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Research Article
Evaluating the Effect of Combined Plant Extracts on α-amylase and α-glucosidase Inhibition Activity as Antidiabetic Agents

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Abstract

Background and Objective: Diabetes mellitus (DM) is the major health and economic problem through the world. The insufficiencies and severe adversarial special effects related with conventional medicines, led to a strong-minded search for alternative natural antidiabetic agents. The study was aimed to screen the *in vitro* antidiabetic activity of seven plant extracts. **Materials and Methods:** The bioactivity of the extracts was correlated through a Gas Chromatography-Mass spectrometry (GC-MS)-based analysis, while extracts with 50% inhibition activity were reported. **Results:** The α-amylase inhibitory activities of the chloroform extracts of *Averrhoa bilimbi*, *Andrographis paniculata*, *Eurycoma longifolia* and *Punica granatum* exhibited more than 50% inhibition (50.55, 68.24, 82.42 and 69.73% respectively), while chloroform extracts of *A. paniculata*, *Orthosiphon stamineus* and *P. granatum* exhibited 90.48, 52.00 and 62.00% of α-glucosidase inhibitory effects, respectively. Only ethanolic and methanolic extracts of *E. longifolia* exhibited α-glucosidase inhibitory effects of 57.80 and 53.31%, respectively. A combination of the ethanolic fraction of *E. longifolia* with the chloroform fraction of *P. granatum* at a ratio of 2:1 exhibited the highest α-glucosidase inhibition rate of 148.06%, while a combination of the ethanolic fraction of *E. longifolia* with the chloroform fraction of *O. stamineus* at the same ratio exhibited α-glucosidase inhibition rate of 137.43%. A phytochemical analysis based on GC-MS revealed the presence of fatty acids, with palmitic acid recording the highest percentage (100%) in the *O. stamineus* extract. **Conclusion:** The observed α-amylase and α-glucosidase inhibitory activities of these plant extracts suggested their potential usage as an alternative sources of antidiabetic agents.

Key words: Antidiabetic, ethnomedicinal plant, α-amylase inhibition, α-glucosidase inhibition, combined extracts, phytochemicals, phytopharmacology

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease that manifests in hyperglycaemia due to either the inefficiency of the body to secrete insulin in response to the presence of high sugar level in the body or due to the insensitivity of the body cells to the secreted insulin or both[1]. A sustained high level of sugar in the body contributes to the organ failures and other conditions prevalent in DM patients[2]. The estimated global prevalence of DM by the International Diabetes Federation (IDF) is shown in Fig. 1.

The use of ethnomedicinal plants in the treatment of several human diseases such as renal stones, diabetes, muscle cramps and warts have been investigated and reported. Despite the waning interest in ethnomedicine due to the recent advances in modern medical tools, it is important to document such medications for knowledge sake and future usage[3]. They are rich sources of anti-diabetic compounds such as flavonoids, alkaloids, phenolic and tannins that improve the efficiency of pancreatic tissues by increasing the insulin secretion or decreasing the intestinal absorption of glucose[4]. In Malaysia, these plants are widely used by the native people to treat certain diseases and are certainly found in several locations such as Kampung Bawong and Perak in Western Malaysia. The abundance of biologically active phytochemicals in these plants makes them ideal for pharmacological consideration[5,6]. A successful management of type 2 DM mainly depends on a successful inhibition of α-amylase and α-glucosidase (starch digestive enzymes) which are involved in starch catabolism. Meanwhile, the side effects of the chemical-based drug currently used for the inhibition of these enzymes have elicited a search for alternative agents (dietary or herbal sources) which could serve equally as α-amylase and α-glucosidase inhibitors[7]. Glycemic control is chiefly focused on inhibitors designed to target glucosidases, contributors of hydrolases located at the level of gastrointestinal tract and whose exo-acting abilities are necessary for carbohydrate digestion[8]. This study was undertaken to investigate the α-amylase and α-glucosidase inhibitory activities of seven medicinal plant extracts prepared with different solvents as well as the activities of their possible combinations as alternative antidiabetic agents.

MATERIALS AND METHODS

Plant materials preparation and chemicals: The current study was carried out at Faculty of Industrial Sciences and Technology (FIST) laboratories, Universiti Malaysia Pahang (UMP), Malaysia, from March, 2018 to February, 2019. Seven plants (*Averrhoa bilimbi* (Ab), *Andrographis paniculata* (Ap), *Orthosiphon stamineu* (Os), *Gynura procumbens* (Gp), *Eurycoma longifolia* (El), *Punica granatum* (Pg) and *Swietenia macrophylla* (Sm)) were purchased from a market in Kuantan, Pahang, Malaysia (Table 1) and transported to the laboratory complex of the Faculty of Industrial Sciences and Technology (FIST), Universiti Malaysia Pahang, Gambang campus for further preparation and investigations. The plants were first washed with running water before finally being rinsed with distilled water (DW). The plants were left to dry for about 7 days at room temperature. Later, the dried plant materials were pulverized in an electric blender and transferred to airtight plastic containers for storage. For the chemicals, α-amylase and α-glucosidase (EC 3.2.1.20) sourced from *Saccharomyces cerevisiae* was bought from Sigma-Aldrich Chemical Co (USA), while all other chemicals and solvents used were of analytical grade.

Ultrasound-assisted extraction of plant inhibitors: The extraction was performed using a modified procedure[10]. Briefly, 50 g of each plant material was extracted with 400 mL

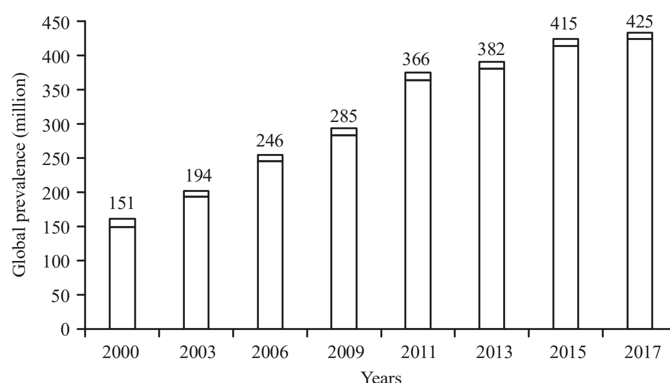

Fig. 1: Increasing global prevalence of diabetes was recorded from 2000-2017
Source: IDF[9]

Table 1: English name, scientific name, code, family and part of all plants used in this study

English names	Scientific names	Codes	Family	Parts used
Tree sorrel	*Averrhoa bilimbi*	Ab	Oxalidaceae	Leaves
King of bitters	*Andrographis paniculata*	Ap	Acanthaceae	Whole plant
Cat's whiskers	*Orthosiphon stamineus*	Os	Lamiaceae	Leaves
Longevity spinach	*Gynura procumbens*	Gp	Asteraceae	Leaves
Longjack	*Eurycoma longifolia*	El	Simaroubaceae	Roots
Pomegranate	*Punica granatum*	Pg	Lythraceae	Peels
Honduran mahogany	*Swietenia macrophylla*	Sm	Meliaceae	Seeds

of the respective solvents (distilled water, 97% ethanol, 99% methanol and 99% chloroform) in a glass beaker. The ultrasonic effect on the extraction process was introduced by immersing the ultrasonic probe of a sonicator into a beaker containing the samples and operated at an ultrasound power of 80 W. The temperature of the process was maintained by setting up the experiments in an ultrasonic bath at a temperature of 45°C. Each extraction bath was sonicated for 15 min at intervals of 1-180 min. After each round of sonication, the samples were filtered before concentrating the filtrate in a rotary vacuum evaporator (Eyela N-1200 Series). The obtained concentrates were dried at room temperature for 48 h and stored at -20°C prior to further analysis.

α-amylase inhibition assay: The α-amylase inhibitory activity of the extracts was evaluated using the procedure described with little modification[11]. Briefly, the plant extracts (50 μL) were added into 50 μL of phosphate buffer (0.02M, pH 6.9) containing 0.5 mg mL^{-1} of α-amylase solution. The mixture was pre-incubated at 25°C for 10 min, after which 50 μL of 1% starch solution in sodium phosphate was added into the mixture and incubated for 10 min at 25°C. The reaction was terminated by adding 100 μL of dinitrosalicylic acid (DNS) reagent into the solution and further heated in a boiling bath for 5 min. After heating, the solution was cooled to room temperature before adding 1 mL of DW. The α-amylase activity was determined by measuring the absorbance of the solution at 540 nm using a microplate reader (Infinite M200PRO). The control solution was prepared in a similar way as the tests except that DW was used in the place of the extracts. The percentage of α-amylase inhibition (%) was determined using Eq. 1:

$$\text{Inhibition (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100 \quad (1)$$

Where, Ac is the control absorbance and As is the sample absorbance. All the analysis were done in triplicate, while the outcomes were presented as the Means ±SD of the observed readings.

α-glucosidase inhibition assay: The α-glucosidase inhibitory activity of the extracts was determined using the procedure earlier described with some modifications[11,12]. A substrate solution of p-nitrophenyl glucopyranoside (pNPG) was first prepared in 20 mM of phosphate buffer of pH 6.9. Then, 100 μL of α-glucosidase (1.0U mL^{-1}) was mixed with 50 μL of the extracts and pre-incubated for 10 min. After the incubation, 50 μL of pNPG (3.0 mM) was added to the solution to initiate the enzyme-substrate reaction. The mixture was incubated for 20 min at 37°C and later terminated by adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the absorbance of the solution at 405 nm. DW was used as the blank solution, whereas the controls were prepared in a similar way as the test solutions without the plant extracts. The percentage of α-glucosidase inhibition (%) was determined using Eq. 1.

Inhibitory effect of combined plant extracts on α-amylase and α-glucosidase activities: The effect of an equal combination of the plant extracts (2, 3 and 4) on the α-amylase and α-glucosidase activities was studied. The extracts that exhibited potent activities were combined and investigated for synergistic inhibitory activities[11,12]. The inhibitory activities of the combined extracts were compared to the activity of the individual extracts.

Phytochemical analysis: The phytochemical content of the extracts was quantitatively analyzed using a GC-MS gas chromatograph (Agilent Technologies) equipped with 30 m×0.25 mm ID DB 5 MS capillary column. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}, splitmode was used in a ratio of 1:60, while the injection volume was 1 μL. The temperature of the system was program as follows initially: 50°C was maintained for 3 min, then, increased to 280°C at a rate of 15°C min^{-1}, held at 280°C for 10 min and the mass range was from m/z 35-800 μ. The appropriate controls were run under the same conditions as the tests. The components were identified by comparing their mass spectra with reference mass spectra hosted by NIST-Wiley 2011 library[13].

Statistical analysis: All the results were expressed as mean±standard error of means (SEM) for 3 samples. The differences between treated and untreated samples were assessed by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Enzyme inhibition assay: The results obtained from 28 different plant extracts for α-amylase and α-glucosidase inhibition assay were presented in Table 2. From the result, only 4 chloroform extracts of El, Pg, Ap and Ab exhibited more than 50% α-amylase inhibition activity with inhibitory percentages of 82.42, 69.73, 68.24 and 50.55%, respectively. The chloroform extracts of Ap, Pg and Os exhibited α-glucosidase inhibitory activities of 90.48, 61.53 and 51.09 % respectively, while the ethanolic and methanolic extracts of El exhibited α-glucosidase inhibitory activities of 57.80 and 53.31%, respectively. The other extracts exhibited α-amylase and α-glucosidase inhibition activities of less than 50% and were not further considered in this study. The results showed chloroform extracts to exhibit better inhibitory activities compared to the other solvents.

Pushparaj *et al.*[14] studied the hypoglycemic and hypolipidemic effects of ethanolic extract of *A. bilimbi* and reported about 50% hypoglycemic effect within 2 weeks but the same extract has been reported to exhibit no inhibitory effect against α-amylase[15]. Extracts from Okra pods have been reported to show a strong inhibitory effect against α-glucosidase compared to α-amylase, showing its suitability for glucose level regulation in diabetic patients. Furthermore, okra pod extracts have been shown to exhibit a competitive inhibitory activity against α-glucosidase and a mixed inhibitory activity against α-amylase, showing that it can be suitable for the management of hyperglycemia in type 2 diabetes cases[16]. The current study, however, compared the inhibitory activities of the different plant extracts (individually and combined) and from the comparison, 7 chloroform extracts were found to exhibit significant levels of inhibitory activities against α-amylase and α-glucosidase, while water, methanol and ethanol extracts showed lower activities ($p<0.05$).

Plant extracts combination: The combined inhibitory activity of 2, 3 or 4 extracts on α-amylase was observed to be in the range of 9.30-77.3, 12.22-51.37 and 3.53%, respectively, while α-glucosidase inhibition was in the range of 4.7-89.62, 0.25-81.30 and 1.96%, respectively (Table 3).

Table 2: α-amylase and α-glucosidase enzyme inhibition (%) of plant extracts using different solvents

	Inhibition (%)							
	α-amylase				α-glucosidase			
Plant codes	Water	Chloroform	Ethanol	Methanol	Water	Chloroform	Ethanol	Methanol
Ab	-	50.55	-	-	-	47.33	-	-
Ap	-	68.24	-	-	-	90.48	-	-
Os	-	-	-	-	-	51.09	-	-
Gp	-	-	-	-	-	17.04	-	-
El	-	82.42	-	-	-	-	57.80	53.31
Pg	-	69.73	-	-	-	61.532	-	-
Sm	-	-	-	-	-	4.050	3.831	-

-: No inhibition

Table 3: Combination effect of 2, triple and quadruple plant extracts on α-amylase and α-glucosidase inhibition (%)

Plant extracts	α-amylase inhibition (%)	Plant extracts	α-glucosidase inhibition (%)
ElC: AbC	9.30[a]	ElE:OsC	82.03[a]
ElC:ApC	77.03[a]	ElE:ApC	89.33[a]
ElC:PgC	64.91[a]	ElE:PgC	89.62[a]
AbC:ApC	16.17[a]	OsC:Apc	4.70[a]
AbC:PgC	31.80[a]	OsC:PgC	47.64[a]
ApC:PgC	55.64[a]	PgC:ApC	83.13[a]
ElC:AbC:ApC	12.22[a]	ElE:OsC:ApC	81.30[b]
ElC:AbC:PgC	51.37[a]	ElE:OsC:PgC	3.033[b]
ElC:ApC:PgC	46.52[a]	ElE:ApC:PgC	0.25[b]
AbC:ApC:PgC	35.71[a]	OsC:ApC:PgC	10.84[b]
ElC:AbC:ApC:PgC	3.53	ElE:OsC:ApC:PgC	1.96

Results expressed as Mean±SD (n = 3), $p<0.05$ vs. control the same conditions, [a]Significant, [b]Non-significant, ElC: Chloroform extract of *E. longifolia*, ApC: Chloroform extract of *A. paniculata*, PgC: Chloroform extract of *P. granatum*, AbC: Chloroform extract of *A. bilimbi*, ElE: Ethanol extract of *E. longifolia*, OsC: Chloroform extract of *O. stamineus*

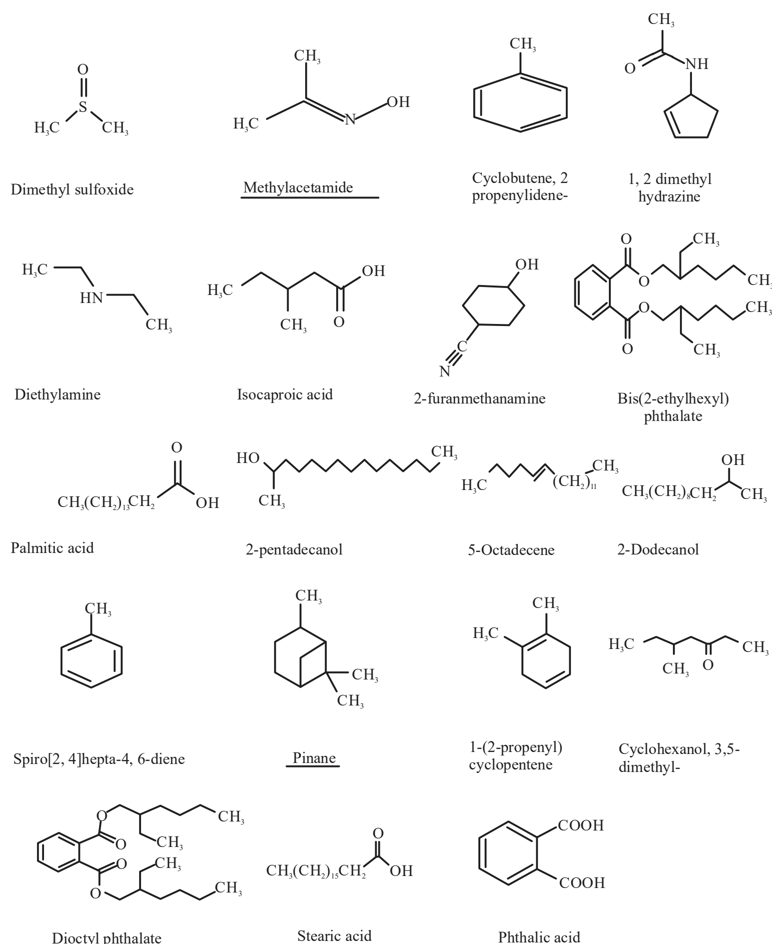

Fig. 2: Chemical structures of the major active constituents' compounds identified by GC-MS

The observed inhibitory effects of a combination of 2 different extracts were as follows: EIC-ApC (77.03%) for α-amylase and EIE-PgC (89.62%) for α-glucosidase. The inhibitory activities of the extract combinations against α-amylase were not significantly better compared to the individual activities of the extracts (Table 2), while the combined extracts (EIE-OsC and EI-PgC) showed significantly better α-glucosidase inhibitory activities (82.03 and 89.62%, respectively) compared to the individual extracts activities (Table 2, 3). Both extracts showed a synergistic inhibitory activity against α-glucosidase enzyme compared to their individual activities. Furthermore, the activity of the combined extracts (EIE-OsC and EIE-PgC) at different combination ratios (1:1, 1:2, 2:1 v/v) was also investigated. The study revealed that both extract combinations at a ratio of 2:1 significantly inhibited α-glucosidase by up to 137.43 and 148.06% respectively, compared to the other combination ratios (Fig. 2, 3). An increase in the combination ratio of EIE to 2 had more significant influence on the enzyme inhibition percentage compared to higher combination ratios of OsC and PgC, suggesting that EIE contributes more to the observed enzyme inhibitory activity of the extract combinations. According to Thu *et al.*[17] studies on the phytochemical composition of different parts of *E. longifolia* extracted with different solvents have shown a range of pharmacological activities, including aphrodisiac, anticancer, anti-osteoporotic, antimicrobial, antioxidant, anti-inflammatory, anti-malarial, anti-diabetic, anxiolytic and anti-ulcer activities. Pomegranate has also been extensively researched as a source of novel compounds with biological activities[18]. Several biological activities have also been attributed to the phytochemical contents of *O. stamineus*. Such activities include diuretic, renal protective, hypouricemic, antioxidant, hepatoprotective, gastroprotective, anti-inflammatory, antihypertensive, antihyperlipidemic, antimicrobial, antidiabetic and anorexic activities. Hence, *O. stamineus* has been used for both traditional and pharmacological purposes for the management of various pathological conditions[19].

Table 4: GC-MS identification of compounds in organic solvent extracts of plants inhibiting α-amylase and α-glucosidase enzyme activity

Plant and solvent used	Identified compound	CP (%)	RT	PA	MF
E. longifolia (Ethanol)	Dimethyl sulfoxide	100.00	4.798	93.09	C_2H_6OS
	Methylacetamide	0.87	3.127	0.81	C_3H_7NO
	Cyclobutene, 2 propenylidene-	1.96	3.556	1.83	C_7H_8
	1,2 Dimethyl hydrazine	1.26	8.614	1.18	$C_2H_8N_2$
	Diethylamine	0.38	11.870	0.36	$C_4H_{11}N$
	Isocaproic acid	0.45	15.744	0.42	$C_6H_{12}O_2$
	2-Furanmethanamine	0.20	16.900	0.19	$C_7H_{11}NO$
E. longifolia (Chloroform)	Bis(2-ethylhexyl) phthalate	100.00	19.257	48.65	$C_{24}H_{38}O_4$
	Palmitic acid	10.33	15.750	5.03	$C_{16}H_{32}O_2$
	2-Pentadecanol	11.94	17.243	5.81	$C_{15}H_{32}O$
	5-Octadecene	15.08	15.984	7.34	$C_{18}H_{36}$
	2-Dodecanol	11.16	13.077	5.43	$C_{12}H_{26}O$
	Spiro[2,4]hepta-4,6-diene	15.25	3.568	7.42	C_7H_8
O. stamineus (Chloroform)	Palmitic acid	100.00	15.747	12.58	$C_{16}H_{32}O_2$
	Pinane	72.41	14.914	9.11	$C_{10}H_{18}$
	1-(2-Propenyl) cyclopentene	81.47	16.905	10.25	C_8H_{12}
	Cyclohexanol, 3,5-dimethyl-	70.98	16.739	8.93	$C_8H_{16}O$
	Phthalic acid, 4,4-dimethylpent-2-yl octadecyl ester	55.80	19.251	7.02	$C_{30}H_{50}O_4$
P. granatum (Chloroform)	Dioctyl phthalate	100.00	19.257	40.76	$C_{24}H_{38}O_4$
	Palmitic acid	23.30	15.750	9.50	$C_{16}H_{32}O_2$
	stearic acid	5.69	17.037	2.32	$C_{18}H_{36}O_2$
	5-Octadecene	2.45	14.605	6.90	$C_{18}H_{36}$
	2-Pentadecanol	11.99	17.243	4.89	$C_{15}H_{32}O$
	Toluene	17.24	3.573	7.03	C_7H_8

CP: Compound percentage, RT: Retention time, PA: Peak area, MF: Molecular formula

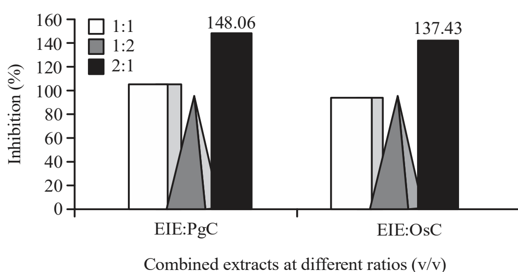

Fig. 3: Effect of potential selected combinations on α-glucosidase inhibition (%) at different ratios (v/v)

Phytochemical analysis: The phytochemical content of the extracts was quantitatively analyzed using GC-MS to identify the probable components that may contributed to the observed enzyme inhibitory activities. The analysis confirmed the presence of different phytochemicals such as palmitic acid, isocaproic acid, stearic acid, etc in the extracts (Table 4). The components were identified by comparing their GC-MS-identified percentages, retention times, peak areas and molecular weights to those of reference standards (Wiley-NIST library). Table 4 showed the identified compounds in the studied extracts along with their calculated compound percentages. The percentage concentration of palmitic acid in the extracts was as follows: *O. stamineus* (100%), *P. granatum* (23.30%) and *E. longifolia* (10.33%), while stearic acid was found in *P. granatum* at a concentration percentage of 5.69%. It was demonstrated that the extracts of *E. longifolia*, *P. granatum* and *O. stamineus* leaves induced hypoglycemic effect by inhibiting α-amylase and α-glucosidase, while *P. oleracea* L. could reduce insulin resistance and inhibit α-amylase and α-glucosidase as well. Therefore, these extracts could regulate body blood sugar levels owing to their rich flavonoid, polysaccharide and polyunsaturated fatty acid contents[20]. Stearic acid contributes majorly to lipotoxicity in the beta cells, implicating its role in beta cell dysfunction and type II diabetes when found in elevated concentrations in the body[9,21-23].

CONCLUSION

This study investigated certain Malaysian medicinal plants for potential antidiabetic activity, focusing mainly on their α-amylase and α-glucosidase inhibitory effects. The combination of ethanolic extracts of *E. longifolia* with chloroform extract of *P. granatum* at a ratio of 2:1 and ethanolic extracts of *E. longifolia* and *O. stamineus* at the same ratio exhibited strong inhibitory activities against α-glucosidase. The improved activity of the combined extracts

indicated that *E. longifolia*, *O. stamineus* and *P. granatum* contained complex compounds that study synergistically at certain combination ratios to bring about the desired enzyme inhibitory effects.

SIGNIFICANCE STATEMENT

This study discovered the effect of different plant extracts and its combinations on α-amylase and α-glucosidase inhibition. This study will help the researcher to uncover the critical area of potential combined medicinal plant extracts as alternative natural therapeutic agents. Thus, a new extract combination has been enhanced and improved the desired enzyme inhibitory effects.

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