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ORIGINAL RESEARCH

In vitro Study of the Inhibitory Potentials of Cold and Hot Aqueous Extract of *Vernonia amygdalina, Calotropis procera,* and *Persea americana* on α-Glucosidase

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Abstract

Background: The surge in the incidence of diabetes mellitus has spurred heightened interest on alternative and complementary therapies, particularly those derived from medicinal plants.
Objective: To assess the α-glucosidase inhibitory activity of cold and hot aqueous extracts of *Vernonia amygdalina* (bitter leaf), *Calotropis procera* (locally known as "bomubomu" leaves), and *Persea americana* (also known as avocado pear) to explore their medicinal applicability.
Method: The leaves of *V. amygdalina, C. procera,* and the seed of *P. americana* were subjected to cold and hot extraction methods using distilled water. These extracts were specifically targeted for their potential inhibitory effects on α-glucosidase activity, a key enzyme involved in carbohydrate digestion.
Results: The α-glucosidase inhibitory assay showed that the extracts from the plants had maximum inhibitory effects at 20 mg/mL. Cold and hot aqueous extracts of *V. amygdalina* exhibited maximal inhibitory activities of 100% and 86%, respectively, at 20mg/mL. Cold and hot aqueous extracts of *C. procera* displayed 100% and 91% inhibitory activities, respectively, at 20mg/mL. Similarly, cold and hot extracts of *P. americana* exhibited the highest inhibitory activities of 77% and 63%, respectively, at 20mg/mL concentration.
Conclusions: The findings demonstrated that cold and hot aqueous extracts of *Vernonia amygdalina, Calotropis procera*, and *Persea americana* exhibited significant inhibitory activities against α-glucosidase. The observed inhibitory effects suggest the therapeutic potential of these plant extracts as natural remedies for managing post-prandial hyperglycaemia in Type 2 Diabetes mellitus.

Key words: a-Glucosidase, Calotropis procera, Diabetes Mellitus, Persea americana, Vernonia amygdalina.

Introduction

Hyperglycaemia is a hallmark of Diabetes mellitus. This chronic endocrine metabolic disease increases the risk of microvascular damage (retinopathy, nephropathy, and neuropathy) as well as macrovascular damage (ischemic heart disease, stroke, and peripheral

vascular disease). [1] Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D) are the two main forms of diabetes mellitus. The autoimmune-mediated loss of pancreatic β cells causes Type 1 diabetes, characterised by a complete lack of insulin, hyperglycaemia, oxidative stress, inflammation, and other metabolic problems. [2] Conversely, Type 2 diabetes, comprising 90–95% of all cases, [3] is characterised by insulin resistance, chronic hyperglycaemia, low-grade inflammation, and dyslipidaemia. [4] Despite the rapid spread of diabetes mellitus, there is currently no established treatment capable of effectively modulating the associated metabolic dysfunctions. The medications used to treat this illness are associated with many side effects such as diarrhoea, flatulence, abdominal discomfort, and liver diseases. [5]

Inhibiting the digestive system's enzymes slows down glucose absorption, which is a therapeutic approach to treating diabetes. [6] Postprandial hyperglycaemia is linked to the activity of the digestive enzyme α-glucosidase, and lowering it may help manage blood glucose levels. Alpha-glucosidase (EC 3.2.1.20) catalyses the hydrolysis of terminal, non-reducing α-1,4-glycosidic linkages of glucose residues from oligosaccharides and disaccharides, producing glucose units as products. [7] Lowering α-glucosidase activity by alpha-glucosidase inhibitors releases less α-glucose and hydrolyses less non-reducing ends of dietary oligosaccharides. [8] This inhibition slows down carbohydrate digestion and the absorption of glucose in the small intestine. This mechanism is crucial in controlling post-prandial hyperglycaemia, representing a modern therapeutic approach to stabilise blood glucose levels, particularly in Type 2 diabetes. [9]

This study evaluated the *in-vitro* inhibitory activity of hot and cold aqueous extracts from three plant species against α-glucosidase: these plants include *Vernonia amygdalina,* commonly known as bitter leaf (Asteraceae family), [10] *Persea americana*, also known as avocado pear (Lauraceae family), [11] and *Calotropis procera,* a wild African bush referred to as apple of Sodom (Asclepiadaceae family). [12] *V. amygdalina* is traditionally used in African medicine for wound healing, anti-inflammatory effects, and the treatment of conditions such as hypertension, malaria, and diabetes mellitus. [13-15] *P. americana* seeds have been traditionally used for medicinal purposes, including treating diarrhoea, dysentery, toothache, intestinal parasites, skin conditions, and for beautification. [16] Various parts of *C. procera* exhibit diverse biological activities such as antimicrobial, [17] antibacterial, [18] antioxidant, [19] and anti-inflammatory effefts.[20]

Methods

Materials

Plant materials

V. amygdalina and *C. procera* leaves were harvested from gardens, while fresh *P. americana* fruits were locally sourced from Ile-Ife, Osun State, Nigeria. These plant materials were identified and authenticated at the herbarium unit of the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Chemicals and Reagents

Sigma-Aldrich, USA, supplied *Saccharomyces cerevisiae* α-glucosidase and p-nitrophenyl glucopyranoside (pNPGP). Hopkin and Williams Chadwell Health, Essex, England, provided the para-nitrophenol, sodium dihydrogen phosphate dihydrate, and disodium hydrogen phosphate dodecahydrate. BDH Chemicals Ltd., located in Poole, England, supplied the sodium carbonate, and Bayer AG Kaiser-Wilhelm-Allee 51368 Leverkusen, Germany, provided the acarbose (Glucobay).

Preparation of Extracts

The preparation of the hot and cold aqueous extracts of *V. amygdalina, C. procera* and *P. americana* involved several steps. Initially, the leaves of *V. amygdalina* and *C. procera*, along with the succulent parts of fresh *P. americana* fruits were thoroughly washed under running water. Subsequently, the leaves and remaining seeds of *P. americana* were sliced into pieces and left to air-dry in the shade. Once dried, the leaves and seeds were finely powdered. Next, 50g of the powder obtained from *V. amygdalina, C. procera,* and *P. americana* were soaked separately in 500ml of both hot and cold distilled water. The mixtures were allowed to stand for 48 hours with intermittent shaking. After this period, the mixtures were filtered using a mesh cloth to remove solid particles. The liquid portions were further processed by centrifugation at 4000 rpm for 20 minutes. The resulting crude extracts were then subjected to oven-drying at a temperature of 45°C, yielding a powdery residue. The residues were carefully stored in a sealed container and refrigerated at 4°C for preservation.

a-Glucosidase Inhibition Assay

Just before usage, 0.1mg of α-glucosidase enzyme was dissolved in 10mL of 20mM phosphate buffer (pH 6.9) to create a new solution of the enzyme. With minor adjustments, the assessment of α-glucosidase inhibition was conducted using the approach outlined by Hong *et al.* [21] Specifically, 100µL of hot and cold aqueous extracts of *V. amygdalina, C. procera,* and *P. americana* were combined with 100µL of phosphate buffer (pH 6.9) at different doses (10, 15, and 20 mg/mL). The mixture was then supplemented with 100 µL of 5mM p-nitrophenyl α-D-glucopyranoside (pNPG). The reaction mixture was gently mixed, and then incubated for 10 minutes at 37°C. After that, 20µL of α-glucosidase solution was added, and the mixture was left to incubate for a further ten minutes at 37°C. Rather than 20µL of α-glucosidase and 20µL of the hot and cold extracts, the blank sample contained 40µL of phosphate buffer. In the control sample, 20µL of the hot and cold extracts were substituted with 20µL of phosphate buffer. Each sample received 100µL of $NaCO_3$ (200mM) to stop the reaction after the 10-minute incubation period. Spectrophotometric measurements of each sample's absorbance were made using Thermoscientific Multiscan (Multiscan Go 1510) at 405 nm. The conventional medications, metformin and acarbose, were formulated using distilled water and these served as positive controls. Using a p-nitrophenol standard curve, the absorbance measurements were translated to the amount of p-nitrophenol emitted, and the following computation was used to find the enzyme activity:

Inhibition (%) = (Absorbance control – Absorbance sample) / Absorbance control X 100

Data Analysis

Every analysis was carried out thrice. The quantitative data was displayed using the standard error mean (SEM) ± mean. The statistical differences ($p<0.05$) between the various inhibitory doses were assessed using a One-Way Analysis of Variance (ANOVA), which was followed by the Tukey HSD post hoc test.

Results

a-Glucosidase Inhibitory Assay

The results of the inhibitory effects of both hot and cold aqueous extracts of leaves of *V. amygdalina, C. procera* and *P. americana* seed on α-glucosidase activity are summarised in Tables I-III and represented in Figures 1-3. The hot and cold aqueous extracts of the different plants demonstrated some inhibitory activities against *α*-glucosidase. Notably, the inhibitory effects of the extracts exhibited a concentration-dependent pattern, showing comparable efficacy with the standard drugs, metformin and acarbose.

In the *V. amygdalina* assay, the cold aqueous leaf extract showed a hypoglycaemic property with inhibitory activity ranging from 90-100% at different concentrations, while the hot aqueous leaf extract had an inhibitory activity that varied from 75-86%. The cold aqueous leaf extract of *C. procera* efficiently inhibited α-glucosidase enzyme *in vitro*, displaying an inhibitory effect of 100% at the highest concentration. The hot aqueous leaf extract had an inhibitory activity of 91%. At the highest concentration tested, the *P. americana* hot aqueous seed extract displayed a substantial inhibitory effect of 63%. The cold aqueous extract exhibited an even higher inhibitory effect of 77%, thus emerging as the most active fraction. Acarbose and metformin, serving as standard drugs, showed slightly higher potency compared to the most active fraction.

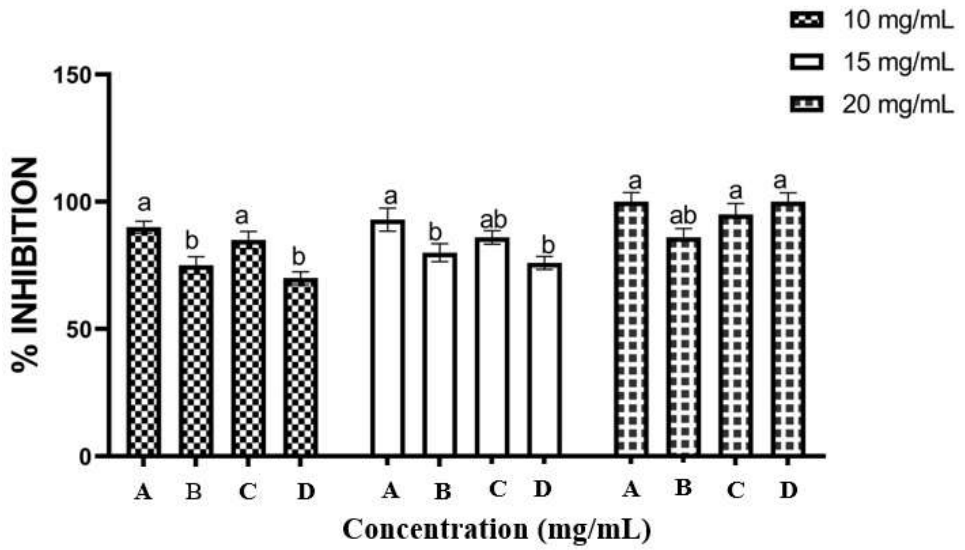

Figure 1: Inhibitory potency of hot and cold aqueous extract of *V. amygdalina* leaf against α-glucosidase activity, compared with the standard drugs, metformin and acarbose.
Legend: Values represent mean ± standard deviation of triplicate readings. Columns with means followed by different alphabets indicate significant differences at $p < 0.05$. A, B, C and D represent *V. amygdalina* cold, *V. amygdalina* hot, Metformin and Acarbose, respectively.

Table I: Alpha-glucosidase inhibitory effects of hot and cold aqueous leaf extract of *Vernonia amygdalina* and the standard drugs, metformin and acarbose

Concentration (mg/ml)	*V. amygdalina* Cold (%)	*V. amygdalina* Hot (%)	Metformin (%)	Acarbose (%)
10	90	75	85	70
15	93	80	86	76
20	100	86	95	100

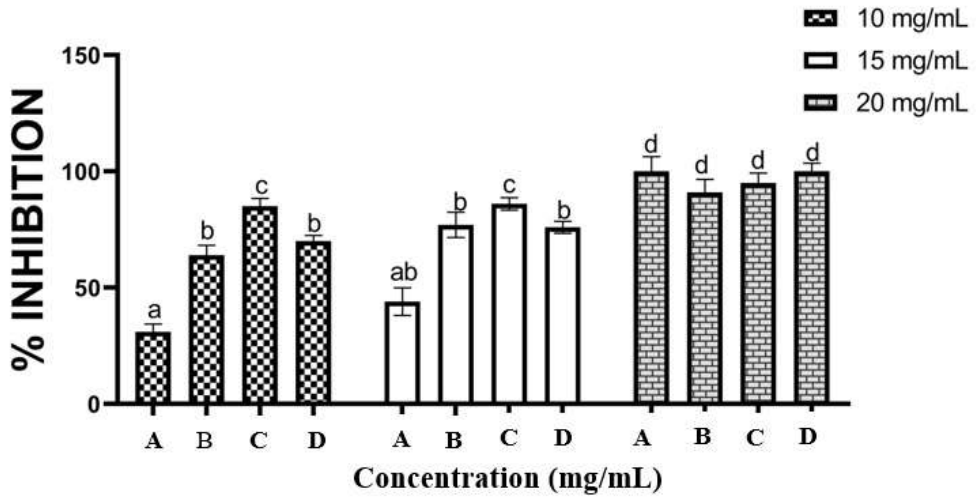

Figure 2: Inhibitory potency of hot and cold aqueous extract of *C. procera* leaf against *α*-glucosidase activity, compared with the standard drugs, metformin and acarbose.
Legend: Values represent mean ± standard deviation of triplicate readings. Columns with means followed by different alphabets indicate significant differences at $p < 0.05$. A, B, C and D represent *C. procera* cold, *C. procera* hot, Metformin and Acarbose, respectively.

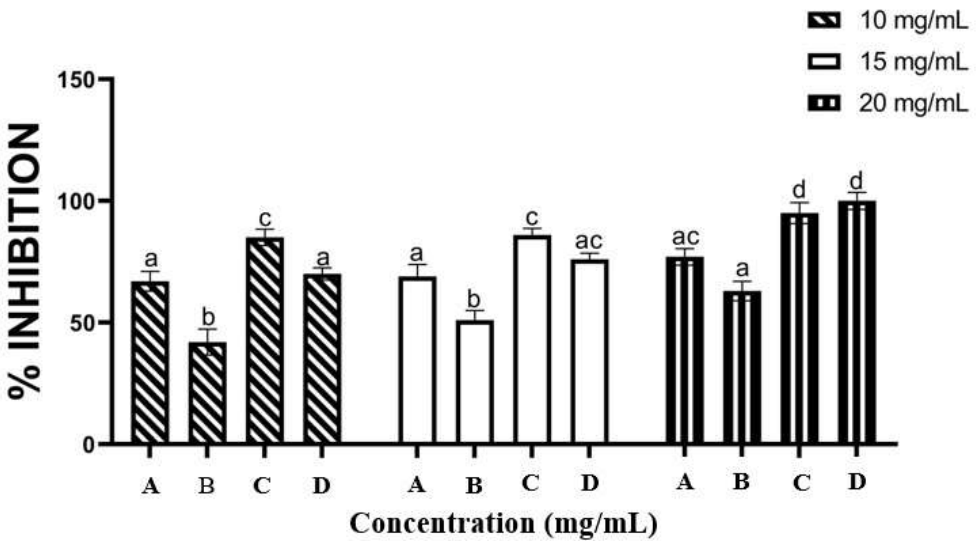

Figure 3: Inhibitory potency of hot and cold aqueous extract of *P. americana* leaf against *α*-glucosidase activity, compared with the standard drugs, metformin and acarbose.
Legend: Values represent mean ± standard deviation of triplicate readings. Columns with means followed by different alphabets indicate significant differences at $p < 0.05$. A, B, C and D represent *P. Americana* cold, *P. Americana* hot, Metformin and Acarbose, respectively.

Table II: Alpha-glucosidase inhibitory effects of hot and cold aqueous leaf extract of *Calotrpis procera* and the standard drugs, metformin and acarbose

Concentration (mg/ml)	*C. procera* Cold (%)	*C. procera* Hot (%)	Metformin (%)	Acarbose (%)
10	31	64	85	70
15	44	77	86	76
20	100	91	95	100

Table III: Alpha-glucosidase inhibitory effects of hot and cold aqueous extract of *Persea americana* seed and the standard drugs, metformin and acarbose

Concentration (mg/ml)	*P. americana* Cold (%)	*P. americana* Hot (%)	Metformin (%)	Acarbose (%)
10	67	42	85	70
15	69	51	86	76
20	77	63	95	100

Discussion

Various strategies have been developed and explored to manage hyperglycaemic conditions, including diabetes mellitus. One significant approach targets post-prandial hyperglycaemia (PPH) by inhibiting α-glucosidase activity, which breaks down oligosaccharides. Reducing post-prandial hyperglycaemia is crucial as it can decrease the formation of advanced glycation end-products (AGEs), a major contributor to cardiovascular issues in diabetes patients. [22] Pharmacological inhibition of α-glucosidase may benefit diabetic patients with impaired insulin response, especially when used alongside other oral hypoglycaemic agents. [23] Combining α-glucosidase inhibitors with insulin, metformin, and sulfonylureas is considered the most effective treatment for uncontrolled hyperglycaemia in diabetics. [24] However, frequent use of these medications can lead to side effects like flatulence, severe abdominal discomfort, and allergic reactions. [25]

Recently, a significant focus has shifted to understanding how herbal extracts with potentials for treating hyperglycaemia in diabetes mellitus work and their chemical compositions.[26] There's also a particular emphasis on studying how phytochemicals found in these extracts inhibit α-glucosidase. The interest in identifying phytoconstituents with pharmacological activity that can inhibit this enzyme stems from the belief that they have fewer side effects and are more cost-effective compared to synthetic drugs like acarbose and metformin. [26, 27]. In this study, we conducted *in vitro* evaluation of the leaves of *V. amygdalina, C. procera* and *Persea Americana* seed, which are traditionally used for managing diabetes mellitus.

Traditional healers commonly employ aqueous macerates of *V. amygdalina* in the empirical treatment of diabetes mellitus. [28] Previous studies have reported the potential anti-diabetic activities of *V. amygdalina.* Asante *et al.* [29] investigated the anti-diabetic effects of ethanolic leaf extracts from both young and old plants against streptozotocin (STZ)-induced diabetes mellitus in mice. Another study by Erukainure *et al.* [30] demonstrated that hot water infusion of *V.*

amygdalina leaves exhibited inhibitory activity against α-glucosidase, reducing intestinal glucose absorption and enhancing muscle glucose uptake. The α-glucosidase inhibitory activity of both hot and cold aqueous leaf extracts of *V. amygdalina* results showed that the cold and hot aqueous extracts of *V. amygdalina* had significant inhibitory effects on α-glucosidase. Interestingly, the cold aqueous leaf extract inhibited the activity of α-glucosidase significantly more, achieving a level of inhibition of 100% at the highest concentration of 20 mg/ml, surpassing the inhibitory effect of the hot aqueous extract. When compared to standard drugs, it exhibited a similar inhibitory effect to the standard drug, metformin. At the highest concentration, there was no significant difference compared with the inhibitory effect of acarbose. The α-glucosidase inhibitory ability of the hot aqueous extract and the standard drug, acarbose, was not significantly different. This observed activity of *V. amygdalina* extracts can be attributed to its phytoconstituents. Luteolin, a bioactive compound isolated from *V. amygdalina* leaves, has been reported to contribute to the plant's alpha-glucosidase inhibitory effect. [31]

The potentials of *C. procera* extracts as an anti-diabetic medication is indicated by their inhibitory effect on α-glucosidase. At lower dosages (10 and 15 mg/ml), the current *in vitro* data showed significant variations in the inhibition of the enzyme between the hot and cold aqueous extracts. At high concentrations, there was no discernible difference between the activities of hot and cold aqueous extracts and efficacy of common medications such as metformin and acarbose, despite the hot extract having somewhat more α-glucosidase inhibitory activity than the cold extract at lower doses. These findings are consistent with those of Kazeem *et al.* [32] who examined the inhibitory properties of several extracts and concluded that the aqueous extract inhibited α-glucosidase *in vitro* most effectively. The phytochemicals—flavonoids, steroids, saponins, and tannins—found in *Calotropis procera* cold and hot aqueous extracts may be responsible for their inhibitory actions on α-glucosidase. Tannins have been shown to cause the translocation of glucose transporter 4 (GLUT4) and phosphorylation of insulin receptors. GLUT4 is a primary mediator of the removal of glucose from the circulation and a critical regulator of glucose homeostasis throughout the body. Furthermore, tannins assist in lowering blood glucose levels without promoting obesity by repressing the primary gene involved in adipogenesis. [33] It has also been demonstrated that flavonoids protect against the advancement of diabetes mellitus by scavenging free radicals in the system, and maintaining β-cell integrity and function. [32]

The seeds of *P. americana* represent more than 16% of the total weight of the fruit. Despite being considered non-edible and under-utilised parts of the fruits, these seeds are rich in phytochemical compounds. [34,35] In the present study, the cold aqueous extract showed a significant increase in the inhibitory potential, reaching 77% at the highest concentration, compared to the 63% observed in the hot aqueous extract. Interestingly, the α-glucosidase inhibitory ability of both hot and cold aqueous extracts differed significantly from that of the standard hypoglycaemic drugs. However, neither plant extract demonstrated superior efficiency in inhibiting α-glucosidase compared to the standard drugs metformin and acarbose. At a concentration of 20 mg/ml, metformin exhibited an inhibitory activity of 95%, while acarbose demonstrated a complete inhibition of 100%. This outcome aligns with the findings reported by Lawal, [36] which showed that the aqueous extract of *P. americana* seed exhibits a noteworthy inhibitory effect on α-glucosidase, reaching its maximum inhibitory activity on α-glucosidase at 56.41% and acarbose at 76.41%. Studies conducted recently have also demonstrated a significant decrease in blood glucose levels in rats

with diabetes mellitus induced by alloxan and treated with crude aqueous extract of *P. americana* seed. [37] Furthermore, it has been found that the crude aqueous extract of *P. americana* seed inhibits α-glucosidase considerably.[1] Certain phytoconstituents, such as cis-11,14-eicosadienoic acid, catechin, and chlorogenic acid that were detected in the GC-MS analysis of the *P. americana* seed aqueous extract have been reported to have hypoglycaemic potentials and to have inhibitory effects against α-glucosidase. [36]

Overall, the study suggests that both the cold and hot extracts of the plants exhibited a significant inhibitory effect on α-glucosidase, which indicates their potential hypoglycaemic effect. Remarkably, the cold aqueous extracts demonstrated higher activity compared to the hot extracts.

Conclusion

This study demonstrates the inhibitory activities of the extracts from *V. amygdalina, C. procera*, and *P. americana* on α-glucosidase activity, indicating their potential effectiveness in patients affected by chronic hyperglycaemia. The study also suggests that the extracts may be used as a possible hypoglycaemic drug. Therefore, further studies are needed to ascertain the possible contributions of the extracts to the management of diabetes mellitus. The present data suggest that these extracts can be developed as effective and economical pharmacological support for diabetes mellitus. This strategy can be used to design novel drugs to manage or treat diabetes mellitus.

Authors' Contributions: FBS and OKT conceived and designed the study. APO carried out the laboratory procedures; SON did the literature review. All the authors analysed and interpreted the data. FBS and OKT drafted the manuscript and revised it for sound intellectual content. All the authors approved the final version of the manuscript.
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