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Inhibition of Carbohydrate Hydrolysing Enzymes, Antioxidant Activity and Polyphenolic Content of *Beilschmiedia* Species Extracts

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Abstract. The goal of the present study was to provide *in vitro* evidence for potential inhibition of carbohydrate hydrolysing enzymes and antioxidant activities of methanol and ethyl acetate extracts from barks of two different *Beilschmiedia* species. These extracts were tested on α-amylase and α-glucosidase inhibitory activities, mode of enzyme inhibition, total polyphenolic content (TPC) and antioxidant capabilities. Methanolic bark extract of *Beilschmiedia insignis* demonstrated optimum inhibitory effects against α-amylase and α-glucosidase with IC_{50} values of 3.233 µg/mL and 12.357 µg/mL, respectively. Further analysis of inhibition mode revealed that the extract demonstrated a mixed inhibition against both enzymes. In comparison to other extracts, methanolic bark extract of *Beilschmiedia insignis* demonstrated the highest TPC content of 420.393 mg GAE/g extract, lowest IC_{50} value of 12.103 µg/mL for DPPH radical scavenging ability and highest FRAP value of 1904.247 µM Fe (II)/mg extracts, indicating the antioxidant potential of the extract. A significant strong correlation coefficient was observed between TPC with FRAP ($r = 0.994$, $p < 0.01$) and TPC with DPPH ($r = -0.860$, $p < 0.01$), signifying that antioxidant activity and reducing capability were contributed by the polyphenolic compounds present in the crude extract. Collectively, methanolic bark extract of *Beilschmiedia insignis* possessed significant carbohydrate hydrolyzing enzyme inhibitory effects and antioxidant activity, suggesting its possible alternative application for diabetes and postprandial hyperglycemia treatment.

1. Introduction

In 2012, it was estimated that 1.5 million deaths worldwide were caused by diabetes mellitus (DM), causing it to be ranked number eight among the leading cause of death among both sexes and the fifth

leading cause of death in women [1]. DM is characterized by high glucose level in the blood (hyperglycemia) associated by disturbances in carbohydrates, proteins and fats metabolism. Such defects in these metabolic pathways may lead to complete or partial insufficiency of insulin actionand/ or insulin resistance with macro-microvascular complications [2]. Previous studies reported that prolonged hyperglycemia is the key to these DM complications as it causes an increase in oxidative stress due to excess generation of free radicals through protein glycation, glucose oxidation and lipid peroxidation [3]. Therefore, the therapeutic approach for DM should be based on targeting glucose metabolism and oxidative stress caused by hyperglycemia-related conditions [3].

There are numerous conventional drugs available for DM, which varies in mechanism of treatment [4]. One of the pharmacological approaches is by using carbohydrate enzyme inhibitor drugs such as acarbose, voglibose and miglitol [5]. These drugs inhibit both α-amylase and α-glucosidase, which are enzymes responsible for the breakdown of carbohydrates. Such inhibition can be achieved by delaying the carbohydrate digestion, which decrease the rate of glucose absorption, thus maintaining blood glucose level as close to normal as possible. However, these current antidiabetic drugs suffer from a number of undesirable side effects, leading researchers to seek traditional medicinal plants as alternatives for diabetic treatment [4].

Beilschmiedia is a genus of approximately 250 species that are trees or shrubs in the family *Lauraceae* [6]. One of the previous phytochemical investigations of plants of the genus *Beilschmiedia* reported the presence of polyphenols which are the secondary metabolites of plants [7,8]. Polyphenols are organic chemicals characterized by the presence of large multiple phenol structural units. They are among the naturally occurring antidiabetic agents because polyphenols exhibit various important biochemical properties including carbohydrate hydrolysing enzyme inhibition and their capabilities to act as antioxidants [9]. In the present study, the methanol and ethyl acetate extracts from barks of *Beilschmiedia lumutensis* and *Beilschmiedia insignis* were evaluated on carbohydrate hydrolysing enzyme inhibition potential, polyphenolic content and antioxidant activities.

2. Materials and method

2.1. Plant material and preparation crude extracts

The bark of *Beilschmiedia lumutensis* and *Beilschmiedia insignis* were collected at Machang, Kelantan on 2006 and at Kuala Lipis, Pahang on 2007, respectively. This plant was identified by T. Leong Eng, a botanist in University of Malaya. The voucher specimens (KL-5269 and KL-5368, respectively) have been deposited at the Herbarium of the Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. The air-dried barks of *Beilschmiedia lumutensis* and *Beilschmiedia insignis* (each 200 g) were sliced, ground and extracted with EtOAc (3 × 0.5 L) followed by MeOH (3 × 0.5 L) at room temperature using maceration method. The solvents were filtered and removed using rotary evaporator to gave the respective extracts. Collectively, four crude extracts were produced for biological evaluation in this study, BLM: *Beilschmiedia lumutensis*-methanol; BLEA: *Beilschmiedia lumutensis*-ethyl acetate; BIM: *Beilschmiedia insignis*-methanol; BIEA: *Beilschmiedia insignis*-ethyl acetate. The extracts were carried out for carbohydrate hydrolysing enzyme inhibition potential, polyphenolic content and antioxidant activities.

2.2. α-amylase inhibition assay

The α-amylase inhibition assay was determined according to Kazeem *et al.*, [10] with minor modifications. Briefly, an amount of 250 µL crude extracts of different concentrations (0.004-0.333 mg/mL) was mixed with 250 µL of 0.5 mg/mL α-amylase solution prepared using 0.02 M sodium phosphate buffer (pH 6.9). The mixture was pre-incubated for 10 minutes at 25°C in a water bath. Then, 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added and further incubated for 10 minutes at 25°C. The reaction was terminated by adding 500 µL of DNS reagent,

followed by incubation in boiling water for 5 minutes. The reaction mixture was diluted with 5 mL of distilled water and the absorbance was measured at 540 nm using the Hitachi U-1900 spectrophotometer. Acarbose was used as a positive control. The percentage of enzyme inhibition was calculated by using the following equation:

$$\text{Inhibition (\%)} = \frac{Abs(control) - Abs(sample)}{Abs(control)} \times 100\% \quad (1)$$

IC_{50} which is defined as the concentration of extracts required to achieve 50% of enzyme inhibition was calculated using GraphPad Prism.

2.3. α-glucosidase inhibition assay

The *α*-glucosidase inhibition assay was performed accordingly to a previous method with slight modifications [11]. Yeast α-glucosidase (1U/mL) and pNPG (3 mM) were prepared in 0.02 M phosphate buffer. An amount of 100 µL of *α*-glucosidase enzyme (1U/mL) was added with 10 µL of plant extracts with different concentrations ranging from 0.004 mg/mL to 0.333 mg/mL and incubated for 10 minutes at 37 °C in a water bath. Then, 50 µL of pNPG was added to start the reaction and further incubated at 37 °C for 20 minutes. The reaction was terminated by adding 2 mL 0.1 M sodium carbonate. The absorbance reading of the yellow *p*-nitrophenol was measured at 405 nm using the Hitachi U-1900 spectrophotometer. Acarbose was used as the positive control. Phosphate buffer was used to replace the enzyme for the blank and for negative control, plant extracts were replaced by phosphate buffer. The percentage of enzyme inhibition for *α*-glucosidase was calculated using equation (1) and IC_{50} was calculated using GraphPad Prism.

2.4. Kinetics for mode of α-amylase inhibition

The extract with the lowest IC_{50} value from α-amylase inhibition assay was evaluated for mode of inhibition [10]. Firstly, 250 µL of the extract was mixed with 250 µL of *α*-amylase enzyme. For control group, instead of using the extracts, 250 µL of pH 6.9 phosphate buffer was used. Both mixtures were pre-incubated for 10 minutes at 25 °C in a water bath. Then, 250 µL of starch at increasing concentrations ranging from 0.30 to 5.0 mg/mL was added to both set of the reaction mixtures to initiate the reaction. The mixture was further incubated for 10 minutes at 25 °C and 500 µL of DNS reagent was added to terminate the reaction. The amount of reducing sugars released was determined spectrophotometrically at 540 nm using a maltose standard curve generated using the maltose assay by DNS method. The mode of inhibition on α-amylase was determined by analyzing the Lineweaver-Burk plot using Michaelis-Menten kinetics.

2.5 Kinetics for mode of α-glucosidase inhibition

The extract with the lowest IC_{50} value from *α*-glucosidase inhibition assay was evaluated for mode of inhibition [10]. In brief, 10 µL of the extract was prepared and mixed with 100 µL of *α*-glucosidase enzyme. The mixture was pre-incubated for 10 minutes at 37 °C in a water bath while pH 6.9 phosphate buffer was used to replace the extracts for control group. Then, 50 µL of pNPG dissolved in 20 mM phosphate buffer (pH 6.9) was added at increasing concentrations ranging from 0.63 to 2.0 mg/mL to both set of the reaction mixtures to initiate the reaction. The mixture was further incubated for 10 minutes at 37 °C and 500 µL of sodium carbonate was addedto stop the reaction. The amount of reducing sugars released was determined spectrophotometrically at 405 nm by using a *p*-nitrophenol standard curve. The mode of inhibition of on α-glucosidase was determined by analyzing the Lineweaver-Burk plot using Michaelis-Menten kinetics.

2.6. Determination of total polyphenolic content (TPC)

The amount of total polyphenolic content (TPC) was determined by using the Folin–Ciocalteu reagent with slight modifications [12]. Each of the methanol and ethyl acetate *Beilschmiedia* extracts was dissolved in DMSO to a final concentration of 0.2 mg/mL. Then, 0.5 mL of the plant extract was dissolved in 2.5 mL of the Folin- Ciocalteu reagent (1/10 dilution) and the mixture was topped up to 10 mL with distilled water. The mixture was left for 5 minutes at room temperature, followed by the addition of 2 mL of 7.5% Na_2CO_3 solution. The final mixture was vortexed and incubated for 2 hours in a dark room at room temperature. Absorbance of the samples were measured at 765 nm using the Hitachi U-1900 spectrophotometer. A gallic acid standard curve was prepared ranging from gallic acid concentrations of 0.2-1.0 mg/mL and TPC was expressed in terms of mg of gallic acid equivalents (GAE) per gram of extract (mg GAE/g extract). The equation below was used for the calculation:

$$\text{TPC (mg GAE/g extract)} = C \times \frac{V}{M} \quad (2)$$

where C represents the concentration determined from the gallic acid standard curve (mg/mL), V represents the volume of extracts used in the assay (mL) and M represents the mass of extracts used in the assay (g).

2.7. DPPH radical scavenging assay

DPPH is a dark- purple coloured crystalline powder composed of stable free-radical molecules. This assay is used to determine the antioxidant ability of the extract being tested by observing the change in colour from purple to yellow by the reduction process caused by antioxidants present in the extract. The DPPH radical scavenging assay was performed accordingly with minor modifications [5]. An amount of 1.5 mL of 0.1 mM DPPH prepared in methanol was added to 0.5 mL of the sample extracts of different concentrations (0.004 to 0.333 mg/mL) in a test tube. The mixture was vortexed for 30 seconds and left to stand in the dark at room temperature for 30 minutes. Then, the absorbance was measured at 517 nm using the Hitachi U-1900 spectrophotometer. The positive control used was ascorbic acid. The negative control was prepared by replacing the extracts with methanol and the blank was prepared by replacing DPPH with methanol. The IC_{50} values were determined using GraphPad Prism and total DPPH radical scavenging ability (%) was calculated using the equation:

$$\text{DPPH radical scavenging ability (\%)} = \frac{Abs(control) - Abs(sample)}{Abs(control)} \times 100\% \quad (3)$$

2.8. Ferric ion reducing antioxidant power (FRAP) assay

The FRAP assay was performed with slight modifications [13]. In brief, an amount of 3 mL of FRAP working solution was added into 100 µL of plant extracts (0.333 mg/mL) and vortexed. The mixture was placed in a water bath at 37°C for 30 minutes in a dark condition and the absorbance was measured at 593 nm using the Hitachi U-1900 spectrophotometer. Ascorbic acid was used as the positive control. A ferrous sulphate standard curve ranging from 5-100 µM was constructed. The absorbance reading obtained was substituted into the standard curve to obtain the concentration of ferrous sulphate and the results were expressed in FRAP values (µM of Fe (II)/mg of extracts).

2.9. Statistical analysis

All experiments were performed in triplicates and data was presented in terms of mean ± standard error of mean (SEM). The IC_{50} values were calculated using GraphPad Prism. The differences between the data obtained and positive control were assessed using t-test and a *p* value of less than 0.05 is considered

as significantly different. Pearson correlation was used to correlate TPC and biological activities of extracts accordingly.

3 Results and discussion

The barks of *Beilschmiedia lumutensis* and *Beilschmiedia insignis* were extracted using two different solvents which were methanol and ethyl acetate. The resulting IC_{50} values of each extracts were calculated and presented in Table 1, where a lower IC_{50} value indicates higher enzyme inhibition activity. The bark methanolic extract of *Beilschmiedia insignis* was observed to exhibit as promising *α*-amylase and *α*-glucosidase inhibitor. For α-amylase inhibition, BIM extract had the lowest IC_{50} value of 3.233 ± 0.512 µg/mL, which explains the better potency of BIM extract in inhibiting α-amylase, thus delaying the breakdown and absorption of carbohydrates and lowering the glucose peaks encountered in postprandial glucose [14]. As for *α*-glucosidase inhibition, BIM extract demonstrated lowest IC_{50} value of 12.357 ± 1.785 µg/mL compared to other extracts.

Plant species are known to contain a broad supply of bioactive compounds or secondary metabolites that are devoted to the beneficial medicinal effects [15]. These bioactive compounds differ in solubility when extracted using different extraction solvents such as methanol and ethyl acetate. Therefore, different active compounds extracted differently by exhibiting different yields when different extraction solvents were used [16]. In the current study, the bioactive compound in the crude extract responsible for *α*-amylase and *α*-glucosidase inhibition was more likely to be extracted by methanol. This indicates that the inhibitory activity was influenced by the choice of solvent used during extraction and the solubility of the bioactive compounds.

Table 1. IC_{50} values for *α*-amylase and *α*-glucosidase inhibitory activity of two *Beilschmiedia* species (*Beilschmiedia lumutensis* and *Beilschmiedia insignis*) extracted using two different solvents (methanol and ethyl acetate).

Extract	IC_{50} values for α-amylase (µg/mL)	IC_{50} values for α-glucosidase (µg/mL)
BLM	7.398 ± 1.091	58.377 ± 6.675*
BLEA	7.905 ± 1.002	21.913 ± 3.933*
BIM	3.233 ± 0.512	12.357 ± 1.785 *
BIEA	19.203 ± 1.892	46.700 ± 4.963*
Acarbose	13.784 ± 3.703	4148.333 ± 650.177

Each value represent the mean IC_{50} value (N=3, mean ± SEM).
* Values are significantly different ($p < 0.05$) as compared to acarbose.

BIM extract with the lowest IC_{50} value was utilized to further evaluate its mode of inhibition against α-amylase and α-glucosidase activities. The results in the form of Lineweaver-Burk plot were shown in Fig. 1 and 2. It was shown that both the V_{max} and K_m were influenced by the presence of BIM extract which indicated a mixed inhibition. The active compounds that contributed to *α*-amylase and *α*-glucosidase inhibition present in BIM extract is believed to bind preferably to the free enzyme instead of the enzyme-substrate complex.

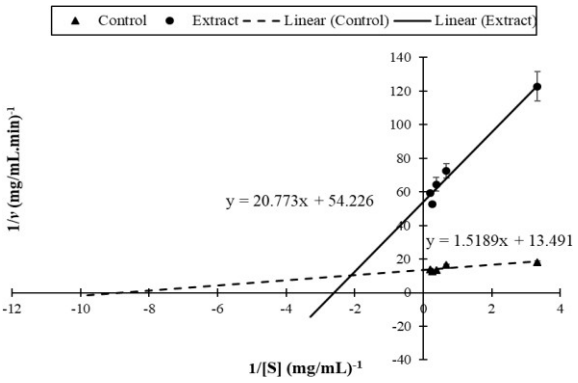

Figure 1. Mode of α-amylase inhibition by BIM extract using Lineweaver-Burk plot.

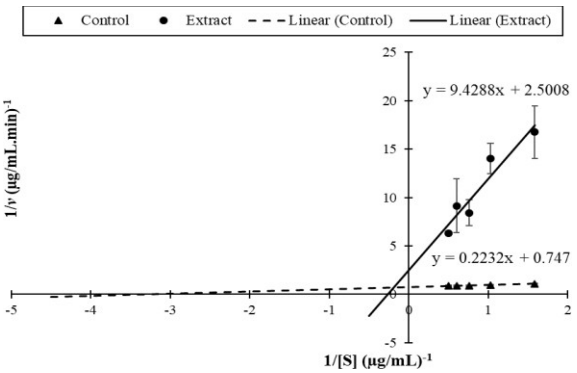

Figure 2. Mode of α-glucosidase inhibition by BIM extract using Lineweaver-Burk plot.

In mixed inhibition, inhibitors tend to attach to the allosteric site of the enzyme instead of the active site. Therefore, it can be concluded that the active compounds in BIM extract interact with the enzyme by binding to the allosteric site of the free enzyme, causing conformational changes to the active site of the enzyme and lowers the enzyme affinity for the substrate, which explains the increase in K_m value. The decrease in V_{max} value observed indicated such inihibition that can be resulted in a retarded breakdown of disaccharides into absorbable monosaccharides, which lowers hyperglycemia peaks in type 2 diabetes [17].

In the present study, the TPC of three *Beilschmiedia* species extracted using two different solvents were determined and shown in Table 2. All four extracts contained polyphenolic compounds. The TPC of all four extracts ranged from 60.439 to 420.393 mg GAE/g extract. The results gathered followed the order: BIM > BLM > BIEA > BLEA. All methanolic extracts had a higher TPC value than their respective ethyl acetate extracts. This result indicated that methanol is a preferred extraction solvent when compared to ethyl acetate in the extraction of polyphenolic compounds due to its higher polarity index. This confirmed that choice of solvent, the polarity index and the solubility of TPC in the solvent affects the recovery of polyphenolic contents [18]. The antioxidant activity of polyphenolic compounds result from their redox properties, enabling them to neutralize free radicals, act as reducing agents, hydrogen donors, quenching singlet and triplet oxygen and metal chelators [12]. Polyphenolic compounds were also reported to contribute towards enzyme inhibition due to their hydroxyl group that is able to bind with proteins, which explains the ability of most *Beilschmiedia* extracts in the current research in inhibiting *α*-amylase and *α*-glucosidase [19].

Table 2. Total polyphenolic content of three *Beilschmiedia* species extracted using two extraction solvents, methanol and ethyl acetate.

Extract	Total phenolic content (mg GAE/g extract)
BLM (bark)	344.202 ± 9.275
BLEA (bark)	60.439 ± 4.684
BIM (bark)	420.393 ± 8.010
BIEA (bark)	82.836 ± 7.928

The resulting IC_{50} values for DPPH radical scavenging ability of each extracts were presented in Table 3 in comparison to ascorbic acid (AA). All extracts were weaker than ascorbic acid in scavenging the DPPH radical and had IC_{50} values that were significantly higher ($p < 0.05$) than ascorbic acid. BIM extract with the lowest IC_{50} value of 12.103 ± 0.312 µg/mL demonstrated the strongest DPPH radical scavenging ability whereas BLEA extract with the highest IC_{50} value of 245.867 ± 35.220 µg/mL demonstrated the weakest DPPH radical scavenging ability. The results of the current study proved that TPC of the extracts were obtained in a more polar solvent with increased level of antioxidants. This finding is in line with previous findings where the antioxidant property of the plant extract was correlated to the levels of polyphenolic content and that antioxidant activity of plants were dependent and influenced by the choice of extracting solvents [20]. This finding is of significant as plant extracts with DPPH scavenging ability are capable to donate their hydrogen atom or electrons, acting as primary antioxidants, thus neutralizing the free radicals in hyperglycemia-induced oxidative stress that leads to complications in type 2 diabetes [21]

Table 3. IC_{50} values for DPPH radical scavenging ability of three *Beilschmiedia* species.

Extracts	IC_{50} values for DPPH (µg/mL)
BLM	13.170 ± 0.935*
BLEA	245.867 ± 35.220*
BIM	12.103 ± 0.312*
BIEA	137.950 ± 15.650*
AA	4.930 ± 0.316

* Values are significantly different ($p < 0.05$) as compared to AA.

The reducing potential of all *Beilschmiedia* extracts were evaluated for their ability to reduce the straw colour TPTZ-Fe (III) complex into blue colour, TPTZ-Fe (II). The results revealed that all of the *Beilschmiedia* extracts possessed reducing power, indicated by their respective FRAP values ranging from 499.212 ± 18.884 to 1904.247 ± 24.588 µM Fe (II)/mg extracts (Fig. 3). The results obtained were in the order: AA > BIM > BLM > BIEA > BLEA, which was almost similar to current findings in TPC and DPPH assay. Based on Figure 3, it was observed that both methanolic extracts exhibited higher FRAP values than their respective ethyl acetate extracts, which reconfirmed the trend in our previous findings in TPC and DPPH assay. Highest FRAP value was exhibited by BIM extract. This result was in parallel with a previous study [22], which discovered that the extraction of active compounds was solvent dependent and polar solvent (methanol) is an efficient solvent for extraction of antioxidants and polyphenolic compounds. Therefore, it can be deduced that the high reducing ability of BIM extract was mostly contributed by the polyphenolic compounds present [23].

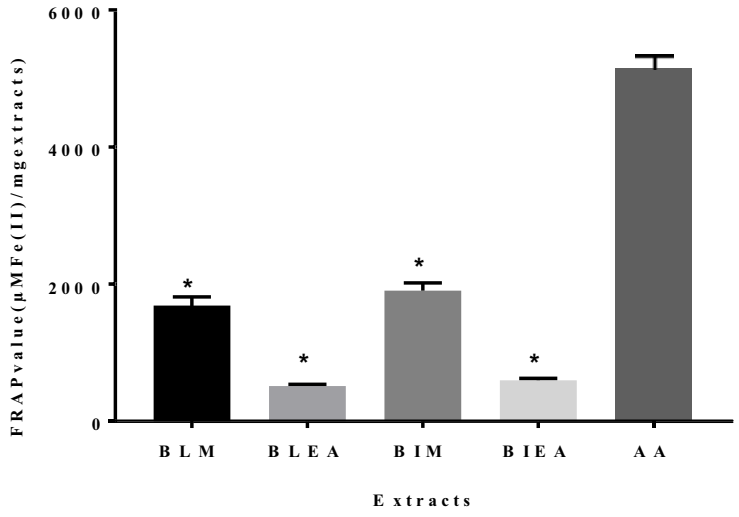

Figure 3. FRAP value of two *Beilschmiedia* species extracted using two extraction solvents, methanol and ethyl acetate. * Values are significantly different ($p < 0.05$) as compared to AA.

Pearson correlation analysis was conducted to analyze the correlations between TPC and some biological activities. Based on Table 4, the highest correlation coefficient, r was found between TPC and FRAP activity and the lowest correlation coefficient was found between TPC and *α*-glucosidase inhibitory activity. TPC had significant strong correlations with both DPPH and FRAP antioxidant assays with respective correlation coefficient of -0.860 and 0.994. Similar results were reported from a previos report [24], where a strong correlation between TPC and both DPPH and FRAP antioxidant assays were observed with correlation coefficients of 0.921 and 0.868 respectively. This indicated that polyphenolic content found in *Beilschmiedia* extracts used in the current research strongly contributed to the radical scavenging capability and reducing strength seen in both antioxidant assays, which is in line with a previous analysis [25]. TPC and enzyme inhibitory activities were also correlated. it was observed that a higher correlation coefficient (-0.589, $p > 0.01$) was seen between TPC and *α*-amylase inhibitory activity compared to TPC and *α*-glucosidase inhibitory activity (-0.191, $p > 0.01$). There is a higher possibility where polyphenolic compounds present in the extracts contributed to α-amylase inhibition than to *α*-glucosidase inhibition. Therefore, *α*-glucosidase inhibition activity demonstrated by *Beilschmiedia* extracts in the current study may be contributed by other phytochemicals or secondary metabolites but not in the form of polyphenolic compounds.

Table 4. Pearson correlation coefficient, r between total polyphenolic content (TPC) and the biological activities of *Beilschmiedia* species.

Biological Activities	Pearson correlation coefficient, r
	TPC
DPPH scavenging ability	-0.860**
FRAP ability	0.994**
α-amylase inhibitory activity	-0.589
α-glucosidase inhibitory activity	-0.191

** significant correlation at $p < 0.01$.

Conclusion

It can be concluded that BIM extract serves as a promising candidate for further evaluation as diabetic drug due to strong inhibition against α-amylase and α-glucosidase by mixed inhibition. Along with promising enzyme inhibition potential, BIM extract also possessed antioxidant activity. BIM extract demonstrated the highest TPC content, the lowest IC_{50} in DPPH radical scavenging assay and highest FRAP value out of all tested extracts. This revealed the strong correlation between TPC and both DPPH and FRAP antioxidant assays. Taken together, all these findings supported the potential of methanolic bark extract of *Beilschmiedia insignis* to be part of the potential medicinal plants that may serve as one of the alternative treatments for hyperglycemia-related conditions.

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