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PHYTOCHEMICAL SCREENING, ANTIOXIDANT AND CARBOHYDRATE DIGESTING ENZYME INHIBITORY POTENTIAL OF *LEUCAENA LEUCOCEPHALA* LEAVES EXTRACT

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ABSTRACT: The study aims to investigate the antioxidant and antidiabetic activity of *Leucaena leucocephala* ethanol leaf extract (LLME). *L. leucocephala* leaves extraction was carried out by the maceration method. Phytochemical analysis was performed using standard analytical procedures. Diphenyl picrylhydrazyl (DPPH) radical and 2, 2'-azinobis (3-ethylbenzathiazoline-6-sulfonic acid) (ABTS) radical scavenging assay; α-amylase and α-glucosidase inhibition assays were performed. The extraction yield of LLME by maceration was 65%. The phytochemical screening confirmed the presence of alkaloids, monosaccharides, proteins, amino acids, steroids, flavonoids, glycosides, tannins, and saponins. The total phenol content of LLME was 970 mg of GAE/g, and total flavonoid content was found to be 550 mg of QE/g. In DPPH and ABTS assay LLME showed good antioxidant activity (IC_{50}: 11.21 ± 0.2 and 11.87 ± 0.05 µg/mL) when compared to the standard drug, ascorbic acid (IC_{50}:11.09 ± 0.05 and 11.01 ± 0.09 µg/mL). Similarly, in α-amylase and α-glucosidase inhibition assay, acarbose revealed enzyme inhibition activity with an IC_{50} value of 12.02 ± 0.02 and 12.11 ± 0.02 µg/mL while LLME showed an IC_{50} value of 12.46 ± 0.06 and 12.47 ± 0.08 µg/mL, respectively. The present study leads to the conclusion that *L. leucocephala* ethanol leaf extract is with tremendous antioxidant and antidiabetic properties.

INTRODUCTION: *Leucaena leucocephala* (Lam.) Seeds (Family: Leguminosae) are commonly known as "White lead tree" or "*Petai belalang*" by Malaysians and Indonesians. The fresh or boiled seeds are consumed in various forms such as salad, sprouts, soup, and are used as a folks remedy to treat diabetes mellitus (DM) [1]. In spite of excellent source of phytochemicals [2] with multiple medicinal benefits to human health, the leaves and seeds of *L. eucocephala* contain a toxic amino acid mimosine, which limits its use as a human/animal food or used as a research plant.

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The presence of toxic constituents in seeds and leaves has severely limited its utilization interest as a research plant. However, mimosine consumption has not shown any toxic signs in humans [3], In Japan, boiling of *L. leucocephala* leaves or seeds served as a tea [4,] and it contains no mimosine or any of its toxic metabolites. It is believed that the toxic amino acid is hydrolyzed when macerated or heated at elevated temperatures and does not cause any toxic symptoms of mimosine to either animals or humans [5].

In spite of an excellent source of nutrients, scientific reports about this plant on human health are limited. Recent advances in science, several research reports have revealed its safety and biological effects on human health and are now gaining interest as a research plant. The antidiabetic and antioxidant activities of its seed extract were attributed due to its high phenolic [6] and flavonoid

content [7, 8]. The oral intake of seeds was safe up to 2000 mg/kg in mice [6]. The seed extracts have shown to exhibit hypoglycaemia [9] and inhibit α-glucosidase [10] and aldose reductase [11]. The seed sprouts are considered a rich source of natural antioxidants [12] Most of the previous reports were on seeds extracts, and recent reports revealed that leave extracts might be of value in the treatment of various diseases arising from oxidative stress [13, 14]. In the present study, we attempted to investigate the phytochemical composition, antioxidant, and carbohydrate digesting enzyme inhibitory properties of *L. leucocephala* leaves extract.

MATERIALS AND METHODS:

Reagents: Standard drug ascorbic acid and diphenyl picrylhydrazyl (DPPH) was obtained from Merck Millipore Co. U. S. A, and acarbose, α-amylase, α-glucosidase, quercetin, and 2, 2'-azinobis (3-ethylbenzathiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma Chemicals Co. St. Louis, USA. All other chemicals were of analytical grade.

Plant Sample Collection: The leaves of *L. leucocephala* were collected from Semeling, Kedah, Malaysia. The herbarium voucher specimen accession number of *L. leucocephala* leaves AIMST/FOP/13.

Extraction of *Leucaena leucocephala* Leaves: The leaves were rinsed in tap water and dried in a hot air oven at 60 °C. After drying, the leaves were blended and extracted by the maceration method. Briefly, the ratio of powdered leaves and ethanol is 1:9. The mixture was macerated for five days on the shaker at 100 rpm. The supernatant liquid was filtered and then subjected to rotary evaporation at 70 °C, and the extract LLME is freeze-dried [15].

Phytochemical Analysis: Phytochemical analysis of LLME was carried as per the standard methods. A series of qualitative phytochemical tests on the extract used to identify the presence of constituents such as alkaloids, flavonoids, phenols, mucilage, tannins, gums, glycosides, non-reducing sugars, saponins, proteins, monosaccharides, steroids, amino acids, carbohydrates, and reducing sugars [16].

Determination of Total Phenolic Content (TPC): Phenolic concentration in the LLME was measured using a modified spectrophotometric method. The ethanolic solution of the LLME with concentration of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL respectively were used for the total phenolic content (TPC) analysis. The reaction solution was prepared by adding the sample (1 mL) to 2.5% aqueous sodium carbonate solution (2 mL) along with, Folin-Ciocalteu reagent (1 mL). The solution was protected for 30 min at 37 ºC from light. The absorbance was measured at 750 nm. The samples were prepared in triplicate, and the average value of absorbance was noted. Blank was concomitantly prepared with ethanol without extract solution. A similar procedure was repeated using standard gallic acid instead of LLME. The calibration curve was constructed from the measured absorbance value. The phenolic concentration was expressed as gallic acid equivalent in mg/g (mg GAE /g of dry extract) [17].

Determination of Total Flavonoid Content (TFC): The total flavonoid content (TFC) of LLME was determined by the aluminum chloride colorimetric method. Both LLME and standard drug (quercetin, QE) were prepared at different concentrations using ethanol ranging from 10 to 100 µg/mL in standard 10 mL volumetric flasks. 1 mL of different concentrations of LLME, 0.7 mL of 5% sodium nitrite and 10 mL of 30% (v/v) ethanol added in 25 mL of volumetric flasks. They are mixed well and allowed to stand for 5 min; then 0.7 mL of 10% aluminum chloride was added and allowed to stand for 6 min 10 mL of 1 mmol/L sodium hydroxide is added and made up volume 25 mL with 30% (v/v) ethanol and allowed to stand for 10 min. The absorbance value of the final solution was measured at 450 nm. The procedure repeated for QE, and a calibration curve plotted. The flavonoid concentration of the extracts expressed as mg of QE equivalent per gram of dry weight (mg QE /g) [17].

Antioxidant Activity:

DPPH Radical Scavenging Activity Assay: The radical scavenging capacity of the LLME against 2, 2-diphenyl-1-picrylhydrazyl radical was conducted using the method described [18, 19] with some modifications. In concise, 0.5 mM solution of DPPH was prepared by dissolving 0.02 g of DPPH crystalline solid in 100 mL of methanol. Different concentrations (1000, 500, 250, 100, 50, 25, and 10 µg/mL) of ascorbic acid (standard) and LLME were

prepared in methanol. 2.4 mL of DPPH solution was mixed with 1.6 mL of LLME in methanol at different concentrations. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm using a UV-VIS Spectrophotometer. The percentage of free radical scavenging was calculated as the equation shown below. The same procedure was recapped for ascorbic acid as standard [20]. The experimental value is expressed as the mean ± Standard error mean (SEM), and all experiments are carried in triplicates (n = 3). The calculation of radical scavenging activity was expressed as the percentage inhibition by using the following formula:

% Inhibition = (Absorbance control - absorbance sample) / Absorbance control × 100..... (Eq. 1)

ABTS Radical Scavenging Assay: The ABTS free radical cation scavenging ability of LLME was determined by the standard method [20]. Varying concentrations (10-1000 µg/mL) of LLME and ascorbic acid were prepared using absolute alcohol. ABTS (7 mmol/L) was dissolved in distilled water, and potassium persulfate (2.45 mmol/L) was added. The mixture was incubated for 12-16 h overnight in the dark at room temperature. ABTS+ solution was added and incubated for 30 min. The absorbance was measured at 734 nm [21]. The percentage of scavenging can be calculated using the equation (Eq.1).

α-Amylase and α-Glucosidase Inhibitory Assay: 0. 5 mL of LLME and acarbose were prepared in concentrations ranging from 0.1 mg/Ml to 1 mg/mL respectively were added to 500 µL of 0.5 mg/mL α-amylase solution in 0.20 mM phosphate buffer (pH 6.9) and were incubated at 25 °C for 10 min. 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer is added then incubated again for 10 min at 25 °C. 1 mL of dinitrosalicylate (DNSA) is added and boiled for 5 min. The tubes were allowed to cool at room temperature. 10 mL of distilled water was added, and absorbance was taken at 540 nm [22]. The inhibitory activity of the extract was calculated using the equation (Eq.1).

In the α-glucosidase assay, LLME was pre-incubated with 0.2 mL of α-glucosidase solution for 5 min. Then 0.2 mL of sucrose was added to the sample, proceeding to 30 min of incubation at 37 °C. The reaction was arrested by heating samples at 90 to 100 °C. The quantity of glucose was determined by the GOD-POD method at 546 nm [23]. Enzyme activity hinges upon the amount of glucose released. The inhibitory activity of the extract was calculated using the equation (Eq.1).

Statistical Analysis: Each experimental values are expressed as the mean ± Standard error mean, and all experiments were carried out in triplicates (n=3). All the IC_{50} values for antioxidant and antidiabetic studies were computed using the graph pad prism software (Version 5.03) by non-linear regression graph plotted between the percentages of radical scavenging or enzyme inhibition (x-axis) versus concentration (y-axis) of the extracts.

RESULTS:

Phytochemical Screening: *L. leuco-cephala* leaf extraction was carried out by the maceration method using absolute ethanol, and the yield was found to be 65%. The phytochemical screening of LLME proved alkaloids, monosaccharides, proteins, amino acids, phenols, steroids, flavonoids, glycosides, and tannins.

Total Phenol Content: As shown in **Fig. 1**, the TPC was denoted in terms of gallic acid equivalent (the standard calibration curve equation: y = 0.0003 + 0.0812, r^2 = 0.9786); LLME exhibited the phenol content of 970 mg of GAE/g.

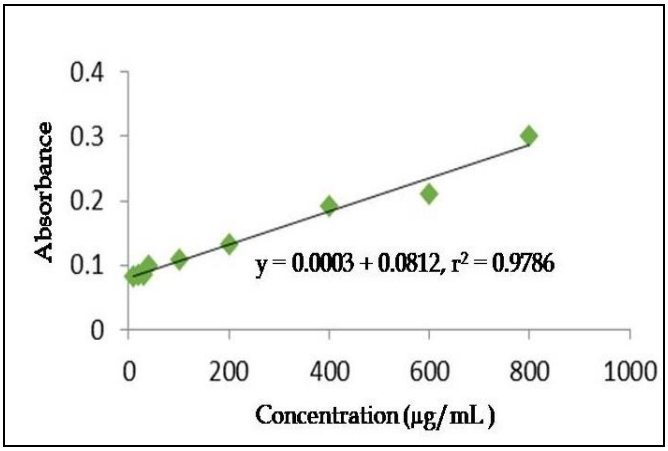

FIG. 1: CALIBRATION CURVE OF GALLIC ACID

Total Flavonoid Content: As shown in **Fig. 2**, the TFC was denoted in terms of quercetin equivalent (the standard calibration curve equation: y = 0.0002 + 0.0323, r^2 = 0.9789); LLME exhibited the flavonoid content of 550 mg of QE/g.

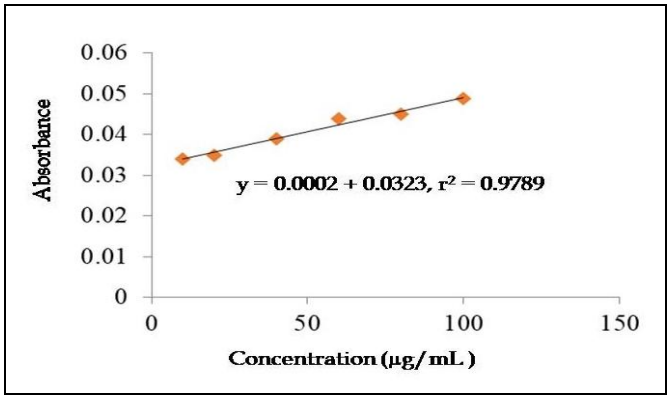

FIG. 2: CALIBRATION CURVE OF QUERCETIN

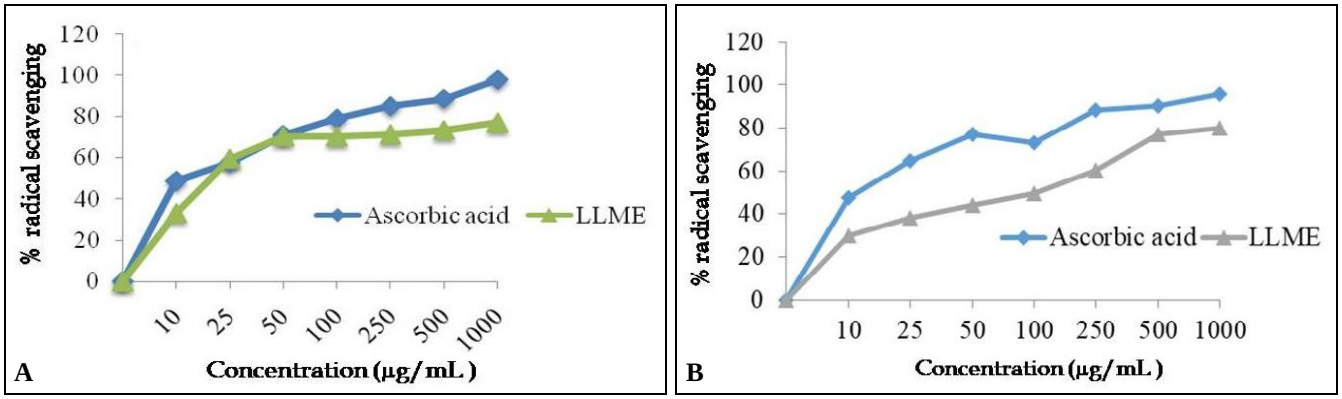

FIG. 3A: ABTS RADICAL SCAVENGING ASSAY OF LLME AND ASCORBIC ACID (N=3); 3B: DPPH RADICAL SCAVENGING ASSAY OF LLME AND ASCORBIC ACID (N=3)

Antioxidant Activity: Using DPPH and ABTS method, LLME was examined for *in-vitro* antioxidant activity and measured for scavenging of free radicals.

The sample and ascorbic acid (standard) were prepared at concentrations ranging from 10 µg/mL to 1000 µg/mL for both methods. As shown in **Fig. 3A**, LLME (IC_{50} ± SEM: 11.87 ± 0.05 µg/mL) has shown good antioxidant activity when compared to the standard drug, ascorbic acid (IC_{50} ± SEM: 11.01 ± 0.09 µg/mL) in the ABTS method. In DPPH method, from the graph in **Fig. 3B**, supported the good antioxidant activity of LLME (IC50 ± SEM: 11.21 ± 0.2 µg/mL) when compared to ascorbic acid (IC_{50} ± SEM: 11.09 ± 0.05 µg/mL).

Anti-diabetic Activity: As shown in **Table 1**, the LLME extract and acarbose (standard) were used at concentrations 100-1000 µg/mL.

TABLE 1: PERCENTAGE INHIBITION OF α-AMYLASE AND α-GLUCOSIDASE ACTIVITY WITH LLME

Concentration (µg/mL)	% inhibition of enzyme activity			
	α-amylase		α-glucosidase	
	Acarbose	LLME	Acarbose	LLME
100	49.02	28.95	43.25	27.82
200	63.45	48.94	62.35	47.82
400	74.45	52.26	72.13	51.76
800	85.91	64.28	84.88	65.75
1000	89.43	74.96	88.75	77.12
IC_{50} ± SEM (µg/mL)	202 ± 0.02	246 ± 0.06	211 ± 0.02	247 ± 0.08

Note: a Mean standard error using GraphPad Prism 5.03 (n=3)

In α-amylase inhibition assay, acarbose revealed α-amylase inhibition activity with an IC_{50} value of 12.02 ± 0.02 µg/mL; meanwhile, LLME showed an IC_{50} value of 12.46 ± 0.06 µg/mL. The sample showed good inhibitory activity against α-amylase.

In α-glucosidase inhibition assay also supports the results of α-amylase inhibition assay of acarbose IC_{50} value of 12.11 ± 0.02 µg/mL followed by LLME with IC_{50} values of 12.47 ± 0.08 µg/mL.

DISCUSSION: *Leucaena leucocephala* is a well-known medicinal food used in different parts of the world for the treatment of various diseases associated with oxidative stress including DM [6, 10]. Various parts of *L. leucocephala* have been reported to possess significant pharmacological properties, including antioxidant and antidiabetic activities [6]. The combination of multiple phyto-constituents by virtue of their synergistic or antagonistic properties might be helpful to combat multiple disease-related drug targets with minimal adverse effects [24]. The extraction of plants using a cold maceration technique is used for the extraction of thermolabile phytoconstituents. Occasional shaking during the extraction process facilitates the diffusion of solvents into the plant materials and also removes the concentrated solution from the plant material surface, and allows the fresh solvent to enter them and achieve a higher extraction yield. Compared to any other plant extraction method, maceration is a more applicable, convenient, and cost-effective method and produces a high yield of bioactive compounds such as polyphenols [25].

In the present study, the phytochemical screening of the LLME revealed the presence of multiple phytoconstituents such as alkaloids, mono-saccharides, proteins, amino acids, phenols, steroids, flavonoids, glycosides, and tannins. The presence of numerous phytoconstituents might benefit to combat oxidative stress and hyper-glycemia by their different modes of action. The TFC determination was conducted using the aluminum chloride colorimetric method. Flavonoids such as glycosyl flavones, flavones, and condensed tannins in which plant secondary metabolites may be formed. The presence of a free OH group determines the presence of antioxidant activity [26]. *In-vitro* antioxidant activity was carried out on LLME using DPPH and ABTS method to measure their ability to scavenge potent free radicals. In the DPPH method, the reagent undergoes reduction by donating an electron or receiving a hydrogen atom, which changes color from violet to changes [27]. As for the ABTS method, a blue or green chromophore is produced due to the reaction between potassium persulfate and ABTS. The presence of complex mixtures of proanthocyanidins in the leaf and fruit of *L. leucocephala* might be responsible for scavenging the potent DPPH and ABTS radicals [28].

Plant-derived phytoconstituents influence glucose metabolism by reducing the α-amylase and α-glucosidase activities and also defend against oxidative stress [29]. *L. leucocephala* was reported to possess high levels of polyphenols and flavonoids[7, 13,] and the presence of polyphenols might be responsible for their antioxidant and carbo-hydrate digesting enzyme inhibitory effect[10]. Similarly, LLME has antioxidant properties in the current study, suggesting that the antioxidant activity could affect its antidiabetic activity in digestive enzymes, α-amylase, and α-glucosidase.

CONCLUSION: This study illustrated that the *L. leucocephala* ethanolic leaves extract containing flavonoids has promising antioxidant effects against ABTS and DPPH. Suggesting *L. leucocephala* extract could be used to prevent oxidative stress induced by free radicals. *The in-vitro* antidiabetic activity was also investigated on the inhibitory effects on α-amylase and α-glucosidase. This study suggests a potential mode of action *L. leucocephala* extract due to the inhibition of digestive enzymes. It could be beneficial for diabetic patients to avoid or reduce diabetic complications. However, further study should be done to isolate the active constituents of *L. leucocephala* and research for their natural bioactivity.

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