



RESEARCH ARTICLE

Free radical scavenging activity, pancreatic lipase and α -amylase inhibitory assessment of ethanolic leaf extract of *Phyllanthus amarus*

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Abstract

Oxidative stress is associated with the pathogenesis of diabetes and obesity. The key enzymes involved in the carbohydrate and lipid metabolisms are α -amylase and pancreatic lipase respectively. The phytochemicals present in medicinal plants are known to be potent free radical scavenging agents and great inhibitors of metabolizing enzymes. *Phyllanthus amarus* leaves have antioxidant, anti-diabetic, and anti-obesity properties. This research is aimed at elucidating the carbohydrate and lipid metabolism inhibitory activities of *P. amarus* leaves through *in-vitro* screening. The phytochemical screening revealed that *P. amarus* is having high phenolic, flavonoid, tannin, and saponin content. The total protein content of *P. amarus* showed 2.720 ± 0.001 mg BSAE/g. Diphenyl-2-picrylhydrazyl (DPPH) assay showed 0.0001 mg/mL, Nitric oxide assay revealed 0.452 mg/mL, Ferric Reducing Antioxidant Power (FRAP) assay showed a value of 0.132 ± 0.010 mg RE/g, Total antioxidant capacity (TAC) indicated a value of 2.217 ± 0.002 mg GAE/g and 2,2-azino-bis(3-ethylbenzotiazoline)-6-sulfonic acid (ABTS) inhibitory activity at the peak concentration (1.2 mg/mL) was 40.29%. The enzyme inhibitory assessment showed a decrease in α -amylase activity as the concentration increased while there was a slight decrease in α -lipase activity but a slight increase at 18.75 mg/mL.

Keywords

Diabetes, medicinal plant, obesity, oxidative stress

Introduction

The etiology of diseases is associated with instability between a generation of free radicals and the antioxidizing power of the body (1). Oxidative stress is regarded as instability between Reactive Oxygen Species and antioxidants that results in oxidative damage of nucleic acids, proteins, fats, and carbohydrates (2,3). Medicinal plants have therapeutic agents as they contain phytochemicals with vast medicinal potential which are employed in the prevention, maintenance, and treatment of diseases (4). Herbs are regarded as the major source of pharmaceuticals in the development of drugs and pharmaceuticals used in the treatment of human diseases as a result of the diversity of the phytochemicals contained in them (5).

Diabetes mellitus is reported to be a serious metabolic disorder affecting 285 million people worldwide affecting those in the age group that ranges from 20 to 79, in 2010. By 2030, it is expected to increase up to 439 million, (6). Type-2 diabetes is caused as a result of insulin resistance and it is mainly

characterized by high level of blood glucose (7). Inhibition of amylase is regarded as a major way of maintaining a reduced blood glucose level. Amylase, secreted in saliva and pancreas is responsible for catalyzing 1,4-glycosidic bonds converting polysaccharides into smaller oligosaccharides (maltose). Thus, glucose absorption rate reduction occurs as a result of inhibition of carbohydrate metabolizing enzymes (8). Some of the examples of inhibitors are miglitol, acarbose and voglibose.

Obesity, as defined by World Health Organization (WHO), is the accumulation of abnormal or excessive fat (9). It tends to affect the physiological state of the body. It poses the risk of developing disorders such as cancer (10), poor mental health (11), cardiovascular diseases (12,13), diabetes (14), and musculo-skeletal disorders (15). Natural products constitute inhibitors of pancreatic lipase developed into clinical products as a remedy for obesity. The major phytochemicals such as polyphenols, flavonoids, saponins, and caffeine are contributed to the inhibition of pancreatic lipase. For instance, anthocyanin (phenolic compound) inhibits pancreatic lipase leading to the reduction in body weight as a result of restriction in calorie intake (16, 17, 18, 19).

In developing countries, plants play a vital role in diabetes treatment because there are limited resources. There is a high demand for the use of alternative medicine in diabetes treatment due to the side effects related to the usage of hypoglycaemic agents (20). WHO exerted an expert group on diabetes which further suggested folklore methods of treatment in managing the disease, due to the increased death rate resulting from the problems caused by the adoption of commonly used anti-diabetic therapeutic agents (citation needed). Various natural products are used in the management of diabetes traditionally but it is vital to validate their mechanism of action (21).

One of the medicinal plants used in the treatment of diabetes is *Phyllanthus amarus* which belongs to the family Euphorbiaceae. It is an annual herb (10-15cm in height) and having green smooth tiny seeds. Its medicinal properties are traditionally attributed to the treatment of diabetes, jaundice and obesity. The common names of *P. amarus* are *Iyin-Olobein* in South-Western part of Nigeria. *P. amarus* contain proteins (6.10%), carbohydrates (45.52%), fiber (24.50%) and mineral elements (22). It has high carbohydrate content, particularly polysaccharide which may be responsible for its anti-diabetic activity. Carbohydrate diet causes a reduced level in blood glucose concentration as an aftermath of increased concentration of blood glucose (23). *P. amarus* is reported to have anti-hypertensive, anti-diabetic, anti-inflammatory, anti-microbial, and many more properties including that of being hepatoprotective. The use of ethanolic extract of *P. amarus* leaf for the prevention and treatment of diabetes and obesity need to be scientifically validated (24). Considering the wide range of medicinal importance of *P. amarus* and the increased morbidity and mortality rate among diabetes patients and the prevalence of obesity in growing countries like Nigeria, it is crucial to validate the therapeutic effect of *P. amarus* as a cheap and accessible medicine over conventional drugs.

Several *in-vitro* and *in-vivo* studies have been carried out on the leaves of this plant but there is still a resurgence of interest due to the carbohydrate and lipase inhibitory agents contained in it. The present study is aimed to assess the amylase and lipase inhibitory action of the phytochemical constituents in the ethanolic leaf extract of *P. amarus*.

Materials and Methods

P. amarus leaves as shown in fig 1. were sourced from specific locations in Covenant University (N6° 39'53.32644" E3°9'35.9496"), Ogun State, Nigeria. The leaves were identified by a botanist in the Department of Biological Sciences, Covenant University. The voucher numbers were deposited at the Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria.



Fig. 1. *P. amarus* (source: Internet).

Chemicals and Reagent

Chemicals of analytical grade and salts of high purity were bought from reliable sources. The list of chemicals include; acetonitrile, sodium phosphate buffer, pancreatic α -amylase, gallic acid, starch solution, dinitrosalicylic acid, porcine pancreatic lipase, 4-nitrophenylbutyrate, p-nitrophenyl- α -Dglucopyranoside, porcine pancreatic α -amylase, ascorbic acid, BHA (butylated hydroxyanisole), (2,2-diphenyl-1-picryl-hydrazyl) DPPH reagent, potassium ferricyanide and ferric chloride and acarbose.

Plant Extracts preparation

Fresh leaves of the selected plant were dried in a cool place, an electric blender was used in pulverizing the dried leaves into powdery form. The pulverized form of *P. amarus* (150g) was steeped into 90% ethanol in the ratio of 1:5 (v/v) for 72 hours, followed by filtration. A rotary evaporator was used to concentrate obtained filtrate at 50°C to achieve the solvent-soluble fractions (25).

Assessment of total alkaloid content

Ten percent of 200cm³ acetic acid was mixed with 2.5g of powdered sample. After a duration of 4 hours, the extract was concentrated to a quarter of its initial volume. Thereafter, 15 drops of concentrated ammonium hydroxide were added to the filtrate until precipitation was finalized. The oven-dried residue was weighed (26).

$$\text{Percentage of alkaloid (\%)} = \frac{\text{weight of residue}}{\text{weight of sample}} * 100$$

.....(1).

Total phenolic content

An extract (1mg/mL) of 0.5 mL and 2.5 mL Folin–Ciocalteu (FC) Assay reagent (1:10 v/v) were mixed together. A 2 mL volume of sodium carbonate (7.5%) was added after 5 minutes of incubation. It was further incubated at 40°C for 30 minutes. Reading was taken using an ultraviolet spectrophotometer at 765 nm. The varying concentration (0.02 - 0.1 mg/mL) of gallic acid and the sample's phenolic content were calculated using the standard curve as mg of gallic acid equivalent (mg GAE/g extract) (25).

Total flavonoid content

An extract (1 mg/mL) of 0.15 mL was added to 2mL of distilled water. After thorough mixing, the same amount of sodium nitrite (5%) was added and was set to incubate for 6 minutes. Further incubation was set up for 5 minutes after adding 0.15mL of AlCl₃ (10%). To the resulting incubated mixture 1 mL of Sodium hydroxide (4%) was added and 1.2 mL of distilled water to make up to a final volume of 5 mL. The absorption rate of the mixture was taken at 420 nm. Rutin was used as standard and dH₂O as blank. The total flavonoid content was illustrated as rutin equivalent mg RE/g extract using the standard curve (27).

Total tannin content

This assay was carried out according to the principle of the Folin Ciocalteu method. A sample (1mg/mL) extract of 0.5 mL, 3.75 mL of dH₂O and 0.25 mL of FC reagent (1:10v/v) were mixed together. Finally, 0.5 mL of Na₂CO₃ (35%) was mixed and the absorbance was read at 725nm. Different concentrations (0.1-0.00625 mg/mL) of tannic acid was used and the assay was conducted in triplicate. The plant extract's tannin content was calculated as tannic acid equivalent mg TAE/g extract (25).

Total saponin content

A sample of 0.05 mL and 0.25 mL of dH₂O were added together. A volume of 0.25 mL vanillin reagent of exactly 800 mg was dissolved in 10 mL ethanol and 2.5 mL of sulphuric acid (72%) added to the initial mixture. The absorbance was measured at 544nm after incubation at 60°C for 10 minutes. The standard used was diosgenin and the assay was conducted in triplicate and the values were calculated as diosgenin equivalents (mg DE/g extract) (28).

Total protein content

To a 0.2mL sample 2mL of alkaline copper sulphate (AlCuSO₄) was added and then incubated for 10 minutes. A further incubation was set for 30 minutes after adding 0.2 mL of Folin Ciocalteu reagent. The mixture was measured at 660 nm in a spectrophotometer. BSA (Bovine Serum Albumin) of varying concentrations of 0.05-1 mg/mL was set as a standard for the assay. The values were illustrated as BSA equivalents (mg BSAE/g extract) (29).

Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay was done following the method according

to Iheagwam *et al.* (2020) (citation needed). 0.5 mL DPPH (0.1 mM) was added to 0.5mL of sample and further incubated in dark for 30 minutes. Different concentrations (0.00125-0.000019 mg/mL) of ascorbic acid were used.

$$\% \text{ inhibition of DPPH scavenging activity} = \frac{Ac-As}{Ac} * 100 \dots\dots$$

.....(2).

Ferric reducing antioxidant power (FRAP) assay

One mL sample was evenly mixed with a volume of 2.5 mL [K₃Fe (CN)₆, 1%] and was put in for incubation for 20 minutes at 50°C. Trichloroacetic acid of 2.5mL was pipetted into the resulting mixture, the exact volume was taken off, and later added 2.5mL of dH₂O. The resulting mixture reading was measured at 700 nm in an ultraviolet spectrophotometer after adding 0.5 mL of FeCl₃. Ascorbic acid was used as the assay standard (25).

2,2-azino-bis(3-ethylbenzotiazoline)-6-sulfonic acid (ABTS) assay

ABTS radical solution was prepared by a thorough mixing of potassium persulfate (2.45 mmol/L) and ABTS^{•+} (7 mmol/L) in the same proportion. The resulting mixture was kept in the dark for 12-16 hours. One mL of the radical solution was diluted with a required amount of ethanol/methanol (1:89 v/v) to achieve an 0.700 ± 0.200 absorbance value just immediately before the use at 745 nm. Thereafter, 0.1 mL of sample/standard was added to a volume of 3.9 mL ABTS solution and was set to incubate at 25°C for 6 minutes. The absorbance of the final mixture was measured at 745 nm in an ultraviolet spectrophotometer and read against methanol as blank, ABTS solution was used as control, and butylated hydroxytoluene (0.0375-1.2 mg/mL) as standard (30).

$$\% \text{ inhibition of ABTS scavenging activity} = \frac{Ac-As}{Ac} * 100$$

.....(3)

Where Ac implies control's absorbance and As is the absorbance of the plant extract.

Nitric oxide (NO) radical scavenging activity

A 10 mM solution of sodium nitroprusside was prepared and a volume of 2mL was dissolved in 0.5 mL of a pH 7.4 phosphate buffer saline and 0.5 mL of varying concentrations of the sample was added. The mixture was set for incubation for 150 minutes at 25°C. An equal volume of 0.5 mL of the resulting mixture and Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylenediamine dichloride were mixed and proceeded for another incubation period of 30 minutes. The absorbance was measured at 546 nm against a blank (phosphate buffer) and the Griess reagent was set as the control (31).

$$\% \text{ inhibition of NO scavenging activity} = \frac{Ac-As}{Ac} * 100$$

.....(4)

Where Ac implies control's absorbance and As is plant extract's absorbance.

Total antioxidant capacity (TAC)

A volume of 1 mL of phosphomolybdate reagent made up of 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate were added to 0.1 mL of extract. After incubating for 90 minutes, at 95°C, the incubated mixture was measured at 695 nm against blank. The control used was gallic acid and distilled water replaced the sample/standard (25).

Carbohydrate Inhibitory assessment

α -amylase solution (2 U/ μ L in phosphate buffer, 6.8) of 500 μ L was mixed with 250 μ L of acarbose/sample and was set to incubate at 37°C for 20 minutes. A volume of 250 μ L starch solution (1% in pH 6.8 phosphate buffer) was mixed and incubated for 1 hour at 37°C. The mixture was measured in the spectrophotometer at 540 nm against buffer as control and DNSA as blank after 1mL of dinitrosalicylic acid (DNSA) reagent was mixed and boiled for 10 minutes (32).

$$\% \text{inhibition of } \alpha - \text{amylase activity} = \frac{Ac - As}{Ac} * 100 \dots (5)$$

Where Ac implies control's absorbance and As is plant extract's absorbance.

Lipase inhibitory assessment

An assay buffer of 164 mL and 6 mL solution of pancreatic lipase were mixed in a 96-well plate. An extract of 20 mL was added and incubated at 37°C for 10 minutes. A 10 mL of the substrate was mixed and further put for incubation at 37°C 15 minutes. The reading of the final mixture was taken at 405 nm (33).

$$\% \text{inhibition of alpha lipase} = \frac{Ac - As}{Ac} * 100 \dots (6)$$

Statistical analysis

The data were analyzed using the GraphPad Instat software (GraphPad Prism Inc., San Diego, CA, USA). All columns of the extracts were compared to the control in an ANOVA with the Dunnet post-test to evaluate levels of significance. The mean SEM \pm was used to express all of the data. Values that are significantly different from each other were indicated by $P \leq 0.05$ values.

Results

Voucher referencing and extraction yield of the selected plants

This study was conducted with aqueous 90% ethanol extract of the *P. amarus* leaves. The percentage yield of this selected plant (Pa/Bio/H841) was calculated at 23.53%.

Phytochemical Screening and Total Protein Content of *P. amarus* leaf extract

The ethanolic extract of the leaf showed the presence of phytochemicals stated in Table 1. These phytochemicals include; phenols, flavonoids, tannins, alkaloids and saponins. The leaves of *P. amarus* are reported to have high nutritive value. This study reported the presence of high

Table 1. Phytochemical screening and total protein content of *P. amarus*

| Phytochemicals and Protein | <i>P. amarus</i> |
|----------------------------|-------------------|
| TPC (mg GAE/g) | 0.212 \pm 0.001 |
| TFC (mg RE/g) | 0.199 \pm 0.020 |
| TSC (mg DE/g) | 2.335 \pm 0.007 |
| TTC (mg TAE/g) | 0.143 \pm 0.065 |
| TAC (%) | 10.00 \pm 0.003 |
| TPC (mg BSAE/g) | 2.720 \pm 0.001 |

protein content (2.720 \pm 0.001 mg BSAE/g) in the leaf extract.

Values are illustrated as means \pm standard error of the mean. Different letters in superscripts vary significantly from each other ($p > 0.05$). GAE, RE, DE, TAE, BSAE, TPC, TFC, TSC, TTC, and TAC represent Gallic acid equivalent, Rutin equivalent, Diosgenin equivalent, Tannic acid equivalent, Bovine serum albumin equivalent, Total phenolic content, Total Flavonoid Content, Total Saponin Content, Total Tannin Content, Total Alkaloid Content.

Antioxidant Analysis

To determine the radical scavenging ability of *P. amarus* leaf extract, antioxidant assays such as DPPH, Nitric oxide, FRAP, TAC, and ABTS were carried out. The result of FRAP and TAC showed 0.132 \pm 0.01 mg RE/g and 2.217 \pm 0.002 mg GAE/g respectively as shown in table 2. The inhibitory percentage of ABTS at the highest concentration (1.2 mg/mL) is 40.29%, as shown in fig 2 below. Table 3 shows the 50% inhibitory concentrations (IC₅₀) value DPPH to be 0.0001 mg/mL and Nitric oxide value of *P. amarus* as 0.0452 mg/mL. The DPPH IC₅₀ value of *P. amarus* showed to have more potent antioxidant power than ascorbic acid (0.0003 mg/mL).

Enzyme Inhibitory assessment

Carbohydrate and lipid inhibitory enzyme assessment il-

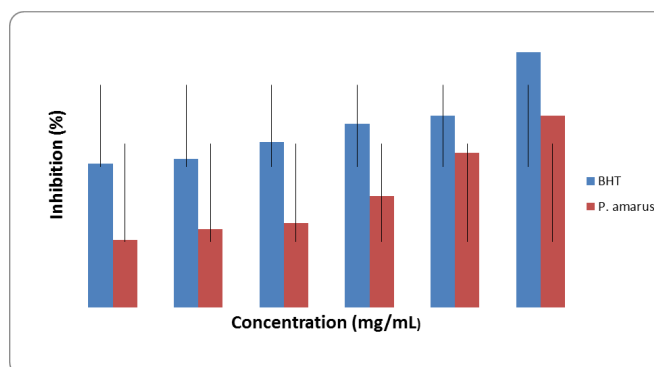


Fig. 2. *P. amarus* scavenging activity on ABTS radical .

lustrated in fig 3 and fig 4 showed that *P. amarus* inhibited α -amylase and pancreatic lipase respectively.

Table 2. Antioxidant activity of *P. amarus*

| Antioxidant Assays | <i>P. amarus</i> |
|--------------------|------------------|
| FRAP(mg RE/g) | 0.132 ± 0.010 |
| TAC (mg GAE/g) | 2.217 ± 0.002 |

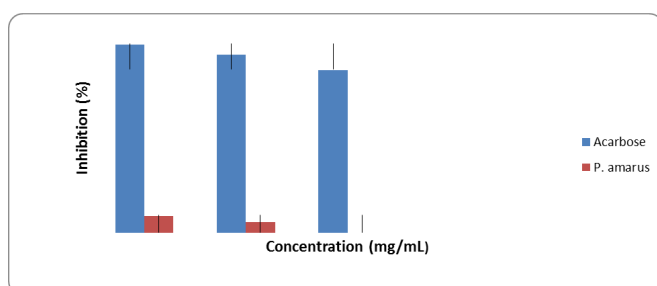
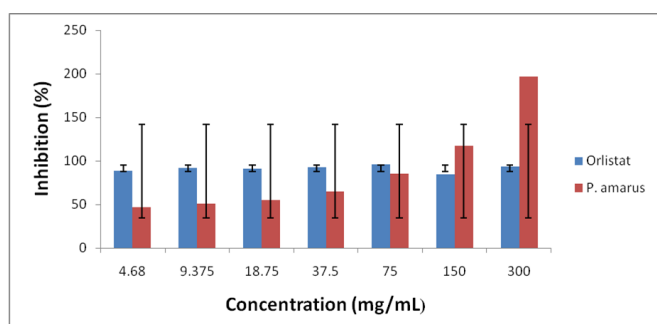
FRAP = Ferric reducing antioxidant power, TAC = Total antioxidant capacity
RE = Rutin equivalent, GAE= Gallic acid equivalent

Table 3. DPPH and Nitric Oxide IC₅₀ values of *P. amarus*

| | DPPH (mg/mL) | NITRIC OXIDE (mg/mL) |
|------------------|--------------|----------------------|
| Ascorbic acid | 0.0003 | 0.0198 |
| <i>P. amarus</i> | 0.0001 | 0.0452 |

Discussion

Medicinal plants are adopted worldwide for the prevention and therapeutic purpose of diseases. A remarkable number of people all over the world are dependent on herbs as drugs (34). *P. amarus* is a common plant and it is used in traditional medicine as a result of its wide-range of medicinal potentialities. *P. amarus* is reported to have various pharmacological activities in both *in vitro* and *in vivo* studies (35).

**Fig. 3.** Inhibitory activity *P. amarus* on α-amylase.**Fig. 4.** Inhibitory activity of *P. amarus* on alpha lipase.

P. amarus is reported to be rich in phytochemicals such as flavonoids, alkaloids, lignans, tannins and phenols (36,37,38). The potent radical scavenging capacity of *P. amarus* is due to the high phenolic content and the presence of other phytochemicals such as flavonoids, tannins, and saponins, as reported in the present study.

Saponins are anti-nutritional factors that reduce glucose and lipid uptake particularly the gut cholesterol *via* physicochemical interaction of intra-lumen. Nwankpa *et al.* (39) reported the presence of saponins in an aqueous extract of *P. amarus*. These phytochemicals have been shown to interact with the key enzymes. Therefore, it can

be concluded that high saponin and other phytochemical contents in the plant may be acting as great inhibitors of α-amylase and pancreatic lipase.

Conclusion

The result in this present study confirmed *P. amarus* to have anti-diabetic and anti-obesity activities. The phytochemicals such as saponin, flavonoids and tannin present in plant leaves are having a great inhibitory effect on α-amylase and α-lipase. This finding can be further validated through clinical trials.

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Authors contributions

AO: Resources investigation, data curation, analysis, methodology, original draft, writing, and analysis; **AB:** Supervision, review, editing; **FI:** methodology, supervision, data curation and analysis; **KA:** review and editing; **OOG:** analysis, methodology, review and editing; **JO:** methodology, supervision, review and editing. **OO:** Conceptualization, analysis, methodology, supervision, review and editing.

Compliance with ethical standards

Conflict of interest: All the authors declared that they have no competing interest related to this article.

Ethical issues: None.

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