 2.5, 3, and 3.5 µg/mL) solutions were prepared as used for the assay. The test sample concentrations were obtained from optimization results. Then, 1 mL of DPPH in methanol were added to the standard and sample solutions up to a final volume of 5 mL.[22] The mixture was vortexed for 10 sec and incubated at room temperature (25°C) for 30 min in the dark. The absorbance was measured at 515 nm. The experiment was done in triplicates, and the percentage inhibition of the DPPH radical for each sample and the standard was calculated using the formula below:

$$\% \text{ Inhibition} = \left(\frac{\text{Blank Abs.} - \text{Sample Abs.}}{\text{Blank Abs.}}\right) \times 100\%$$

Where; Blank Absorbance = solvent + DPPH
Sample Absorbance = solvent + DPPH + sample

The IC_{50} (concentration of the extract/standard that can inhibit 50% of the activity of DPPH free radicals) was determined using the linear regression equation based on the absorbance value to determine the relationship between absorbance and antioxidant activity. The concentrations of standard solutions were entered as x variables, while the inhibition percentages were entered as y variables. Based on the linear regression equation; $y = a + bx$, IC_{50} was calculated using the following formula:

$$IC_{50} = \frac{50 - a}{b}$$

Samples with IC_{50} values <50 µg/mL is regarded as having strong antioxidant activity.

2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) diammonium Assay

Determination of the antioxidant activity using the ABTS assay was done according to the procedure described by Ernawati *et al.* (2013),[26] with some modifications. Briefly, Potassium persulfate and ABTS stock solution were combined to produce the ABTS radical, which was then allowed to stand in the dark at room temperature for 12 to 16 h before use.[27]

Then, the test solution was prepared by mixing a specific quantity of ABTS radical stock solution with 95% ethanol in a ratio of 1:15. The following concentrations of the extracts and positive controls were prepared; MBE (1, 2, 3, 4, and 5 µg/mL), MLE (20, 40, 60, 80, and 100 µg/mL), Ascorbic acid and quercetin (1, 1.5, 2, 2.5, and 3 µg/mL). The ABTS test solution (1 mL) and ethanol were added to the samples up to a final volume of 5 mL. The mixture was incubated in the dark for 30 min at room temperature. The absorbance of the sample was then determined at 752 nm using a UV-Vis spectrophotometer. The test was performed in triplicates.

A calibration curve was created in the same way as the DPPH assay. The percentage inhibition of ABTS radical was calculated using the following equatfiion:

$$\%\text{Inhibition} = \left(\frac{\text{Blank Abs.} - \text{Sample Abs.}}{\text{Blank Abs.}}\right) \times 100\%$$

Where; Blank Absorbance = solvent + ABTS
Sample Absorbance = solvent + ABTS + samples

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out according to the procedure previously described by Arianti *et al.* (2020)[28] and Srakeaw *et al.* (2021)[3] with slight modifications. The FRAP II reagent was prepared by combining 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ in 0.3 M acetate buffer (pH 3.6) at a 1:1:10 ratio, while in the preparation of FRAP I reagent for the calibration curve, $FeCl_3$ was replaced with double-distilled water. The ammonium ferrosulphate (AFS) solution was used to prepare the calibration curve. Ascorbic acid and quercetin were used as the positive controls. The extract and positive control solutions were combined with 1 mL of FRAP II reagent and made up to 5 mL with distilled water. The mixture was incubated in the dark at 37°C for 30 min. The absorbance was measured at 596 nm using a UV-Vis spectrophotometer.

Based on the preliminary results from optimization, the test concentrations of MBE and MLE used were 5 µg/mL and 60 µg/mL, respectively. The calibration curve was prepared from iron (II) sulfate heptahydrate solution (7.19 - 35.97 µM) from which a linear regression equation of the form $y = bx + a$ was obtained. The FRAP value was expressed as µM ferrous sulphate equivalent (FSE)/g sample.[29] The FRAP value was determined using the following equation:

$$C = \frac{\Delta A596\text{ nm} - a}{b}$$

$$\text{FRAP value } (\mu M/g) = \frac{C \times V \times Fp}{m}$$

Where;
C = Sample Concentration (µM)
V = Sample Volume (mL)
Fp = Dilution Factor
M = Sample Weight (mg)

Statistical Analysis

All the determinations were done in triplicates and data expressed as mean ± standard deviation. The data were analysed using the statistical programme Microsoft Excel 365.

Results and Discussion

Extracts Yeild

The leaves and stem bark of *Diospyros malabarica* were extracted by Ultrasound-Assisted Extraction (UAE) using methanol as solvent. Previous study which compared the conventional maceration and UAE extraction methods showed that UAE produced the highest yield in the shortest time. The UAE method is an extraction technique that uses ultrasonic waves that form cavitation bubbles that damage cell membranes to accelerate the penetration and movement of solvents, as well as components in the cells; thus, facilitates the extraction process.[30,31] The extraction process uses methanol as the solvent, because methanol has the ability to extract non-polar, semi-polar, and polar compounds.[32,33] Methanol is a universal solvent that has polar groups in the form of hydroxyl groups (OH) and non-polar groups in the form of methyl groups (CH_3).[34] Methyl groups in methanol will bind non-polar and semi-polar compounds, while hydroxyl (OH) groups will bind polar compounds. For this reason, methanol is an effective solvent for extracting phenolic compounds.[35,36] From 25 g each of dried powdered leaves and stem bark, the MLE and MBE obtained were 1.569 g and 2.538 g, respectively. The percentage yields were calculated to be 6.28% for MLE and 10.15% for MBE (Table 1).

Table 1: Weight and percentage yield of *Diospyros malabarica* extracts

Plant Parts	Plant Powder Weight (g)	Extract Weight (g)	Yield (%)
Leaves	25	1.569	6.28
Stem Barks	25	2.538	10.15

Phytochemical Constituents of Diospyros malabarica extract

Phytochemical screening aims to determine the group of compounds as a preliminary information on the compounds contained in the sample. The phytochemical screening detected the presence of alkaloids, flavonoids, glycosides, saponins, tannins, and terpenoids group of compounds in the leaves and stem bark extracts of *D. malabarica*, while anthraquinone group was absent (Table 2). This finding is in accordance with the study conducted by Zreen *et al.* (2022),[15] which found out that *Diospyros malabarica* extract contains tannins, saponinss, terpenoid, and flavonoids.

Total Phenolic and Total Flavonoid Contents of Diospyros malabarica extract

The total phenolic and flavonoid contents were determined based on the colorimetric method using Folin-Ciocalteau reagent (FCR) and Aluminium chloride. The results were expressed as gallic acid equivalent and quercetin equivalent for TPC and TFC, respectively. In determining total flavonoid content, the addition of $AlCl_3$ will form an acid complex with a C-4 ketone group and a neighboring hydroxyl group on the C-3 or C-5 atom of the flavonoid. $AlCl_3$ will form a stable acid complex with the orthodihydroxyl group on the A or B ring of flavonoid compounds with a maximum absorbance at 432 nm.[37]

The determination of total phenolic content with FCR depends on the electron transfer reaction between the FCR (oxidant) and electron donor species (antioxidant). It is necessary to add sodium carbonate solution (Na_2CO_3) as an agent to deprotonate the OH group on the phenolic ring and increase the pH up to 10, because the group of phenolic compounds only reacts with the FCR reagent under alkaline conditions. The FCR contains a mixture of phosphotungstic and phosphomolybdic acids. When these acids are reduced in alkaline media, tungsten blue chromophore and molybdenum oxide are produced, which have maximum absorbance at 760 nm.[38,39] Determination of TPC and TFC was carried out using a calibration curve of gallic acid and quercetin, with the regression equations $y = 0.0904x + 0.0155$ ($R^2 = 0.9983$) and $y = 0.1344x - 0.0143$ ($R^2 = 0.9993$).

The results showed that MBE had total phenolic and flavonoid contents of 621.31 ± 0.74 mg GAE/g extract and 32.86 ± 0.31 QE/g extract, respectively which were 14 times higher than that of MLE which had total phenolic and flavonoid contents of 42.83 ± 0.15 mg GAE/g extract and 2.37 ± 0.02 QE/g extract, respectively (Table 3). These outcomes are consistent with a study conducted by Zreen *et al.* 2022,[15] which reported that *Diospyros malabarica* stem bark had the highest content of total phenolics and flavonoids compared to other parts of the plant including the leaves.

Anti-diabetic Activity of Diospyros malabarica extract
Alpha-Glucosidase Inhibitory Activity

Evaluation of α-glucosidase inhibitory activity was carried out using p-nitrophenyl-α-D-glucopyranoside (p-NPG) which acts as a substrate. The α-glucosidase enzyme breaks down p-NPG into α-D-glucose and p-nitrophenol, which produces a yellow colour.[40]

The results of the α-glucosidase inhibitory activity of the extracts and the positive control (acarbose) are shown in Figure 2 and Table 4.

Inhibition of α-glucosidase activity was measured using a microplate reader by measuring the amount of p-nitrophenol produced from p-nitrophenyl-α-D-glucopyranoside. If a sample has the ability to inhibit α-glucosidase, the concentration of p-nitrophenol released will decrease. More p-nitrophenol is formed when the yellow color of the solution is more intense.[41] Testing the inhibition of α-glucosidase enzyme activity by the positive control (acarbose) aims to validite and ensure the reliability of the test. Acarbose is a pseudo-tetrasaccharide with a structure similar to an oligosaccharide, with a nitrogen atom present between the first and second glucose molecules, this increases the affinity of acarbose for α-glucosidase enzyme. Owing to the presence of an imino group (>C=NH), acarbose cannot be hydrolyzed by digestive enzymes.[42]

Acarbose has been widely used as a standard in previous studies because it is a reversible and competitive inhibitor of α-glucosidase enzyme. The IC_{50} value of 56.31 ± 5.70 µg/mL for acarbose was obtained from the regression equation $y = 0.233x + 36.879$ ($R^2 = 0.9795$), this was lower than the IC_{50} value of MLE which was 429.89 ± 3.39 µg/mL. This indicates that MLE has a lower α-glucosidase inhibitory activity than acarbose.

Table 2: Phytochemical Constituents of *Diospyros malabarica*

No.	Phytochemical Group	MLE	MBE
1	Alkaloids	+	+
2	Anthraquinones	-	-
3	Flavonoids	+	+
4	Glycosides	+	+
5	Saponins	+	+
6	Tannins	+	+
7	Terpenoids	+	+

Key: + indicates presence of compound; - indicates absence of compound
MLE = Methanol Leaf extract; MBE = Methanol stem bark extract

Table 3: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of *Diospyros malabarica* Extracts

Extracts	TPC (mg GAE/g)	TFC (mg QE/g)
MLE	42.83 ± 0.15	2.37 ± 0.02
MBE	621.31 ± 0.74	32.86 ± 0.31

Values are Mean ± Standard Deviation. MLE = Methanol Leaf extract; MBE = Methanol stem bark extract

However, MBE showed a higher α-glucosidase inhibitory activity than acarbose and MLE with an IC_{50} value of 14.36 ± 0.21 µg/mL. This observation may be attributed to the higher TPC and TFC in MBE than in MLE. These findinds are in agreement with the results of the experiments conducted by Mondal *et al.* (2008),[43] which showed that the stem bark extract of *Diospyros malabarica* has blood glucose lowering effect in Swiss albino rats. In the same vein, the work of Zreen *et al.* (2022)[15] also showed that the methanol extract of the stem bark of *Diospyros malabarica* had higher α-amylase enzyme inhibitory activity than the methanol extract of the leaves.

Dipeptidyl peptidase 4 (DPP-4) Inhibitory Activity

DPP-4 inhibitory activity can be done colorimetrically using chromogenic substrates like Glycyl-prolyl-para-nitroanilide (Gly-Pro-pNA), Alanyl-prolyl-para-nitroanilide (Ala-Pro-pNA), or Arginyl-prolyl-para-nitroanilide (Arg-Pro-pNA)[44,45] or fluorometrically with the use of the non-fluorescent substrate Glycyl-prolyl-7-amino -4-methylcoumarin (Gly-Pro-AMC), which is based on the principle of peptide bond breakage from the Gly-Pro-AMC substrate by DPP-4 enzyme to release the fluorescent substrate 7-Amino -4-Methylcoumarin (AMC).[46]

Glycyl-prolyl-para-nitroanilide (Gly-Pro-pNA) is a chromogenic substrate that is commonly used to evaluate the DPP-4 enzyme inhibitory activity of a compound.[47] It is based on the principle of peptide bond breakage by DPP-4 to release the chromogenic substrate para-nitroanilide (pNA) which is an indicator of enzyme activity. By monitoring the rate of pNA release from the chromogenic substrate, DPP-4 activity is determined kinetically. In this study, DPP-4 inhibitory activity of MLE and MBE was measured as the inhibition percentage of the extracts compared to the inhibitory activity of the positive control (sitagliptin) at known concentrations.

Evaluation of the inhibition of DPP-4 enzyme activity using a positive control (sitagliptin) aims to ensure that the enzyme works properly. Sitagliptin is a DPP-4 inhibitor that acts competitively to prevent the degradation of Glucagon-Like Peptide-1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP). GLP-1 and GIP inactivation due to the administration of sitagliptin will increase insulin in the body, suppress glucagon release, and improve glucose tolerance.[2]

The results of the DPP-4 enzyme inhibitory activity assay for sitagliptin, MLE, and MBE are shown in Table 5 and Figure 3. The IC_{50} value of MLE was 2630.53 ± 55.87 µg/mL, which was ten times higher than that obtained for MBE (IC_{50} = 205.39 ± 2.94 µg/mL). This indicates that MBE has a higher DPP-4 inhibitory activity *vis a vis* a higher anti-diabetic activity than MLE. However, the DPP-4 inhibitory activity of MBE was lower than that of the positive control (sitagliptin) which had an IC_{50} value of 0.09 ± 0.00 µg/mL.

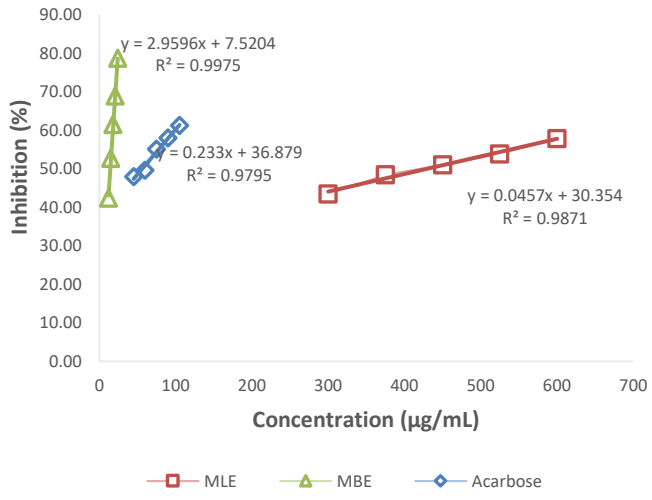

Figure 2: Calibration curves of α-glucosidase Enzyme Inhibition for Acarbose, MLE, and MBE

Antioxidant Activity of Diospyros malabarica extract

DPPH Free Radical Scavenging Activity

The DPPH radical scavenging assay is commonly used to determine antioxidant activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical is a stable nitrogen free radical with a delocalized spare electrons on the molecule. The delocalization results in a dark purple colour in ethanol, acetone-water, methanol, alcohol-water, and benzene solvents.[48] The DPPH assay estimates antioxidant activity by determining free-radical scavenging potential; a mechanism by which antioxidants prevent lipid oxidation.[49] Mixing DPPH solution with antioxidant compounds that can donate hydrogen atoms leads to the formation of reduced compound DPPH-H (hydrazine), which is a non-radical and is characterized by a colour change from purple to pale yellow.[50]

Table 4: Alpha-glucosidase Enzyme Inhibitory Activity of *Diospyros malabarica* Extracts and Standard

No.	Extract	Concentration (µg/mL)	Percentage Inhibition (%)	IC_{50} (µg/mL)±SD
		45	47.95±1.66	
		60	49.57±1.20	
1	Acarbose	75	55.08±0.57	56.31 ± 5.70
		90	57.96±1.19	
		105	61.23±1.23	
		300	43.42 ± 0.58	
		375	48.41 ± 0.37	
2	MLE	450	51.02 ± 0.24	429.89 ± 3.39
		525	53.83 ± 0.72	
		600	57.83 ± 0.86	
		12	42.39 ± 1.13	
		15	52.62 ± 0.20	
3	MBE	18	61.43 ± 1.40	14.36 ± 0.21
		21	68.89 ± 0.75	
		24	78.64 ± 0.79	

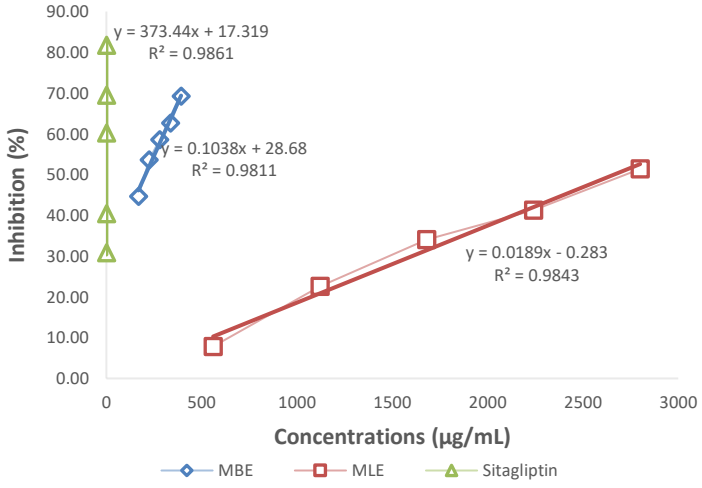

Figure 3: Calibration curves of DPP-4 Enzyme Inhibition for Sitagliptin, MLE, and MBE

Ascorbic acid and quercetin were the standard antioxidants used in this assay. The linear regression equations of the plot of percent inhibition of DPPH versus the concentrations of the samples are shown in Figure 4. The regression equations are y = 16.24x + 6.5473 (R^2 = 0.9989), y = 16.15x – 4.8494 (R^2 = 0.9954), y = 0.4738x - 4.686 (R^2 = 0.9962), and y = 6.8406x - 4.9848 (R^2 = 0.9983) for ascorbic acid, quercetin, MLE, and MBE, respectively. The results showed that ascorbic acid had an IC_{50} of 2.68 µg/mL, which indicated a stronger antioxidant activity than quercetin with an IC_{50} value of 3.40 µg/mL. The IC_{50} value of MLE was 115.42 µg/mL, which indicated a lower antioxidant activity compared to MBE with an IC_{50} value of 8.04 µg/mL (Table 6). In the present study, the antioxidant activity of both MLE and MBE in terms of their DPPH radical scavenging activity was lower than that of the positive controls (Ascorbic acid and Quercetin). The results of the present study agrees with that obtained from the study of Mondal *et al.* (2006),[9] which showed that the methanol stem bark extract of *Diospyros malabarica* have potent antioxidant activity, with an IC_{50} value of 9.16 µg/mL.

ABTS Free *Radical Scavenging Activity*

ABTS assay is a colorimetric assay based on the formation of ABTS cation radicals. The ABTS-colou Figure 2: Calibration curves of α-glucosidase Enzyme Inhibition for Acarbose, MLE, and MBE red cation radical is produced by ABTS oxidation (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid). The modified technique for producing the ABTS cation radical includes the direct production of the green-blue ABTS chromophores via the ABTS and potassium persulfate reaction. An electron transfer mechanism is involved in the ABTS radical elimination process. The degree of discoloration allows for the calculation of the percentage inhibition of the ABTS cation radical, which is dependent on the antioxidant concentration and reaction time.[51]

The ABTS free radical scavenging assay results are presented in Table 7. The linear regression equations for ascorbic acid, quercetin, MLE, and MBE are y = 23.262x + 5.3814 (R^2 = 0.9984), y = 24.726x + 0.6649 (R^2 = 0.9942), y = 0.703x - 0.6373 (R^2 = 0.9945), and y = 10.27x + 23.208 (R^2 = 0.997), respectively (Figure 5).

Based on the outcomes of the antioxidant activity evaluation using the ABTS assay, MBE was found to have strong antioxidant activity with IC_{50} value of 2.61 ± 0.03 µg/mL, while MLE had moderate antioxidant activity with IC_{50} value of 72.03 ± 0.04 µg/mL (Table 7). This finding indicates that MBE is more effective as an antioxidant than MLE, however, when compared to the positive controls, the the antioxidant activity of MBE is lower.

Ferric Reducing Antioxidant Power (FRAP) Activity

The FRAP values for the extracts and standards were obtained from the equation [(y = 0.0207x + 0.0088 (R^2 = 1)] of the iron (II) sulfate heptahydrate calibration curve. The FRAP values of ascorbic acid, quercetin, MLE, and MBE were 18045.09 µMFSE/g, 24099.84 µMFSE/g, 363.29 µMFSE/g, and 6803.86 µMFSE/g, respectively (Table 8). From the results, MBE had a higher FRAP value than MLE, indicating that MBE has a higher antioxidant activity than MLE, because the higher the FRAP value, the greater the potential antioxidant activity of a sample. Instead of hydrogen atom transfer, the ferric-reducing antioxidant power (FRAP) mechanism is based on electron transfer. To maintain iron solubility, the FRAP reaction was conducted at an acidic pH of 3.6. The reaction at low pH increases the redox potential and decreases the ionization potential that drives hydrogen atom transfer, which is the dominant reaction mechanism. When 2,4,6-trypyridyl-s-triazine is present during the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to ferrous-tripyridyltriazine complex (Fe^{2+}-TPTZ), an intense blue coloured complex is formed with maximum absorption at 593 nm.[52] Antioxidants are essential in mitigating the complications of diabetes by scavenging the free radicals created by oxidative stress or through dual mechanisms that target the causes of metabolic syndromes/diseases and minimize free radical formation. By eliminating the oxidants and correcting the damage that oxidants inflict on the body, antioxidants defend cells against damaging oxidants (ROS and RNS).[53]

Qualitative phytochemical screening results show that MLE and MBE have the same chemical group of compounds. However, from the TPC and TFC results, MBE showed a higher amount of phenolics and flavonoids than MLE, and this may have contributed to the much stronger antioxidant and anti-diabetic activities of MBE compared to MLE. In addition, the position and number of hydroxyl groups (OH) in phenolic compounds can affect their radical scavenging activity[3] as well as their inhibitory activity on α-glucosidase enzyme.[54] The results obtained from this study are supported by the findings of Famuyiwa *et al.* (2019)[55] which stated that most of the plants with anti-diabetic activity had high antioxidant activity. Alkaloids, flavonoids, glycosides, and phenolic acids act as α-glucosidase and DPP-4 inhibitors, along with their antioxidant properties.[56] Thus, MBE is more effective as scavenging free radicals and therefore a strong antioxidant agent owing to its phytochemical contents. Further studies should be done to identify, isolate and characterize the active compound(s) from the stem bark of *Diospyros malabarica*.

Conclusion

The results of this study have shown that the stem bark of *Diospyros malabarica* has greater anti-diabetic and antioxidant activities than the leaves which correlated with their total phenolic and flavonoids contents. The IC_{50} value for α-glucosidase, and DPP-4 inhibitory activities of MBE were 14.36 ± 0.21 µg/mL, and 205.39 ± 2.94 µg/mL, respectively, while the IC_{50} values for antioxidant activity were 8.04 ± 0.05 µg/mL, 2.61 ± 0.03 µg/mL, and 6803.86 µMFSE/g for DPPH radical scavenging, ABTS radical scavenging, and FRAP activities, respectively. On the basis of its strong antioxidant, α-glucosidase and DPP-4 inhibitory activities, the stem bark of *Diospyros malabarica* could be a potential source of anti-diabetic agent that could help lower blood glucose levels in diabetic patients by delaying the digestion of complex carbohydrates and therefore reduce intestinal glucose absorption.

Table 5: DPP-4 Enzyme Inhibitory Activity of *Diospyros malabarica* Extracts and Standard

No.	Extract	Concentration (µg/mL)	Percentage Inhibition (%)	IC_{50} (µg/mL)
1	Sitagliptin	0.035	30.84 ± 2.35	0.09 ± 0.00
		0.07	40.44 ± 0.65	
		0.105	60.23 ± 2.70	
		0.14	69.46 ± 4.19	
		0.175	81.68 ± 2.49	
2	MLE	560	7.85 ± 0.55	2630.53 ± 55.87
		1120	22.62 ± 1.18	
		1680	34.05 ± 1.74	
		2240	41.30 ± 0.80	
		2800	51.38 ± 1.14	
3	MBE	168	44.68 ± 0.38	205.39 ± 2.94
		224	53.62 ± 0.54	
		280	58.58 ± 0.60	
		336	62.63 ± 0.87	
		392	69.25 ± 0.60	

Values are Mean ± Standard Deviation. MLE = Methanol Leaf extract; MBE = Methanol stem bark extract

Table 6: DPPH Radical Scavenging Activity of *Diospyros malabarica* Extracts and Standards

No.	Sample	Concentration (µg/mL)	Percentage Inhibition (%)	IC_{50} (µg/mL)
1	Ascorbic Acid	1	22.93 ± 1.94	2.68 ± 0.01
		1.5	30.52 ± 2.59	
		2	39.00 ± 0.78	
		2.5	47.78 ± 0.82	
		3	54.90 ± 0.45	
2	Quercetin	1.5	18.92±0.43	3.40±0.09
		2	27.80±0.87	
		2.5	35.25±1.30	
		3	44.91±1.19	
		3.5	50.75±1.40	
3	MLE	50	20.05 ± 0.22	115.43 ± 0.39
		75	30.66 ± 0.27	
		100	41.19 ± 0.27	
		125	53.92 ± 0.16	
		150	67.65 ± 0.18	
4	MBE	2	9.01 ± 0.68	8.04 ± 0.05
		4	22.35 ± 0.65	
		6	36.21 ± 0.38	
		8	48.29 ± 0.56	
		10	64.44 ± 0.32	

Values are Mean ± Standard Deviation. MLE = Methanol Leaf extract; MBE = Methanol stem bark extract

Table 7: ABTS Radical Scavenging Activity of *Diospyros malabarica* Extracts and Standards

No.	Sample	Concentration (µg/mL)	Percentage Inhibition (%)	IC_{50} (µg/mL)
1	Ascorbic Acid	1	28.07 ± 0.34	1.92 ± 0.00
		1.5	40.70 ± 0.30	
		2	51.94 ± 0.57	
		2.5	54.49 ± 0.34	
		3	74.33 ± 0.46	
2	Quercetin	1	25.02 ± 0.77	2.00 ± 0.02
		1.5	39.49 ± 1.63	
		2	49.10 ± 1.43	
		2.5	60.78 ± 0.62	
		3	76.19 ± 1.05	
3	MLE	20	12.06 ± 0.35	72.03 ± 0.04
		40	28.41 ± 0.19	
		60	41.81 ± 0.19	
		80	57.70 ± 0.33	
		100	67.72 ± 0.14	
4	MBE	1	32.45 ± 0.66	2.61 ± 0.03
		2	44.36 ± 0.40	

No.	Sample	Concentration (µg/mL)	Percentage Inhibition (%)	IC_{50} (µg/mL)
		3	55.11 ± 0.51	
		4	64.39 ± 0.32	
		5	73.79 ± 0.25	

Values are Mean ± Standard Deviation. MLE = Methanol Leaf extract; MBE = Methanol stem bark extract

Table 8: FRAP Values of *Diospyros malabarica* Extracts and Standards

No.	Samples	FRAP (µmol FSE/g Extract)
1	Ascorbic Acid	18045.09 ± 228.30
2	Quercetin	24099.84 ± 362.59
3	MLE	363.29 ± 0.81
4	MBE	6803.86 ± 25.56

Values are Mean ± Standard Deviation. MLE = Methanol Leaf extract; MBE = Methanol stem bark extract

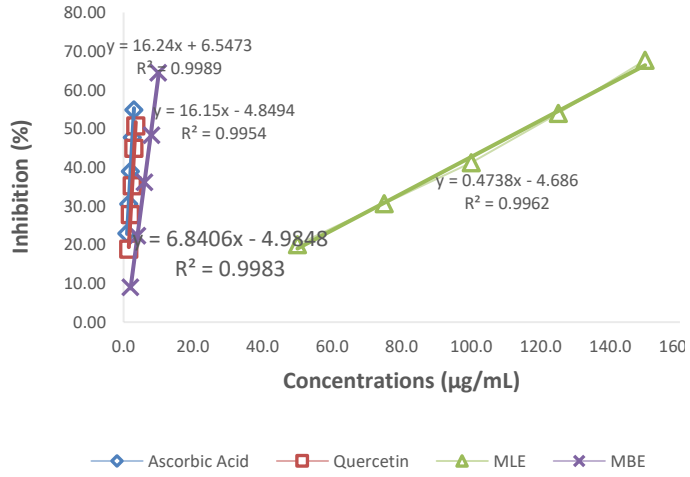

Figure 4: Calibration Curve of DPPH Inhibition for Ascorbic Acid, Quercetin, MLE, and MBE

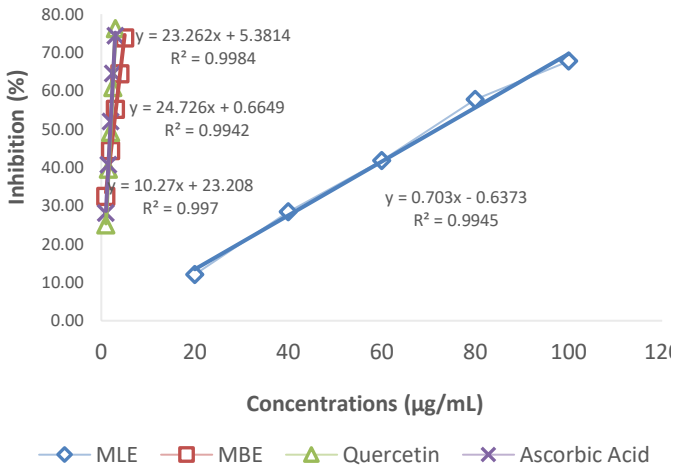

Figure 5: Calibration Curve of ABTS Inhibition for Ascorbic Acid, Quercetin, MLE, and MBE

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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