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Research Article

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In-Vitro antidiabetic potential of *Piliostigma thonningii* leaf column chromatographic fractions

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ABSTRACT

Diabetes mellitus is a metabolic disease characterized by high blood sugar that profoundly affects human health and economy. Although there are a plethora of antidiabetic drugs in the market, the efficacy shown by some medicinal plants sometimes outperforms that of synthetic drugs while being more affordable and less toxic. This study was conducted to evaluate the anti-diabetic potential of column-chromatographic fractions of *Piliostigma thonningii* leaf. Phytochemical screening, column, and thin layer chromatography were respectively conducted following standard laboratory protocols. The *in-vitro* antidiabetic activity of the extracts was assessed using alpha-glucosidase and hemoglobin glycosylation inhibitory methods. A total of 175 column fractions (CF1-CF175) were eluted respectively. Thin layer chromatography protocols employed pooled a total of 11 (A-J) fractions. The result showed significant ($P>0.05$) inhibition of alpha-glucosidase in only fraction J2, which was significantly greater when compared to standard drug (Voglibose) and crude extract. Also, there was no significant hemoglobin glycosylation inhibitory effect in all fractions compared with crude extract and standard drugs (Voglibose). In conclusion, *P. thonningii* leaf exhibited alpha-glucosidase and hemoglobin glycosylation inhibitory potentials that further support its usage as a hypoglycemic agent.

KEYWORDS: Diabetes mellitus, *Piliostigma thonningii*, Alpha-glucosidase, Hemoglobin glycosylation, Column chromatography

INTRODUCTION

Diabetes mellitus is a progressive metabolic disease that is characterized by high blood sugar and is a great threat to human health (Lu & Zhao, 2020). According to the World Health Organization (WHO), there are currently more than 536 (10.5%) million people suffering from diabetes worldwide and that number is projected to reach 783 (12.2%) million by 2045 (WHO, 2021). As of 2021, it was estimated that about 3.6 million people suffer from diabetes in Nigeria which placed it second to South Africa in the African continent (Cho *et al.*, 2018). Diabetes lowers the quality and life expectancy of the affected person due to its chronic nature and associated complications (Jamison *et al.*, 2006). It can result in serious long-term complications such as renal failure, blindness, and amputation. It is also associated with increased morbidity from cardiovascular disease, including myocardial infarction and stroke (Davies *et al.*, 2008).

Natural products, especially those derived from plants have been used to help mankind and sustain health since the dawn of medicine (Moghadamtousi *et al.*, 2015). Several species of herbal drugs with potential antidiabetic activity have been prescribed due to their good efficacy, fewer side effects in clinical experience and relatively low costs. Medicinal and natural herbal plant products have been traditionally used for a long time in many countries for the treatment of diabetes mellitus. *P. thonningii* is a multipurpose tree of high priority in Nigeria, almost all its parts are used in traditional medicine and its seeds are good sources of antioxidants, and micronutrients, rich in crude protein and carbohydrates (Deshi *et al.*, 2014). The roots are used locally in the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin infections in Northern Nigeria while the leaf extract has been used for various purposes including the treatment of malaria all over Nigeria (Kwaji *et al.*, 2010). Column chromatography plays a crucial role in separating plant components and can significantly impact bioactivity by isolating the active compounds, removing interfering substances, exploring synergistic effects and optimizing the desired therapeutic effect (Ighodaro *et al.*, 2012). Hence this work aimed to evaluate the in vitro anti-diabetic potential of column chromatographic fractions of *P. thonningii*

leaf of methanol extract by studying their effects on inhibition of glycosylation and alpha-glucosidase activity.

MATERIALS AND METHODS

Chemical and Reagents

All the chemicals and reagents used were of analytical grade and were used without any further purification. They are methanol (JHD-china), n-hexane (JHD-china) and ethyl acetate (Loba Chemie Pvt. Ltd).

Sample Collection, Identification, and Preparation

Fresh leaves of *Piliostigma thonningii* (Schumach.) Redheads were collected in September 2021 from Giro village, Suru Local Government Area, Kebbi state, Nigeria. The plant was identified and authenticated to be *P. thonningii* at the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aliero (KSUSTA). A voucher specimen (KSUSTA/PSB/H/VOUCHER NO109) was deposited at the Herbarium Department of Biological Science, KSUSTA. The leaves were cleaned and air-dried under shade at room temperature for three days, pulverized using mortar and pestle and subjected to the following analysis.

Plant Extraction

One thousand four hundred grams (1400 g) of the powdered leaves was extracted using 5500 mL of methanol. The extract was concentrated to dryness by evaporation process at room temperature and stored in the lab. They were weighed, labeled, and kept until required.

Phytochemical Screening

Phytochemical screening of the extracts was performed following standard protocols procedure to the presence of tannins (Evans, 2009), saponins (Sofowora, 1993), alkaloids (Harborne, 1998), phenols (Harborne, 1998), flavonoids (Kumar *et al.*, 2011) terpenoids (Evans, 2009), anthocyanins (Sofowora, 1993), glycoside (Sofowora, 1993) and cardiac glycoside (Evans, 2009).

Solvent System

The crude leaf methanol extract of *P. thonningii* was reconstituted in absolute methanol and spotted on a pre-coated (Silica gel F254) thin layer chromatographic (TLC) aluminium plate. Various solvent systems: Hexane/ethyl acetate, chloroform/ethyl acetate, ethyl acetate/methanol, and methanol/acetone were used in different combination ratios as mobile phase to determine the most suitable eluent for column fractionation of the extract. After each separation, the TLC plates were exposed to H_2SO_4 in a chamber. The eluent (solvent-system at a particular ratio) which gave the best separation or resolution was adopted.

Chromatographic Separation of Bioactive Component

The silica gel (100-200 mesh) (120 g) was mixed with 100% n-hexane and the wet silica gel was loaded into the column using additional n-hexane. The dried form of the crude extract (3g) was then added to the packed column. The column was eluted with hexane: ethyl acetate (100:0, 98:02, 95:05, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100 %v/v, 100 mL each).The column was also eluted with ethyl acetate: methanol (100:0, 99:01, 98:02, 95:05 %v/v, 100 mL each) respectively, followed by methanol: acetone (99:01, 98:02, 95:05 %v/v, 100 mL each).The eluted fractions were collected into small sample bottles (10 mL each).The fractions were allowed to evaporate to dryness.

TLC Pooling of Column Fractions

A commercially prepared aluminium back TLC plate of 20×20cm activated silica gel of $60F_{254}$ (Merck Germany) was cut to sizes of 7, 7, and 6 cm. The dried column chromatographic fractions obtained were reconstituted in methanol and spotted on TLC plates, then developed with a hexane: ethyl acetate (70:30) solvent system. The developed plates were allowed to dry and detection of compounds was aided by dipping the plates in vanillin reagent (1 g vanillin powder in 90 mL distilled water and 10 mL conc. H_2SO_4).The plates were heated at 100 °C for 3-5 minutes for optimal colour development. In this study, a total of 175 bottle fractions were pooled and categorized into groups labeled A to J, based on their similarities. Fraction J was further divided into sub-labels J1 and J2, as it consisted of two distinct immiscible layers. The 11 pooled fractions obtained from thin-layer chromatography (TLC) were subsequently subjected to in vitro analysis.

In Vitro Antidiabetic Potential of *P. thonningii* Leaf of TLC Pooled Fractions

Inhibition of alpha-glucosidase

The inhibitory activity was determined by incubating a solution of starch substrate (2% w\v maltose or sucrose) 1 mL with 0.2 M Tris buffer pH 8.0 and various concentrations of plant extract for 5 minutes at 37 °C. The reaction was initiated by adding 1 mL of alpha-glucosidase enzyme (1 U/mL) to it followed by incubation for 40 minutes at 35 °C. Then the reaction was terminated by the addition of 2 mL of 6 N HCl. Then the intensity of the color was measured at 540 nm (Krishnaveni *et al.*, 1984).

Evaluation of Hemoglobin Glycosylation Inhibitory Effect

The blood was collected from a healthy human volunteer and transferred into a bottle containing an anticoagulant. Hemolysate was prepared based on the principle of hypotonic lysis (Adisa *et al.*, 2005). The red blood collected was washed thrice with 0.14 M NaCl solution and one volume of red blood cell suspension was lysed with two volume of 0.01 M phosphate buffer, pH 7.4 and 0.5 volume of CCl_4. The hemolysate was

then freed from the debris by centrifugation at 2300 rpm for 15 minutes at room temperature. The hemoglobin rich fraction (i.e., the upper layer) was separated and dispensed into a sample bottle for storage and refrigerated until required for use (Adisa *et al.*, 2005).To 1 mL of hemoglobin solution, 5 µL of gentamicin and 25 µL of the plant extract (30 µg/ML) were added. The reaction was started by the addition of 1 mL of 2% glucose in 0.01 M phosphate buffer (pH 7.4) and incubated in the dark at room temperature. The concentration of glycated hemoglobin at the incubation period of 0, 24 and 72 hours was estimated spectrophotometrically at 443 nm (Adisa *et al.*, 2005).

RESULTS

Extraction and Phytochemical Screening of *P. thonningii* Leaf

Plant extraction using methanol yielded 12.18% and the extract is soluble in water, dark brown in color and with a fine texture. The preliminary phytochemical screening test of *P. thonningii* methanol leaf extract revealed the presence of alkaloids, flavonoids, tannins, saponin, glycoside, phenols, terpenoids, anthocyanins and cardiac glycosides (Table 1).

Results of Chromatographic Separation and TLC Pooled Fractions of *P. thonningii* Leaf

P. thonningii leaf crude extract separation using column chromatography yielded 175 fractions. Pooling together of the fractions with similar TLC mobility profiles, afforded 11 fractions with percentage yields ranging from 1.37% to 9.47%. The colour of the fractions varies from colourless, black, dark yellow, yellow, dark brown, and light brown. It was observed that fractions JI (11.54%) and J2 (9.47%) had the highest percentage yield among all the fractions (Table 2).

Alpha-glucosidase Inhibition

Column fraction J2 exhibited significantly ($P<0.05$) the highest α-glucosidase percentage at concentration of 250 µg/mL (61.76%), 500 µg/mL, (80.38%) and 750 µg/mL (37.42) compared to crude extract. The α-glucosidase inhibitory effect of J2 and standard drug (Voglibose) was comparable at 750 µg/mL only while significantly ($P<0.05$) at 250 µg/mL-500 µg/mL respectively (Table 3).

Hemoglobin Glycosylation Inhibition

There is no significant difference in all fractions compared with crude extract at 250 µg/mL except fraction A, and also no significant difference in all fractions when compared with drug (Voglibose). Also, there was no significant difference in fractions A, B, C, D, E, F, H and E when compared with crude extract and drugs at 500 µg/mL and 750 µg/mL except the most potent fraction i.e. J1 and J2. But there's a significant decrease in fraction G at 500 µg/L and 750 µg/mL when compared with crude extract and drug (Voglibose) (Table 4).

Table 1: Phytochemical constituent of *P. thonningii* methanol leaf extract

Phytochemicals	Results
Alkaloids	+
Flavonoids	+
Tannins	+
Saponin	+
Glycoside	+
Phenols	+
Terpenoids	+
Anthocyanins	+
Cardiac glycosides	+

+ = Present

Table 2: Percentage yield of TL-C Pooled Column Fractions

Column Fraction	Color	Yield (mg)	Percentage Yield (%)
A	Colorless	41.24	1.37
B	Black	72.24	2.41
C	Dark yellow	39.24	1.31
D	Yellow	57.24	1.91
E	Yellow	61.24	2.04
F	Dark yellow	39.30	1.30
G	Dark yellow	61.24	2.04
H	Dark yellow	32.24	1.07
I	Yellow	23.24	0.77
J1	Dark brown	346.30	11.54
J2	Brown	284.24	9.47

Table 3: Alpha-Glucosidase inhibitory activity of *P. thonningii* pooled column fractions

Treatment	Percentage Inhibition		
	250 µg/mL	500 µg/mL	750 µg/mL
Fraction A	10.08 ± 3.45^{b}	18.17 ± 1.04^{bcd}	20.42 ± 8.37^{cde}
Fraction B	19.52 ± 3.64^{b}	5.00 ± 0.95^{bc}	-18.88 ± 1.61^{ab}
Fraction C	11.51 ± 7.23^{b}	18.78 ± 4.65^{bcd}	32.59 ± 22.25^{de}
Fraction D	14.53 ± 1.46^{b}	16.89 ± 4.85^{bcd}	17.89 ± 4.22^{cd}
Fraction E	21.79 ± 1.64^{b}	21.61 ± 1.36^{cd}	20.52 ± 5.75^{cde}
Fraction F	30.06 ± 7.81^{bc}	19.43 ± 0.89^{bcd}	22.34 ± 4.13^{cde}
Fraction G	16.34 ± 5.72^{b}	15.89 ± 3.58^{bcd}	19.34 ± 1.75^{cde}
Fraction H	18.98 ± 4.20^{b}	21.62 ± 1.97^{cd}	10.17 ± 3.91^{cd}
Fraction I	24.61 ± 0.55^{b}	3.45 ± 2.25^{bc}	-1.72 ± 4.03^{bc}
Fraction J1	11.35 ± 1.62^{b}	-10.35 ± 2.12^{b}	-23.34 ± 5.67^{ab}
Fraction J2	61.76 ± 1.90^{c}	80.38 ± 0.83^{e}	37.42 ± 1.67^{de}
Crude Extract	37.33 ± 13.99^{a}	77.83 ± 31.03^{a}	30.33 ± 4.67^{a}
(Viglibose)	43.59 ± 7.93^{b}	39.32 ± 2.10^{d}	43.59 ± 1.14^{e}

Values are presented as mean±SEM (n=3). Values in column having same superscript are not significantly different with standard drug at ($P<0.05$) using one-way ANOVA, followed by Duncan multiple comparison test with SPSS version 20.0

DISCUSSION

There is a possibility of undesired side effects or herbal toxicity as a result of the presence of certain components in the plants whole extracts. This explains why column chromatography separation of crude extracts is a significant step in optimizing the therapeutic effectiveness of medicinal plants and in overcoming herbal toxicity which is a major concern in the medicinal application of herbs or plant extracts. In this regard the separating plant component is not applicable to the potential of crude extract. For instance in the present

Table 4: Hemoglobin glycosylation inhibitory activity of *P. thonningii* pooled column fractions

Treatment	Percentage Inhibition		
	250 µg/mL	500 µg/mL	750 µg/mL
Fraction A	73.30±20.36^{a}	94.16±0.33^{bc}	93.73±0.25^{bc}
Fraction B	93.46±0.19^{b}	93.20±0.51^{bc}	95.23±0.14^{bc}
Fraction C	98.66±3.08^{b}	94.43±0.56^{bc}	91.20±2.79^{b}
Fraction D	94.38±0.18^{b}	94.59±0.53^{bc}	98.23±4.23^{c}
Fraction E	99.03±2.89^{b}	93.95±1.55^{bc}	97.48±3.68^{bc}
Fraction F	89.24±0.83^{ab}	92.08±0.62^{b}	95.82±0.18^{bc}
Fraction G	94.64±0.70^{b}	86.13±2.03^{a}	77.63±1.95^{a}
Fraction H	93.46±0.05^{b}	92.56±0.14^{b}	91.54±0.11^{bc}
Fraction I	94.96±0.66^{b}	95.45±0.14^{cd}	91.70±2.36^{bc}
Fraction J1	96.09±0.75^{b}	97.220±0.14^{d}	94.13±1.04^{bc}
Fraction J2	95.58±0.14^{b}	97.16±0.14^{d}	94.38±0.41^{bc}
Crude Extract	91.70±0.11^{b}	93.04±0.14^{bc}	94.32±0.60^{bc}
(Viglibose)	90.85±1.16^{ab}	95.23±0.43^{cd}	96.51±0.14^{bc}

Values are presented as mean±SEM (n=3). Values in column having same superscript are not significantly different with standard drug at ($P<0.05$) using One-way ANOVA, followed by Duncan multiple comparison test with SPSS version 20.0

study, some column fractions obtained from *P. thonningii* leaf showed some degree of hypoglycemic activity. The results are in agreement with the work of (Asuzu & Nwaehujor 2015). However, in this study column fraction J2 showed higher significant inhibitory activity in α-glucosidase compared to crude extract and drug. No significant difference in all fractions except fraction A, compared with drug and crude extract in glycosylation, and significant reduction only in the G fraction. This result also suggests that the hypoglycemic capability of *P. thonningii* leaf extract is present with separation of the extract into its component fractions. The Findings of this study suggest that the anti-diabetic potential of column fraction J2 (most potent) could be possibly due to the higher percentage yield contained in it. Therefore, this research corroborates with the work of Patil *et al*. (2011), who identified amentoflavone as the antidiabetic principle in *Biophytum sensitivum*. Lupeol also has been reported to be one of the antidiabetic compounds in *Coccinia indica* (Kumar *et al*., 2013) and two other *Coccinia species* (Ocvirk, 2013).

CONCLUSION

The present column and *in vitro* investigation support the usage of *P. thonningii* as a traditional antidiabetic herb. In this study, the most potent bioactive compound is shown to be present in fraction J2 which was able to inhibit α-glucosidase and hemoglobin glycosylation. It is therefore recommended that further work should be aimed at the identification of antidiabetic agents using in silico, molecular docking, and target prediction so that they can be considered in pharmaceutical formulation for the successful management of diabetes.

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