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Research Article
Phytochemical Analysis and *in vitro* Evaluation of Antidiabetic Activity of *Diospyros buxifolia*

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Abstract

Background: Diabetes mellitus is a chronic disease and is one of the main public health concerns worldwide. To date, the potential of many natural products in its treatment are not fully explored. **Materials and Methods:** In this study, major phytochemicals present in the leaves and stems of *Diospyros buxifolia* were determined following extraction using distilled water and 70% of methanol. The antidiabetic activities of the extracts were determined *in vitro* by measuring α-amylase and amyloglucosidase inhibitory activities at four different extract concentrations (0.125, 0.250, 0.500 and 1.000 mg mL^{-1}). **Results:** Methanolic extracts of *D. buxifolia* stems found to possess significant α-amylase inhibitory activity (84.68±0.03%) followed by methanolic extract of the leaves (84.00±0.08%), aqueous extracts of the stems (49.44±0.05%) and aqueous extract of the leaves (40.71±0.03%). Methanolic extracts of the stems showed significant inhibition of amyloglucosidase (62.72±0.15%) followed by methanolic extract of the leaves (58.93±0.08%), aqueous extracts of the stems (36.65±0.05%) and finally aqueous extract of the leaves (20.57±0.03%). **Conclusion:** It can be concluded that methanolic stem extract of *D. buxifolia* displayed the presence of phytochemicals and also significant α-amylase and amyloglucosidase inhibition. Further analysis needs to be made to find out the mechanism of action of antidiabetic activities of the extracts used in this study.

Key words: Diabetes, medicinal plants, *Diaspyros buxifolia*, α-amylase

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

INTRODUCTION

Diabetes mellitus is one of the leading public health concerns worldwide. In 2014, 387 million people have been diagnosed with diabetes with 4.9 million fatalities which means that for every 7 sec, a person dies from diabetes or its related complications[1]. To date, traditional treatments based on medicinal plants rather than synthetic drug are getting more popular due to the high cost and side effects of the latter[2,3]. In fact, generally the drugs derived from medicinal plants tend to be safer and are more cost effective[4,5]. Studies have shown that many plants are used in the treatment of diabetes with different plant extracts having the ability to inhibit carbohydrate digesting enzymes activities therefore yielding the antidiabetic effects[5].

Diospyros buxifolia which belongs to the Ebenaceae family and native to Asia. This plant is a dioecious tree which produces berry type fruits and can grow up to 30 m height[6]. According to Maridass *et al.*[7], phytochemical screening on fruits extracts of *D. buxifolia* indicated the presence of essential oils, saponins, terpenoids and alkaloids with Ebenaceae listed as one of the plant family which have antidiabetic properties. A recent study reported that administration of ethanolic leaves and bark extract of *D. virginiana* at 500 mg kg^{-1} for 15 days reduce not only blood glucose level but also the body weight, glucose and ketone levels of urine and pancreatic tissues of streptozotocin-induced diabetic rats.

Phytochemicals play an important role in ameliorating various types of diseases and their complications. Medicinal plants are abundant with different types of phytochemicals including polyphenols and flavonoids. In this scenario, the present study aimed to investigate the phytochemical content and *in vitro* antidiabetic potential of *D. buxifolia*.

MATERIALS AND METHODS

The α-amylase (from porcine pancreas) and amyloglucosidase (from *Aspergillus oryzae*) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals and reagents were of analytical grades.

Collection of plant material: Fresh leaves and stems of *D. buxifolia* were collected in April, 2015 from Agro Park University Malaysia Kelantan Jeli Permanent Forest Reserve and were authenticated by Dr. Shamsul Khamis, Universiti Putra Malaysia to be of the correct species.

Preparation of the plant extract: The plant extract was prepared using a modified method as previously described by Norhayati *et al.*[8]. The *D. buxifolia* samples were cleaned under a running tap water before being dried at room temperature. The leaves and stems were separately grounded into a fine powder by using a grinder. A total of 50 g of sample (either the leaves or the stems) was immersed overnight (12 h) in 500 mL methanol (70%) contained in an Erlenmeyer flask. Similarly, another 50 g of the leaves and stems samples were similarly treated albeit using 500 mL of distilled water. The Erlenmeyer flasks containing different types of samples were mixed in an orbital shaker at 30°C, 150 rpm for 48 h. The extract was filtered through a Whatman No.1 filter paper followed by evaporation at 45°C using a rotary evaporator at a minimum pressure. The two different solvent extracts for the two sample types were kept in Duran bottles at -20°C until further analysis.

Phytochemical screening of the extract: Phytochemical analysis of the four different types of samples were determined based on the different modified methods as reported by Yusuf *et al.*[9] and Kazeem *et al.*[10]

Borntrager`s test for anthraquinones: Chloroform (5 mL) was added to 0.5 g of the samples. The resulting mixture was then shaken for 5 min before filtration using a Whatman No. 1 filter paper. The filtrate was mixed with equal volume of 10% ammonia solution. The presence of pink, red or violet colour confirmed the presence of anthraquinones.

Test for flavonoids: The plant extract (0.5 g) was heated with 10 mL of ethyl acetate over a steam bath for 3 min. The mixture was filtered using a Whatman No. 1 filter paper. The filtrate (4 mL) was shaken with 1 mL of diluted ammonia solution. The formation of yellow colour indicates the presence of flavonoids.

Test for terpenoids: The plant extract (0.5 g) was mixed with 2 mL chloroform followed by the careful addition of 3 mL concentrated sulphuric acid to form a layer. The formation of reddish brown colour on the interface indicates the presence of terpenoids.

Test for reducing sugars: The plant extract (1.0 g) was mixed with 10 mL of distilled water and was boiled for 5 min. The mixture was filtered using a Whatman No. 1 filter paper while the solution was still hot. The cooled filtrate was made alkaline by using a litmus paper by using 20% sodium hydroxide (5 M). The resulting solution was boiled with an equal volume of

Benedict qualitative solution in a water bath. The formation of a brick-red precipitation indicates the presence of a reducing compound.

Test for steroids: A small portion of the extract was dissolved in 1 mL of chloroform before filtration using a Whatman No. 1 filter paper. To the filtrate, 1 mL of acetic acid was added on ice, followed by the addition of a few drops of concentrated sulphuric acid to the sides of the test tube. The appearance of blue, bluish green or a rapid colour change from pink to blue colour confirmed the presence of steroids.

Test for saponins: Approximately 0.2 g of the extract was mixed with 5 mL of distilled water. The solution was then heated to boiling. Frothing or the appearance of a creamy mist of small bubbles indicated the presence of saponins.

Test for tannins: The plant extract (0.5 g) was boiled in 20 mL of water and the solution was filtered using a Whatman No. 1 filter paper. A few drops of 0.1% ferric chloride was added. A brownish green or blue black colour indicates the presence of tannins.

Test for phlobatannins: The plant extract (0.5 g) was dissolved in 1.0 mL of water followed by filtration using a Whatman No. 1 filter paper. The filtrate was boiled with 2% hydrochloric acid solution (0.6 M). The formation of a red precipitate indicates the presence of phlobatannins.

Test for glycosides: The plant extract (0.5 g) was dissolved in 1.0 mL of water followed by the addition of an aqueous solution of sodium hydroxide (5 M). The formation of a yellow colour indicates the presence of glycosides.

Test for alkaloids (Mayer's Test): Approximately 0.5 mL of the extracts was separately treated with a few drops of 1 mL 2 N hydrochloric acid followed by filtration. Mayer's reagent was prepared by dissolving 1.36 g of mercuric chloride in 60 mL distilled water, while 5 g of potassium iodide was dissolved in 10 mL of distilled water. The two solutions were mixed and was made up to 100 mL of distilled water. A few drops of the reagent were added to 1.0 mL of the acidic aqueous solution of the samples. The formation of a white or a pale precipitate indicates the presence of alkaloids.

α-amylase inhibition assay: The α-amylase assay that was used in this study was according to the method as described by Kazeem *et al.*[10]. A total of 250 µL of extract (0.125-1.000 mg mL^{-1}) was placed in a tube followed by the addition of 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution (0.5 mg mL^{-1}). The solution was pre-incubated at 37°C for 10 min, followed by the addition of 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9). Then, the mixture was further incubated at 37°C for 10 min. The reaction was terminated by adding 500 µL of DNS reagent (3,5-dinitro salicylic acid reagent) (1%) and 12% sodium potassium tartrate in 0.4 M sodium hydroxide. Then, the tubes were incubated in boiling water for 5 min before being cooled to a room temperature.

The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using a UV spectrophotometer (Model: HACH DR/2000, USA). A control was prepared based on a similar procedure but this time replacing the extract with a phosphate buffer. The α-amylase inhibitory activity was calculated as percentage of inhibition based on the formula:

$$\text{Inhibition (\%)} = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{extracts}}]}{\text{Abs}_{\text{control}}} \times 100$$

Where:
Abs = Absorbance

Amlyloglucosidase inhibition assay: The amyloglucosidase assay method was based on[11] with some modifications (in terms of the volume of reagents used). A total of 250 µL extract (0.125-1.000 mg mL^{-1}) was placed in a tube. This was followed by the addition of 50 mM acetate buffer (pH 5.5) containing amyloglucosidase solution (0.5 mg mL^{-1}) into the mixture. The solution was pre-incubated at 50°C for 10 min followed by the addition of 250 µL of 1% starch solution in 50 mM sodium phosphate buffer (pH 5.5). Then, the mixture was further incubated at 50°C for 10 min. The reaction was terminated by adding 500 µL of DNS reagent. After that, the tubes were incubated in boiling water for 10 min before being cooled to room temperature. The reaction mixture was diluted with 5 mL distilled water and the absorbance was measured at 540 nm using a spectrophotometer. A control was prepared using a similar procedure but this time replacing the extract with acetate buffer. The result was expressed as percentage of the blank control. The percentage inhibition was calculated as described previously.

Statistical analysis: All data are shown as Mean±SD and statistical analysis using a one-way analysis of variance (ANOVA) followed by Tukey test with a p-value <0.05 accepted

as statistically significant. Statistical analysis was conducted using an SPSS version 16.0 (Armonk, New York).

RESULTS AND DISCUSSION

Qualitative phytochemical analysis: Phytochemical screening indicated that all of the extracts (Table 1) contained tannins. A recent study, the presence of tannins in methanolic extract of *D. buxifolia* fruit has been reported[7]. Tannin is a compound that is able to react with protein to appear as stable water insoluble compound. Since enzymes are made up of proteins, tannin can also interfere α-amylase activity[12]. In addition, phlobatannin a subtype of tannin is also confirmed to be present in all extracts. Thus, it is plausible that the secondary metabolite may also induce *D. buxifolia* antidiabetic activity via a similar route. A previous study showed the presence of tannins in *D. malabarica* which was postulated to be responsible for the antidiabetic activity[13] and also supported by a previous study[14], whereby *Momordica charantia* which can be used to treat diabetes was also found to contain a similar type of tannin which is phlobatannin.

Besides tannin and phlobatannin, all of the investigated plant extracts also contained flavonoids and terpenoids. These components have been reported to be present in *D. mespiliformis*[15]. Other studies confirmed that terpenoids and flavonoids produce antidiabetic activity[16,17] possibly by their antioxidant effects. All plant extracts were negative for anthraquinone which was also similarly shown by another study on the antidiabetic effects of *Gossypium arboreum*[18]. Shanmugam *et al.*[19] also reported that anthraquinone was absent in the methanolic and aqueous extracts of *Phyllanthus niruri* traditionally-used in the treatment of diabetes. Besides anthraquinone, alkaloid was also absent in all of the extracts which was similar to a preliminary phytochemical screening of *D. montana* and *D. sylvatica*[20].

Glycosides were only observed in aqueous and methanolic extracts of the leaves. A recent study[21] revealed that glycoside is a phytochemical which contributes to one of the hypoglycaemic effects. In addition, all of the extracts, except for the aqueous extract of stems, contained some reducing sugars. Steroid compound is only present in the aqueous extract of the leaves. Saponins are present in both leaves and stems methanolic extracts. In a recent study saponins were found to be responsible for α-glucosidase and α-amylase inhibitory activities[22]. Another study reported that saponin can enhance the hypoglycaemic activity and can be used in diabetes treatment[23]. Therefore, it is plausible that saponins also may contribute to the higher inhibition of α-amylase and amyloglucosidase as seen in this study.

Table 1: Phytochemical composition of aqueous and methanolic extracts of *D. buxifolia* leaf and stem

	Leaf extract		Stem extract	
Phytochemicals	Aqueous	Methanol	Aqueous	Methanol
Anthraquinones	-	-	-	-
Flavonoids	+	+	+	+
Terpenoids	+	+	+	+
Reducing sugars	+	+	-	+
Steroids	+	-	-	-
Saponins	-	+	-	+
Tannins	+	+	+	+
Phlobatannins	+	+	+	+
Glycosides	+	+	-	-
Alkaloids	-	-	-	-

+: Present and -: Absent

α-amylase and amyloglucosidase inhibition: The enzyme inhibiting activities of the leaves and stems extracts of *D. buxifolia* and their possible antidiabetic activities were also compared between the different solvent extracts of each part. Additionally, the dependency of inhibition on the concentration of the different extracts was also determined. All extracts were investigated at four different extract concentrations (0.125, 0.250, 0.500 and 1.000 mg mL^{-1}) in triplicate.

The inhibition of carbohydrate-digesting enzymes is one of the *in vitro* approaches utilized to determine the antidiabetic potential of plant extracts. Amyloglucosidase is a carbohydrate-digesting enzyme that has the ability to split mainly α-1, 4 glucosidic bonds as well as α-1,6 glucosidic bonds in glucan. However, amyloglucosidase inhibitors competitively inhibit amyloglucosidase enzyme thus decreasing the rate of carbohydrate hydrolysis causing less glucose to be absorbed since carbohydrates are not digested into glucose molecules.

Thr α-amylase is a noticeable enzyme found in the pancreatic juice and saliva which breaks α-1, 4 glycosidic bonds in the primary compounds of starch resulting in the formation of smaller sugar molecules. The α-amylase inhibitors inhibit the α-amylase enzyme which delay glucose absorption into the bloodstream and is thought to be one of the best treatments for type 2 diabetes[24].

The α-amylase inhibitory activity of all the extracts occurs in a dose-dependent manner (Fig. 1). This finding is similar to another study reported by Kazeem *et al.*[10] on *Morinda lucida*. In addition, all extracts inhibit amyloglucosidase to a lesser extent when compared to α-amylase enzyme (Fig. 2). Nevertheless, the percentage inhibition of amyloglucosidase of all the investigated plant extracts is significantly different at each concentration. Among all of the extracts, the aqueous extract of leaves exhibited the lowest amyloglucosidase

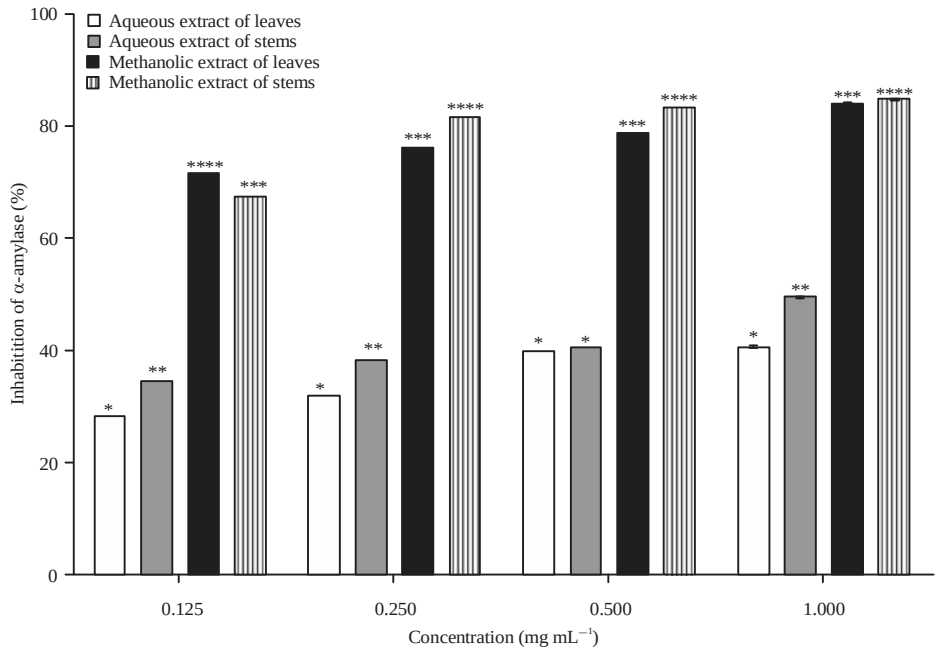

Fig. 1: α-amylase inhibitory activity of different extracts of *D. buxifolia*. The values are presented as Mean±Standard Deviation of triplicate experiments. *,**,***,****Bars with different superscripts at the same concentration are significantly different ($p<0.05$)

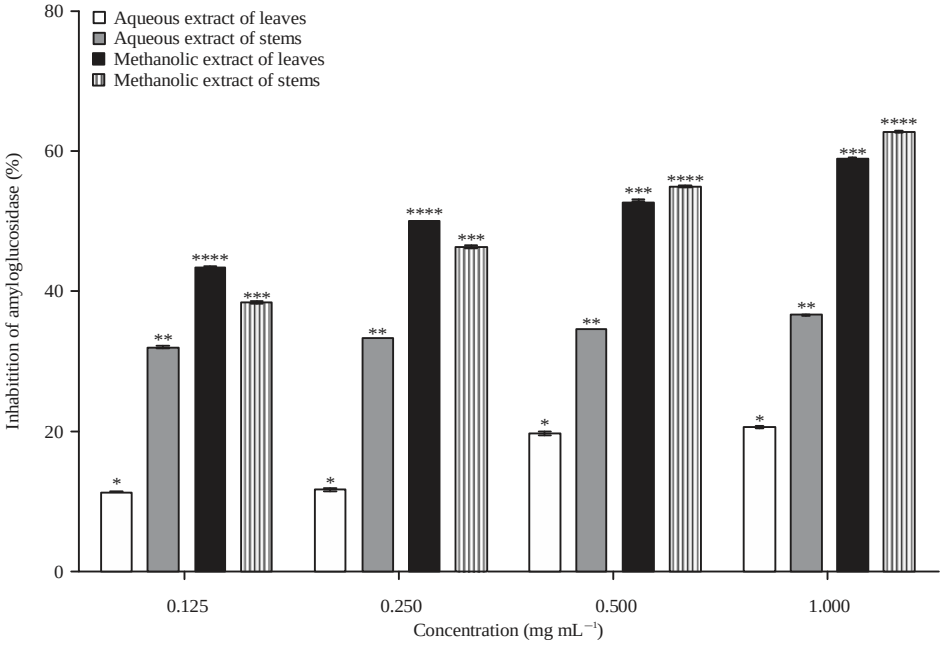

Fig. 2: Amyloglucosidase inhibitory activity of different extracts of *D. buxifolia*. Values represent Mean±Standard Deviation of triplicate experiments. *,**,***,****Bars with different superscripts at the same concentration are significantly different ($p<0.05$)

inhibition when compared to other extracts. The stem extracts showed higher inhibitory activities when compared to the leaf.

Inhibition of α-amylase and amyloglucosidase by stem methanolic extract were high (84.68±0.03 and 62.72±0.15%, respectively) when administered at the highest concentration. *D. buxifolia* stem extract showed better α-amylase and amyloglucosidase inhibitory potentials when compared to the leaves extract. This is supported by another study, where some

of the selected Fabaceae plant extracts including *Bauhinia acuminate*, *Bauhinia purpurea* and *Senna siamea* stem extracts showed higher inhibitory activities towards a carbohydrate-digesting enzyme, α-glucosidase when compared to the leaf extracts[25].

On the other hand, when the type of solvents were compared, methanolic extracts showed significant α-amylase and amyloglucosidase inhibitory activities whereas aqueous extracts showed moderate inhibition activity. Apart from that, the percentage of inhibition exhibited by aqueous extracts of leaves and stems was less than 50% even when used at the highest concentration indicating that the active constituents of *D. buxifolia* is less soluble in water. Another study revealed that methanol is one of the primary alcohols which can inhibit bacillus α-amylase in a non-competitive way[26]. The extracts (arranged in decreasing order of enzyme inhibiting activities are (1) Methanolic extract of stems, (2) Methanolic extract of leaves, (3) Aqueous extract of stems and (4) Aqueous extract of leaves which was also similarly reported by previous studies, where methanolic extracts have been found to show better *in vitro* antidiabetic activity as compared to the aqueous extracts[27,28]. The deferral in carbohydrate absorption with the medicinal plant based α-amylase inhibitors provides a potential opportunity for the treatment of diabetes mellitus.

CONCLUSION

The methanolic stem extract showed the significant inhibition against both enzymes indicating that its active constituents are more soluble in organic solvent. The present study reveals that *D. buxifolia* stem and leaves contains major phytochemicals including tannins, phlobatannins, flavonoids and terpenoids which may contribute to the α-amylase and amyloglucosidase inhibition and antioxidant activities which occur in a dose-dependent manner. It may be concluded that the plant extracts can be with the constituents of biopharmaceutical importance. Further investigations are warranted to isolate the active fractions and also to evaluate the *in vivo* antidiabetic activities with mechanism of action elucidation.

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