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## Antidiabetic and Antioxidant Activities of Bay, Pandan, Citrus Leaves and their Combination in Vitro

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The study aimed to evaluate the effects of bay (B), pandan (P), citrus leaves (C) and their combinations against starch hydrolysis enzymes ( $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes) and antioxidant activity and to examine the role of polyphenol compounds in enzyme inhibition and antioxidant activity. Three single leaves extracts and five of their combinations were applied to inhibit  $\alpha$ -glucosidase hydrolyzing *p-nitrophenyl- $\alpha$ -D-glucofuranoside* or  $\alpha$ -amylase hydrolyzing starch solution as well as to scavenge free radicals. The leaf extracts and their combination showed inhibition activities against  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes with range of inhibition activities were between 17.63% to 26.04% and 20.14% to 35.30% respectively. There is no significant difference among the extracts in modulation of  $\alpha$ -glucosidase activity, but each extract exhibited different effect on  $\alpha$ -amylase or antioxidant activities. Mixing P with B and C increases the inhibitory activity of the extract against  $\alpha$ -amylase as seen that percent of inhibition of BPC is significantly higher than P, even though their total phenolic content was not different. The synergism or antagonism effect was not observed when the extracts were combined as the enzyme inhibition or antioxidant activities are not dependent on the proportion of the extract in the mixtures. The role of polyphenol compounds on inhibition of the starch digestion enzymes and on antioxidant activity was not observed. Further study is required to fully elucidate the effect of the leaf or their combinations on diabetic animal models or diabetic patients.

**Keywords:** Bay leaf, pandan leaf, citrus leaf, antidiabetic, antioxidant, phenolic compound.

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Rice is staple food of most Asia countries including Indonesia. As rice is good source of starch, therefore, consumption of rice is suggested as risk factor of diabetes mellitus<sup>1 and 2</sup>. Reducing of starch digestibility of the rice is one of promising strategies to reduce hyperglycemic effect of the rice<sup>3</sup>.

Starch, a polysaccharide composed of alpha 1,4-linked glucose units (amylose) and alpha 1,4-1,6-linked branched structure (amylopectin), is cleaved in the duodenal cavity involved several hydrolytic enzymes such as pancreatic alpha-

amylase and brush border glycosidase<sup>4</sup>. Inhibition of these enzymes is not only considered as a strategy to reduce the digestibility of the starch but also a treatment of carbohydrate uptake disorder, such as diabetes and obesity<sup>5 and 6</sup>. Plants are an important source of phytochemical compounds that have inhibition activities against the enzymes, therefore, they have potentiality for therapeutic drug or functional food for the diseases<sup>7</sup>.

Rice is prepared by cooking (steaming or boiling) rice soaked in water. Addition of aromatic or flavouring ingredients such as Indonesian bay

(*Eugenia polyantha* Wight), pandan (*Pandanus amaryllifolius* Roxb.) and citrus (*Citrus hystrix*) leaves is a common practice in Indonesian rice cooking. Whether the leaves have beneficial effects on rice starch digestibility when they are mixed with rice remain unexplored. However, the therapeutic benefits of these ingredients for diabetes have been reported.

Aqueous extracts of the bay leaves improve glucose and insulin metabolism in *in vitro* model<sup>8</sup>. Consumption 1 to 3 g a day of ground bay leaves by type 2 diabetes patients reduced serum glucose with significant decreases ranging from 21 to 26% after 30 d and improved lipid profile of the subjects<sup>9</sup>. Moreover, methanolic extract of bay leaf displayed scavenging activity against superoxide and hydroxyl radicals in a concentration-dependent manner<sup>10</sup>.

Pandan leaf is a tropical plant which is used mostly as a flavoring agent for certain rice and bread recipe<sup>11</sup>. Water extract of pandan leaves reduced blood glucose level as well as improvement the insulin resistance of obese mice<sup>12</sup>. In healthy subjects, drinking pandan leaf tea effectively decreased postprandial blood sugar through inhibition of  $\alpha$ -glucosidase enzyme and induction of insulin production in pancreatic cell<sup>13</sup>.

Citrus leaf is an aromatic Asian leaf most often used in Indonesian recipes including cooked rice recipe<sup>14</sup>. The leaf extracts exhibited anticancer activity through reduction of cancer cell line viability<sup>15</sup>. Fresh juice from Citrus fruits contains phenolics, tannins and flavonoids and exhibited anti-alpha amylase and alpha-glucosidase *in vitro*<sup>16</sup>.

Mechanism of the protective effect of dietary antioxidants has been hypothesized through inhibition of oxidation chain reactions<sup>17</sup>. Thus, consumption plant foods rich in antioxidant compounds could reduce incidence of chronic diseases, such as diabetes, through down regulation of oxidative stress<sup>17,18</sup>. In this study, we aimed to evaluate the effects of bay, pandan, citrus leaves and their combinations against starch hydrolysing enzymes ( $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes) and antioxidant activity. Additionally, due to the leaves are rich in polyphenol<sup>19, 20, 21</sup>, the role of polyphenol compounds in enzyme inhibition and antioxidative effect was also elucidated.

## MATERIALS AND METHODS

### Plant material

Bay, pandan and citrus leaves were collected from local market in Bandar Lampung, Indonesia. The leaves, immediately after collection were thoroughly washed with water and dried in oven at 60°C. Dried leaves were powdered using grinder to produce coarsely powder.

### Preparation of extracts

The dried leaf powder of bay, pandan, citrus or their combination (10 g) were boiled in 100 mL water for 20 minutes. The extract was filtered (extract 1), and the residue was reboiled in 100 mL for 20 minutes, then filtered to get extract 2. Extract 1 and extract 2 were then mixed and considered as 100% extract. Proportion of each type of leaves in the combination was 50.0 % when the combination contained two types of plants, and 33.3 % when the combination contained three types of plants (Table 1).

### Assay of $\alpha$ -glucosidase inhibitory activity

The slightly modified method described by Rao *et al.*<sup>22</sup> was applied to measure the effect of the leaf extracts on 500U- $\alpha$ -glucosidase activity using  $\alpha$ -glucosidase crude enzyme (Shandong Longda Bio-Products Co., Ltd.). The substrate solution p-nitrophenyl gluco-pyranoside (pNPG) (Sigma Aldrich, Switzerland) was prepared in aquades (0.03012 g/100mL). Briefly, sample of 200 500U leaf extracts were preincubated with 2 mL of  $\alpha$ -glucosidase crude enzyme for 10 min at 37°C. The reaction was initiated by addition of 1 mL of pNPG substrate and incubated at 37°C for 30min. The reaction was stopped by adding 2 mL of 2 %  $\text{Na}_2\text{CO}_3$  (Merck, Germany). The 500U- $\alpha$ -glucosidase activity was determined by measuring the yellow-colored p-nitrophenol released from pNPG at 405 nm (Thermo Scientific Genesys 20, USA). Percentage inhibition is calculated as %Inhibition = [(Abscontrol - Absextract) / Abscontrol]  $\times$  100.

### Assay of $\alpha$ -amylase inhibitory activity

$\alpha$ -amylase inhibitory activity assay was performed at 37°C using  $\alpha$ -amylase crude enzyme (Shandong Longda Bio-Products Co., Ltd.). A mixture containing 1 ml  $\alpha$ -amylase crude enzyme, 0.1 ml phosphate buffer 0.1 M and 0.2 extract (A) or water (B) was incubated for 10 min at 37°C.

Then, 3 mL of 4% starch solution (wheat starch) was added to the mixture and incubated for 60 min at 37°C<sup>24</sup>. Reducing sugar released from the starch hydrolysis was measured using DNS method.  $\alpha$ -amylase inhibitory activity was calculated by the following formula:

$$\alpha\text{-amylase inhibitory activity} = \frac{(B-A)}{B} \times 100\%$$

Where A and B represent sugar concentrations in the reaction mixture with and without an addition of leaf extract, respectively.

#### Antioxidant activity measurement

The antioxidant activity assay of the extracts was performed according to protocol describe by Xu *et al.*<sup>25</sup> using DPPH (2,2-Diphenyl-1-Picrylhydrazyl) (Merck, Germany) radical scavenging activity methods. Leaf extract (0.25 mL) was mixed with 2 mL DPPH solution (3.3 mg of DPPH in 100 mL methanol) and 8 mL ethanol (JT Baker), vortexed gently, then incubated at room temperature for 30 min in the dark, and the absorbance (A1) was measured at 517 nm (Thermo Scientific Genesys 20, USA). The absorbance (A0) of a control sample (distilled water instead of leaf extract) was also recorded at the same wavelength. Radical scavenging activity (%) was calculated by using the formula =  $[(A0 - A1)/A0] \times 100$ , where A0 was the absorbance of control sample and A1 was the absorbance of extracts.

#### Total phenolic analysis

Total phenolic content of the extract was measured using the Folin-Ciocalteu (Merck, Germany) reagent<sup>26</sup> with slight modification. 0.2 mL of leaf extract was mixed with 0.2 mL of aquades and 0.2 mL of Folin-Ciocalteu reagent (1 N). Then

4 mL of sodium carbonate (Merck, Germany) solution ( 2% ) was added and then allowed to stand for 30 min in the dark for incubation. The absorbance was measured at 760 nm in a spectrophotometer (Thermo Scientific Genesys 20, USA). A standar curve was prepared using Gallic acid (Tokyo Chemical Industry Co., Ltd) (0.00-0.01 mg/mL). The total phenolic contents were expressed in terms of gallic acid equivalents (GAE) (mg of per mL extract).

#### Statistical analysis

Results are expressed as the mean of 3 replicates. Statistical analysis was carried out with a statistical program Minitab version 18. One way-Anova with Fisher test was used. Results were considered significant if  $p < 0.05$ .

## RESULTS AND DISCUSSION

Alpha glucosidase enzyme is located in the brush border of the small intestine and is involved in starch digestion to release monosaccharide. Inhibition of the enzyme leads to retardation of starch digestion in small intestine. Study of  $\alpha$ -glucosidase inhibitor activity of extracts of bay, pandan, citrus leaves or their combination might contribute to the understanding of their potentiality for diabetic management. Commercially, inhibitor of this enzyme is available for glucose-lowering medications for diabetic patients<sup>27</sup>.

The  $\alpha$ -glucosidase inhibitory activity of the extracts of single or mixture of leaves is not depend on the type of extracts (Fig 1). Percent of inhibition of B, P and C or their combinations against  $\alpha$ -glucosidase was around 32%. Synergism or antagonism effect was not observed when the

**Table 1.** Proportion of leaves in dried leaf combination

Treatments	Proportion of each leaf in combinations (%)		
	Bay	Pandan	Citrus
BPC	33.3	33.3	33.3
BC	50.0	0.0	50.0
PC	0.0	50.0	50.0
BP	50.0	50.0	0.0
B	100.0	0.0	0.0
P	0.0	100.0	0.0
C	0.0	0.0	100

**Table 2.** Correlation coefficients between total phenolic with antihglucosidase, antiamylase and antioxidant activities of the extracts

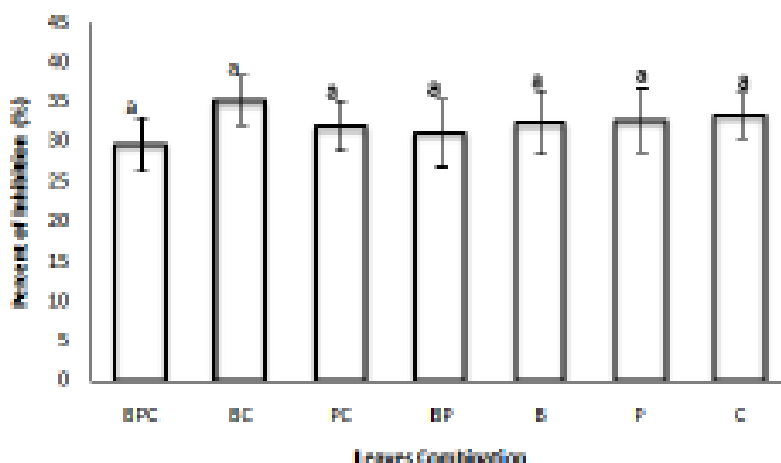
Activities	Cell Contents	Total Phenol
Antihglucosidase	Pearson correlation	0,104
	P-Value	0,654
Antiamylase	Pearson correlation	-0,112
	P-Value	0,630
Antioxidant	Pearson correlation	-0,527
	P-Value	0,014

\*Correlation is considered significant when  $p < 0.05$

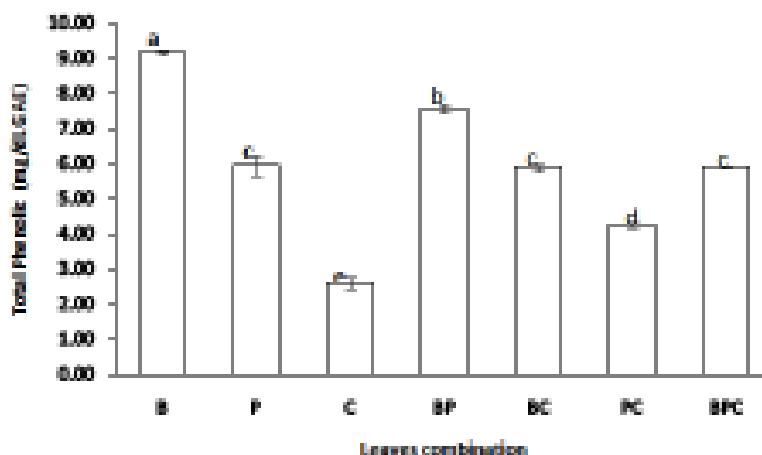
extracts were combined as the inhibition activity is not changed when these leaves were mixed.

It has been reported that the phenolic compounds of plant extracts have an ability to inhibit  $\alpha$ -glucosidase<sup>20,21</sup>. B, P and C extracts were suggested containing different type polyphenol compounds with different affectivity against  $\alpha$ -glucosidase. Although the total amount of the phenolic compounds in Indonesian bay leaves

is the highest among other tested leaves (Fig.2), its inhibitory activity against  $\alpha$ -glucosidase is similar as others (Fig. 1). Our results are in line with the previously reported results whereas not all phenolic compounds or fractions in plant extracts showed similar inhibition activity against  $\alpha$ -glucosidase enzymes<sup>20,21</sup>. The phenolic compounds of *Artemisia* species extracts showed inhibitory activity against  $\alpha$ -glucosidase enzymes



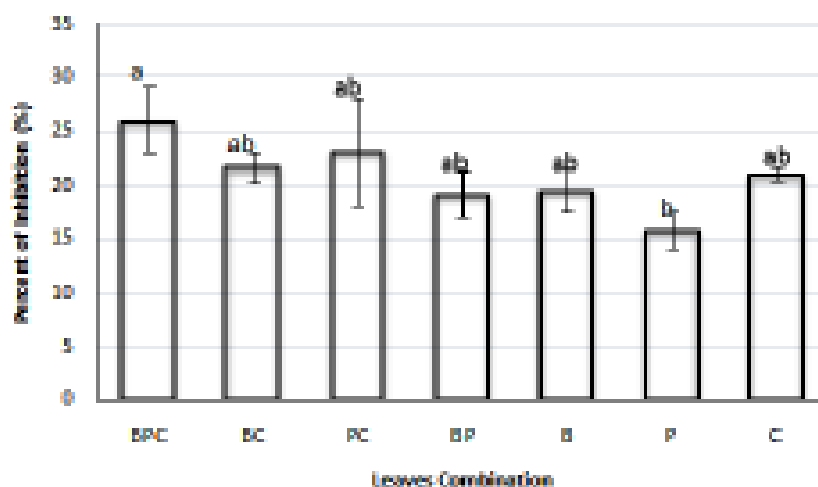
**Fig. 1.** Effect of leaf extracts on  $\alpha$ -glucosidase inhibitory activities. Each value represents a mean  $\pm$  SEM ( $n = 3$ ). BPC, BC, PC and BP were combination B, P and C, B and C, P and C, B and P respectively. B, P and C were Bay, Pandan, and Citrus leaves extracts, respectively. The bars represent the mean of three replicates. Data points denoted by different superscripts (letters on the bar) differ significantly with  $p < 0,05$ .



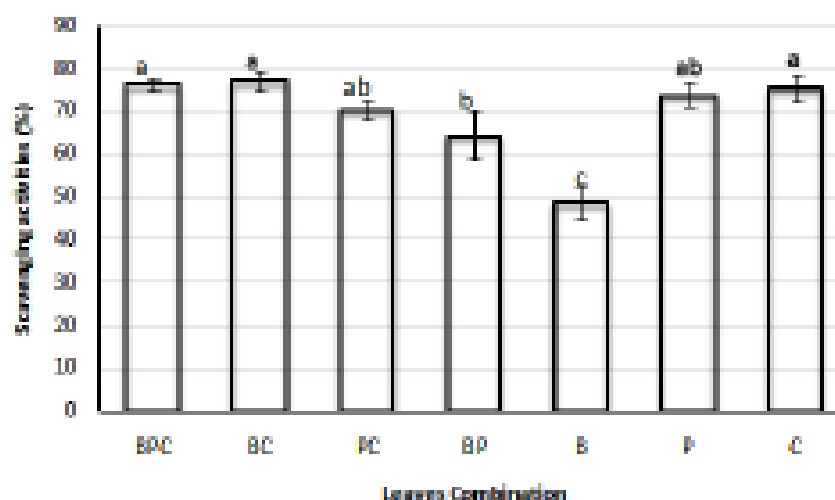
**Fig. 2.** Total phenolic content of leaves extracts, single or combination. BPC, BC, PC and BP were combination B, P and C, B and C, P and C, B and P respectively. B, P and C were Bay, Pandan, and Citrus leaves extracts, respectively. The bars represent the mean of three replicates. Data points denoted by different superscripts (letters on the bar) differ significantly with  $p < 0,05$ . Total phenol content of each combination was calculated based on content of single extracts.

( $IC_{50} = 214.43-734.12 \mu\text{g/mL}$ ) but not all individual phenolic compounds in the extract exhibited high inhibition. Extract of *Artemisia* containing high caffeoylquinic acids was the most pronounced<sup>11</sup>. The ethyl acetate extract of *Citrospodium axifolium* (Kunth) Govaerts (Lamiaceae) showed stronger inhibitory activity against  $\alpha$ -glucosidase than the methanolic and the hexanic extracts where urolic acid contained in the three extracts was the

individual phenolic compound that showed a strong inhibitory activity<sup>12</sup>. However, similar inhibition of 2 different fractions ( $P < 0.05$ ) against  $\alpha$ -glucosidase also has been observed for fraction of methanol (rich in phenolic and flavonoid compounds) and ethyl acetate (rich in proanthocyanidins) from *Ehretia cymosa* Thonn<sup>13</sup>. Therefore, it is suggested that phenolic compounds contained by B extract are less effective in inhibiting of  $\alpha$ -glucosidase than



**Fig. 3.** Effect of plant extracts on  $\alpha$ -amylase inhibitory activities. Each value represents a mean  $\pm$  SEM ( $n = 3$ ); BPC, BC, PC and BP were combination B, P and C, B and C, P and C, B and P respectively. B, P and C were Bay, Pandan, and Citrus leaves extracts, respectively. The bars represent the mean of three replicates. Data points denoted by different superscripts (letters on the bar) differ significantly with  $p < 0.05$ .



**Fig. 4.** Effect of plant extracts on antioxidant activities. Each value represents a mean  $\pm$  SEM ( $n = 3$ ); BPC, BC, PC and BP were combination B, P and C, B and C, P and C, B and P respectively. B, P and C were Bay, Pandan, and Citrus leaves extracts, respectively. The bars represent the mean of three replicates. Data points denoted by different superscripts (letters on the bar) differ significantly with  $p < 0.05$ .

the phenolic compounds in P or C extract, but their total phenolic concentration have no correlation with anti- $\alpha$ -glucosidase activity ( $p = 0.654$ ) (Table 2).

Alpha amylase is a carbohydrate hydrolyzing enzyme secreted by salivary glands and pancreas that can hydrolyze starch into oligosaccharide and simple sugars. Inhibition of this enzymes inhibits starch digestion and reduce the rate of glucose absorption in small intestine. Our result shows that extracts of B and C exhibit  $\alpha$ -amylase inhibition higher than P but the difference is statistically not significant (Fig. 3). As the total phenolic concentration of the three extracts was difference (Fig 2), it is suggested that the effect of the extract on  $\alpha$ -amylase activity merely depend on phenolic composition than on the total phenolic concentration. Phenolic compounds extracted from different plant species or cultivar has different composition and anti  $\alpha$ -amylase activity<sup>28</sup>. Less than 15% of 126 extracts gained from 17 plants posed inhibition activity against  $\alpha$ -amylase with varying degree, whereas 3 of them, those contain different compounds, had inhibition level more than 50%<sup>28</sup>. Chemical analysis of 2 Oat varieties (Amalal and F11-5) revealed the phenolic composition of their extracts was different, where  $IC_{50}$  for amylase inhibition of F11-5 was higher than Amalal, 1027.14  $\mu$ g/mL and 723.91  $\mu$ g/mL respectively<sup>29</sup>.

Inhibition pattern of the extracts (single or mixture) against  $\alpha$ -amylase enzyme (Fig 3) was not similar as the pattern of the extracts against  $\alpha$ -glucosidase (Fig 1). There is no significantly differ among the extracts in modulation of  $\alpha$ -glucosidase activity, but each extract exhibited different effect on  $\alpha$ -amylase activity. Mixing P with B and C increases the inhibitory activity of the extract against  $\alpha$ -amylase as seen that percent of inhibition of BPC is significantly higher than P, even though their total phenolic content was not different. P and BPC extracts reduce the  $\alpha$ -amylase activity by 16% and 26%, respectively. The increasing of inhibition activity of P due to mixing with B and C (BPC) was not due to increasing of total phenolic concentration (Fig 2) as no correlation between its phenolic concentration and inhibition activity was observed ( $p=0.630$ ; Table 2). It is suggested that synergism effect was occurred when P was mixed with B and C as shown that mixture of plant extracts show superior

effect when compared to single extract at the equivalent concentration<sup>30</sup>. Previously Lau *et al.*<sup>31</sup> identified a synergism effect of a mixture of extracts of *Astragalus membranaceus* and *Rehmanniae glutinosa* roots in wound-healing of a diabetic foot ulcer animal model.

It has been shown that potency of 50 $\mu$ D-glucosidase and 50 $\mu$ D-amylase inhibition of plant extracts are related to the presence of phenolic compounds<sup>28,32,33</sup> those have antioxidant activity<sup>34</sup>. Therefore, in the current study we investigated the antioxidant power of the extracts with DPPH radical scavenging assay. Figure 4 shows a variation in DPPH radical scavenging activities of the extracts ranging from 49% to around 77%. The C extract exhibited a stronger DPPH scavenging ability than the P extract, though the difference is not statistically significant (76% and 74%, respectively). On the contrary the B extract had the weakest scavenging activity (49%). A Mixture of the C and B extracts was proven to be more effective in the enhancement of the antioxidant activity of the B extract than any other combinations with the scavenging activity of 77% (Fig. 4).

The antioxidant activity patterns of the extract combinations does not depend on the antioxidant activity of the single extract or their total phenolic content. For example, the total phenolic compound of the C extract was lower than of the P extract. However, both extracts exhibited a similar antioxidant activity. Additionally, the BC extract mixture showed higher antioxidant activity than the BP extract mixture (21.68 and 19.18%, respectively), though the total phenolic compound of the BP mixture is higher than the BC mixture (7.57 and 5.90 mg/dL respectively). Therefore, accumulation rather than synergism effect on antioxidant activity was detected when the extracts were mixed. Negative correlation (-0.527) between total phenol concentration of the extracts with antioxidant activity was observed ( $p = 0.014$ ), in which plant extracts with higher concentration of total phenol have lower antioxidant activity (Fig. 2 and Fig. 4). Similar findings have been reported showing that total phenolic compounds in the extracts of ginger, curcuma, cinnamon<sup>35</sup> and Korean propolis<sup>36</sup> have negative correlation with their antioxidant activity. Antioxidant activity of the plant extracts may not always positively



correspond to the total phenol concentration, but may be determined by the composition of phenolic compounds<sup>49</sup>.

Furthermore, the molecular interactions between major phenolic compounds in plant extracts determine the antioxidant capacity of the extracts<sup>49</sup>. A mixture of plant extracts with high total phenol compounds will have low antioxidant capacity when major phenolic compounds in the extracts have antagonistic interactions. A mixture of chlorogenic acid and caffeic acid is an example of this description whereas a combination of gallic acid and caffeic acid that showed synergistic interaction, has high antioxidant capacity<sup>49</sup>. Therefore, antioxidant activity of mixture of phenolic-rich plant extracts may not always the sum of antioxidant activity of individual extract or individual phenolic compound present in the extracts<sup>49</sup>.

## CONCLUSION

The leaf extracts and their combination showed inhibition activities against  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes and scavenging activity against free radicals. The synergism or antagonism effect was not observed when the extracts were combined as the enzyme inhibition and scavenging activities are not depend on the proportion of the extract in the mixtures. Additionally, the role of polyphenol compounds on inhibition of the starch digestion enzymes or scavenging of free radicals was not observed. Further study is required to fully elucidate the effect of the leaf or their combinations on diabetic animal models or diabetic patients.

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