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Alpha Amylase Inhibitory and Antioxidant Activities of Leaf Extract and Fractions of *Sorindea warneckei* Engl. (Anacardiaceae)

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ABSTRACT

Postprandial hyperglycemia is characteristic of type 2 diabetes. The development of alternative medicines containing secondary metabolites with ability to delay or prevent glucose absorption will be a great therapeutic approach in the management of diabetes. The aim of the present study was to evaluate the *in-vitro* α-amylase inhibitory and antioxidant activities of the leaf extract and fractions of *Sorindea warneckei.* The antidiabetic activity of the extract and its fractions were examined by α-amylase bioassay. The antioxidant activity was measured using 2, 2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging and reducing activities. The HPLC analysis, total phenol and total flavonoid contents were also determined. The extract demonstrated substantial concentration-dependent α-amylase inhibitory (IC_{50} = 400 µg/mL) and DPPH radical scavenging (IC_{50} = 40 µg/mL) activities. The ethyl acetate fraction showed the highest activity among the fractions with highest phenolic and flavonoid contents. Statistical analysis showed that total phenolic and flavonoid contents had significant ($p < 0.05$) effect on α-amylase inhibitory and antioxidant activity. HPLC analysis revealed the presence of quercetin, rutin and gallic acid in the active ethyl acetate fraction. This study shows that ethanol leaf extract of *Sorindea warneckei* exhibits significant α-amylase inhibitory and antioxidant activities which localize mainly in the ethyl acetate fraction. The plant is a veritable source of α-amylase inhibitors.

Keywords: *Sorindea warneckei, α*-amylase inhibition, antioxidant, phenols, flavonoids, HPLC analysis.

Introduction

Diabetes mellitus (DM) is a chronic endocrine disorder characterized by hyperglycemia and disruption of carbohydrates, protein and fat metabolisms due to defects in insulin secretion, insulin action or both.[1,2] The increase in incidence of the disease constitutes a global public health concern and according to WHO, 366 million people was estimated to have DM in 2011 and it is likely to rise to 552 million or more by 2030.[3] In the development of type 2 diabetes, postprandial hyperglycemia plays a key role. Thus, its management can be achieved by decreasing blood glucose rise after meal by delaying the activities of the enzymes α-amylase and α-glucosidase which are responsible for the digestion of carbohydrates and absorption of glucose in the digestive tract.[4] Postprandial hyperglycemia depends mainly on absorbed monosaccharides and velocity of absorption in the intestine through the activities of these carbohydrates metabolizing enzymes.[5,6] Recently, emphasis has been placed on complementary and alternative medicine especially medicinal plant treatments for diabetes due to unwanted side effects of currently available synthetic derivatives.[7,8]

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Over a thousand plant species are being used in herbal medicine for the management of diabetes.[9] Medicinal plants with antidiabetic activity have different mechanisms of action-mimic insulin, act on insulin secreting beta cells or modify glucose use. Plants that control glucose use work by changing the viscosity of gastrointestinal contents, delaying gastric emptying or glucose absorption.[10]

Sorindea warneckei Engl. (Anacardiaceae) is a scandent shrub, understory or jungle tree from Sierra Leone to Southern Nigeria. It is also found in Western and Southern parts of Cameroon. The plant is used to make blue colour tattooing and also used as chewing stick in Western Nigeria. Its fruits are edible and can be made into juice.[11,12] Extract of the plant exhibited strong activity against broad spectrum of bacteria including medically and dentally relevant bacteria. It showed potent activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, multidrug-resistant *Burkholderia cepacian* and *Pseudomonas aeruginosa*.[13] Various plants in the Anacardiaceae family have been reported to have antidiabetic activity.[14] In Type 2 diabetes, hyperglycemia induces generation of free radicals including reactive oxygen species (ROS), hydroxyl and nitric oxide (NO) radicals which are responsible for oxidative stress induced pancreatic ß-cell destruction as well as the activation of all major pathways underlying the different components of chronic vascular diabetic complications such as glycation and sorbitol pathways among others.[15,16] The foregoing, thus suggest the need for agents with potent antioxidant properties that could decrease postprandial hyperglycemia . Keeping these facts in mind, the present study was designed to evaluate the *in-vitro* α-amylase inhibitory and antioxidant activities of the leaf extract and fractions of *S. warneckei*.

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Materials and Methods

Collection of plant material
The leaves of *S. warneckei* were collected in May 2016 from Iwere-Ile, Oyo, Nigeria and identified by Mr. Tola Oyebanji, a plant taxonomist in the Herbarium Unit, Department of Botany, University of Lagos, Lagos after comparing with voucher specimen no LUH 7532.

Extraction and fractionation of plant material
The air-dried leaves of *S. warneckei* (640 g) were powdered and macerated three times, each for 24 h using 1.5 L of absolute ethanol (EtOH). The combined extracts were filtered and concentrated to dryness using rotary evaporator. About 30 g of the dried extract was then dispersed in methanol: water (1:9) and fractionated gradient-wise three times with 400 mL each of n-hexane, ethyl acetate, and n-butanol respectively. Each fraction was also concentrated to dryness and kept in the freezer until use.

Preliminary phytochemical screening
The ethanol leaf extract and fractions of *S. warneckei* were subjected to chemical test to identify various classes of bioactive compounds present in the samples using standard methods.[17,18]

α-Amylase inhibitory activity
The amylase inhibition assay was performed using previously described method.[19] A total of 0.5 mL of various concentrations of the leaf extract of *S. warneckei* (20-2000 µg/mL) and 0.5 mL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing porcine pancreatic α-amylase (0.5 mL, Sigma Chemical Company, MO) were mixed at room temperature (25^{o}C) for 10 min, then 0.5 mL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each test tube at timed intervals. The reaction mixtures were incubated for 10 min. The reaction was then stopped with 1.0 mL of dinitrosalicylic acid (DNS) color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled. The mixture was diluted with 10 mL distilled water and absorbance measured at 540 nm. The negative control experiment was carried out with 1% dimethylsulphoxide (DMSO) used as solvent in place of extract. The positive control experiment followed the same procedure using acarbose (20-200 µg/mL). The above procedure was repeated for various fractions in triplicate separately at 1000 µg/L. The α-amylase inhibitory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{Abs_{control} - Abs_{test}}{Abs_{control}} \times 100$$

Antioxidant activity of S. warneckei leaf extract
DPPH radical scavenging activity
Free radical scavenging activity of the extract was measured using DPPH assay as in Adesegun *et al*.[20] The plant extract (20-1000 µg/mL) at different concentrations (1.0 mL) was introduced into test tubes and 3.0 mL ethanol and 1.0 mL of freshly prepared ethanolic solution of 1.0 mM DPPH was added. The mixtures were shaken and allowed to stand for 30 min and the absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Each of the experiment was done in triplicate. The positive control experiment followed the same procedure using ascorbic acid (5-200 µg/mL). The above procedure was repeated for various fractions in triplicate separately at 100 µg/mL. The antioxidant activity of the extract and its fractions were expressed in percentage inhibition of DPPH and was calculated using the following equation:

$$\%\,\text{DPPH Scavenging Activity} = \frac{Abs_{control} - Abs_{test}}{Abs_{control}} \times 100$$

Reducing power activity
The reducing power of the sample was determined by the method of Fejes *et al*.[21] Various concentrations 20-1000 µg/mL) of the extract (2 mL) were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 mL of potassium ferricyanide (10 mg/mL). The mixture was incubated at 50^{o}C for 20 min followed by addition of 2 mL trichloroacetic acid (0,1 g/mL). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution. A volume of 2 mL from each of the mixture was then mixed with 2 mL of distilled water and 0.4 mL of 0.1% fresh ferric chloride. After 10 min of reaction, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates a higher reducing power. The positive control experiment followed the same procedure using butylhydroxytoluene (BHT) (20-1000 µg/mL). The above procedure was repeated for various fractions in triplicate separately at 100 µg/mL.

Determination of total phenols
The total phenolic content of the extract was determined spectrophotometrically using Folin-Ciocalteu's reagent.[22] The ethanolic extract (0.5 mL, 1 mg/mL) was mixed with 5 mL Folin-Ciocalteu's phenol reagent, kept for 4 min and 4 mL (75 g/L) sodium carbonate was added to the reaction mixture then kept in a dark room for 1 h. The absorbance was measured at 765 nm using UV-spectrophotometer. A calibration graph was prepared using gallic acid as standard and the total phenolic content in the sample was expressed as milligrams of gallic acid equivalent (GAE) in one gram of sample. Extract and fractions were analyzed in triplicates.

Determination of total flavonoid content
The total flavonoid content was estimated by aluminum chloride method.[23] To 1 mL of sample (1 mg/mL), 5 mL of water and 0.3 mL of $NaNO_2$ (5%) were added. After 5 min, 0.6 mL of $AlCl_3$ (10%) was added followed by 2 mL of NaOH (1 M). The final volume was made up to 10 mL with H_2O and the solution was well mixed. The absorbance was read at 510 nm. Quercetin was used as the standard. The total flavonoid content was expressed as milligrams of quercetin equivalent (QE) in one gram of sample extract and fractions were analyzed in triplicates.

HPLC analysis
The analysis was carried out on the active ethyl acetate fraction using Agilent technologies HPLC 1100 series with a suitable analytical column with stationary phase Zorbax eclipse XDB RP C8 (150 x 4.6 mm, 5µm) with UV detection, set at 257 nm. The injection volume was 20 µL for each technical repeat. The analysis was performed with a column temperature and flow rate of 40^{o}C and 1.0 mL/min, respectively using two mobile phases, solvent A (CH_3CN) and solvent B (H_2O with 0.2% acetic acid). The isocratic elution of the mobile phases (A:B, v/v) was programmed as 50:50 at 30 min. The standard stock solution (1000 µg/mL) was prepared by dissolving each standard compound in MeOH and preserved at less than 4^{o}C. As standard compounds, quercetin, rutin, ferulic acid and gallic acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). The working standard solution was prepared by serial dilutions of the standard stock solution.

Statistical analysis
All results are expressed as mean ± standard error (SE) and differences between means were statistically analyzed using one-way analysis of variance (ANOVA) followed by Turkey's multiple range post-hoc test, with $p < 0.05$ considered to indicate statistical significance.

Results and Discussion

Diabetes is a major health challenge affecting population worldwide. Management of blood glucose level is an important method of controlling diabetes and its complications. One approach for reducing postprandial hyperglycemia is to minimize dietary carbohydrate digestion which involves enzymes like α-amylase and α-glucosidase. Alpha amylase inhibitors belong to one of the classes of antidiabetic drugs and are commonly used in the treatment of type-2 diabetes but causes gastrointestinal side effects. However, there is an increasing interest in the use of plant-derived hypoglycemic agents for the

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management of this disease due to easy accessibility and lower negative effects.[24] Bioactive compounds from these plants may also be a veritable source of new α-amylase inhibitors.

In this study, the average yield of ethanol extract from solvent extraction of 650 g powder leaves of *S. warneckei* by maceration technique was found to be 70.0 g (10.93%). The fractionation of the crude extract produced different amounts of hexane, ethyl acetate, butanol and residual aqueous fractions as shown in Table 1. Phytochemical investigations showed the presence of steroidal compounds, terpenoids, saponins, phenolic compounds and flavonoids in the leaf extract. Cardiac glycosides, alkaloids, and anthraquinones were not detected. The phytochemical constituents of the fractions are shown in Table 2. These compounds present in the extract may have contributed to the observed activities.

The α-amylase inhibitory potential of *S. warneckei* leaf extract and acarbose are shown in Figure 1, The result indicates that the extract demonstrated significant ($p < 0.05$) concentration-dependent α-amylase inhibitory potential with IC_{50} value (400 µg/ml) which was found to be significantly is less than that of acarbose (40 µg/mL) a known α-amylase inhibitor (Figure 1). The α-amylase inhibition of various fractions (1000 µg/mL) was ranked as follows: ethyl acetate > butanol > residual aqueous > hexane (Figure 2). The most potent inhibitor among the fractions was ethyl acetate fraction (71.13%) (Figure 2) and its activity was significantly less ($p < 0.05$) than that of acarbose at the same concentration. Thus, they are starch blockers as they prevent or slow the absorption of starch into the body by preventing the cleavage of 1, 4-glycosidic linkages of starch and other oligosaccharides. This finding suggests that the constituents responsible for the α-amylase inhibition localize mainly in the ethyl acetate fraction.

Antioxidants fight free radicals and protect the body system from various diseases. They act either by scavenging reactive oxygen species or protecting antioxidant defense mechanisms.[25]

Table 1: Amount and % yield of fractions from *S. warneckei* leaves.

Extract	Amount (g)	Yield (w/w)
Hexane	13.3	0.020%
Ethyl acetate	29.86	0.045%
Butanol	4.66	0.007%
Aqueous	11.2	0.017%

Epidemiological, clinical and experimental studies have indicated a relationship between oxidative stress and low-grade inflammation in the development of type-2 diabetes.[26] Also, the production of reactive oxygen species (ROS) was reported to increase due to insulin resistance and hyperglycemia.[27] Various methods are commonly used to assess antioxidant potential in-vitro for rapid screening of substances. Low antioxidant activity in-vitro has been reported to be an indicator of little or no activity *in vivo*.[28]

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution.[20] The technique involves the scavenging of DPPH through the introduction of radical species that decolorizes the DPPH solution. The change depends on the concentration and potency of the antioxidants. A decrease in absorbance of the reaction mixture shows free radical scavenging ability of the sample under test[29]. The *S. warneckei* extract produced a concentration-dependent inhibition of DPPH induced oxidation (IC_{50} = 40 µg/mL) which was significantly less ($p < 0.05$) than that of ascorbic acid (20 µg/mL) (Figure 3). The scavenging effect of the fractions at 100 µg/mL was in the order ethyl acetate > butanol > hexane > residual aqueous (Figure 4). Among the fractions tested, ethyl acetate and butanol fractions showed significant ($p < 0.05$) percentage inhibition of DPPH and positively correlated with total phenolic and flavonoid contents. These findings are in agreement with earlier report which suggested antioxidant activity of phenolic compounds.[30] This suggests that extract and fractions of *S. warneckei* contain plant secondary metabolites such as phenols, flavonoids, as well as saponins (Table 2) which have ability to discolour DPPH solution by their hydrogen donating ability.

In the reducing power assay, presence of reductants in the sample leads to reduction of Fe^{3+}/ferricyanide complex to ferrous form thus Fe^{2+} can be monitored by measurement of absorbance at 700 nm. Increase in absorbance indicates an increase in reducing ability. The reducing properties have been reported to produce antioxidant effect by donating hydrogen atom to quench the free radical chain.[20] Figure 5 shows the concentration response curves for the reducing powers of *S. warneckei* leaf extract and BHT used as positive control. It was found that the reducing power increased with concentration but the activity of the extract was significantly ($p < 0.05$) less than that of BHT. The ranking order for reducing power of fractions was ethyl acetate > butanol > hexane > residual aqueous. Significantly high ($p < 0.05$) reducing power (1.52 ± 0.16 at 100 µg/mL) was evident in ethyl acetate fraction (Figure 6). The antioxidants in extract and fractions of *S. warneckei* produced reduction of Fe^{3+}/ferricyanide complex to ferrous form.

Table 2: Phytochemical content of ethanol extract and fractions of *S. warneckei* leaf.

Phytochemical test	Crude Extract	Hexane fraction	Ethyl acetate fraction	Butanol fraction	Aqueous fraction
Alkaloids	-	-		-	-
Anthraquinones	-	-	-	-	-
Phenolic compounds	+	+	+	+	+
Flavonoids	+	_	+	+	+
Saponins	+	+	+	+	+
Terpenoids	+	+	+	-	-
Cardiac glycosides	-	-	-	-	-

+ Detected; - Not detected

Table 3: Total phenolic and flavonoid contents of extract and fractions of *S. warneckei* leaf.

Test	Extract	Hexane	Ethylacetate	Butanol	Aqueous
Total phenolics	41.24 ± 1.18	23.69 ± 0.21	61.12 ± 0.84	46.55 ± 4.35	35.8 ± 4.34
Total flavonoids	25.54 ± 0.47	6.84 ± 0.36	30.07 ± 1.19	24.94 ± 0.63	15.98 ± 0.76

Data are expressed as mean ± SEM, n = 3

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Plant phenolics are one of the most abundant plant secondary metabolites and are known for their antioxidant and radical scavenging activity and control oxidative stress-related degenerative diseases.

There is an increasing demand for medicinal plants rich in phenolic compounds in food industry due to their ability to reduce oxidative damage of lipids thus improving the quality and nutritional value of food.[31] The total phenolic content (TPC) of the extract and fractions were expressed in terms of Gallic acid equivalents (GAE) (Table 3). The TPCs were calculated using linear regression equation obtained from the standard plot of gallic acid.

$y=26.78x + 0.198$, $r^2= 0.994$ Where, y =absorbance; x= amount of gallic acid (mg)

The results revealed a comparative rate of polyphenols in the extract, hexane, ethyl acetate, butanol and aqueous residue fractions in terms of GAE (Table 3). The total flavonoid content (TFC) of the extract and fractions were reported in terms of quercetin equivalent (QE) (Table 3). The TFC was calculated using regression equation obtained from standard plot of quercetin; $y = 0.993x + 0.006$, $r^2= 0.996$ where, y =absorbance; x = amount of quercetin (mg)

A significant difference ($p < 0.05$) in the phenol and flavonoid contents separately in the extract and fractions was observed.

The crude extract of *S. warneckei* demonstrated high phenolic content whereas among the fractions, ethyl acetate exhibited highest total phenolic content. Plant phenolics have been shown to be effective scavengers of oxidizing molecules including singlet oxygen and various free radicals implicated in many diseases including cancer, diabetes, inflammation and allergy. The results from this study suggested that phenolics and flavonoids may be the major contributors to the α-amylase inhibition and antioxidant activity of the crude extract and active fractions. The study also revealed a significant ($p < 0.05$) correlation between TPC and TFC with α-amylase inhibitory activity of the samples ($r^2= 0.7884$ and 0.7698, respectively). This is in agreement with previous studies which indicated that phenolic rich samples exhibited high antioxidant and α-amylase inhibitory activities.[32] The results also revealed a direct correlation between antioxidant and α-amylase inhibitory activities ($r^2 = 0.7208$). Higher phytochemical constituents (Table 2) and antioxidant activity significantly influenced the α-amylase inhibition of the samples ($p < 0.05$).

Reversed phase HPLC was used to separate individual chemical constituents of most active ethyl acetate fraction of *S. warneckei* leaf extract. The chromatographic profile was compared with the retention times of reference standards used (Figure 7). In reversed phase systems, more polar compounds are eluted from the column faster than less polar compounds. Thus, peaks at the beginning of the chromatogram indicate less polar ones. The chromatographic profile revealed that the ethyl acetate fraction had high content of polar molecules and showed peaks corresponding to gallic acid (56.94% from total peak area, retention time 3.01 min), rutin (41.59% from total peak area, retention time 2.21 min), quercetin (0.93% from total peak area, retention time 15.37 min) while ferullic acid was not detected.

The HPLC analysis revealed that rutin is one of the active components present in the ethyl acetate fraction of *S. warneckei* (Figure 7). Previous reports showed rutin exhibited significant antidiabetic activity by reduction of carbohydrates absorption from small intestine, inhibition of tissue gluconeogenesis, increase in tissue glucose uptake, stimulation of insulin secretion from beta cells and protection of Langerhans islet against degeneration.[33]

The results also indicated the presence of gallic acid in the ethyl acetate fraction. It has been reported that phenolic compounds including gallic acid played a significant role in prevention of chronic diseases including diabetes. The antidiabetic action of gallic acid was reported to be due to enhancement of insulin receptor sensitivity and antioxidant activity.[34]

Quercetin a flavonoid with antioxidant properties was also present. Its oral supplementation has been shown to improve the antioxidant status and prevent oxidative damage, thereby promoting the regeneration of pancreatic islets and increasing insulin release in diabetes-induced rats. It is also beneficial in normalizing blood glucose levels, segmenting liver glycogen content and enzyme and reducing serum cholesterol.[35]

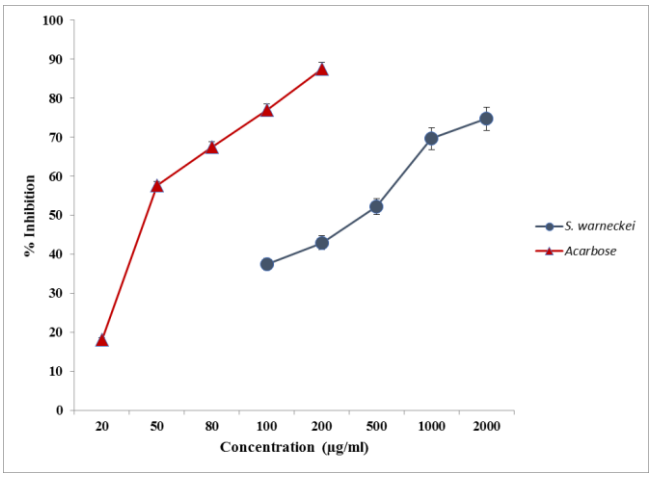

Figure 1: Alpha amylase Inhibitory Activity of Ethanol Extract and Acarbose.

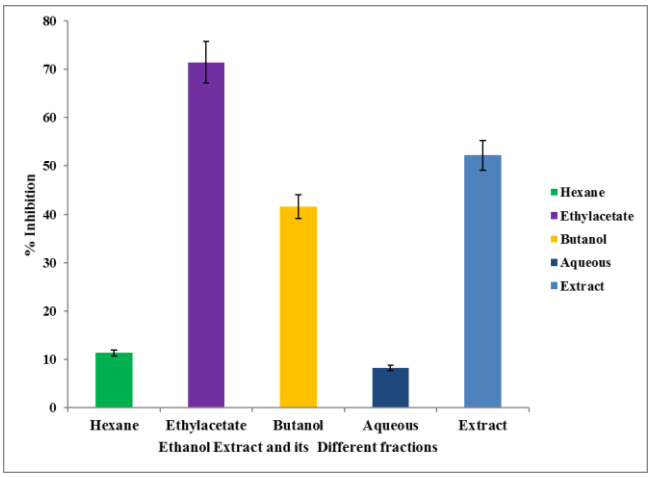

Figure 2: Alpha Amylase Inhibitory Activity of extract and its Fractions.

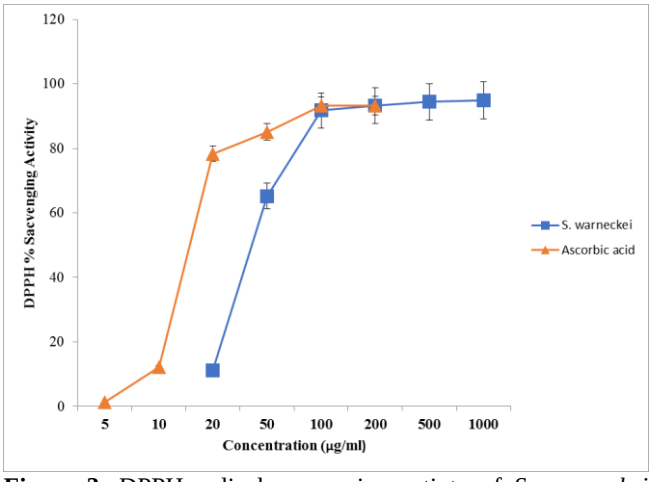

Figure 3: DPPH radical scavenging activty of *S. warneckei* leaf extract.

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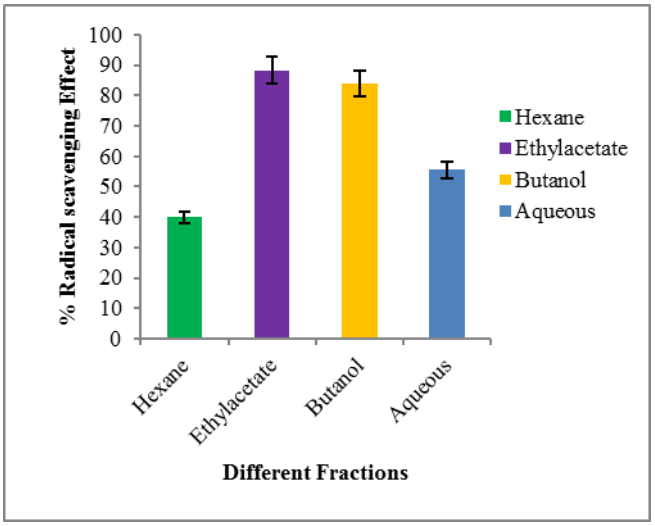

Figure 4: DPPH radical scavenging activity of *S. warnekei* fractions (1000 µg/mL).

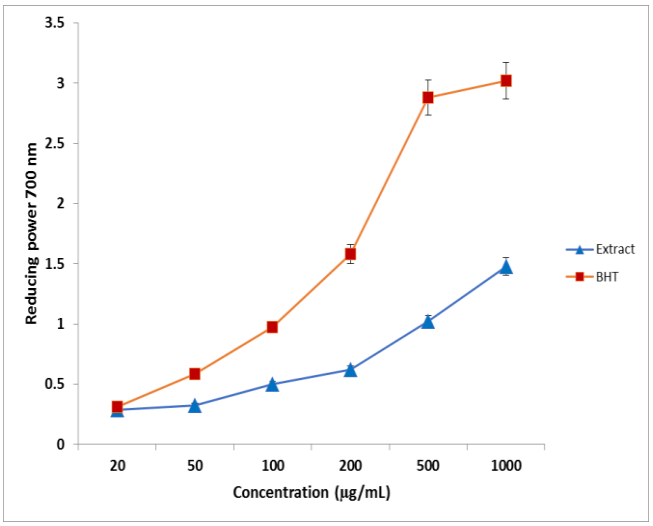

Figure 5: Reducing power of *S. warnekei* leaf extract.

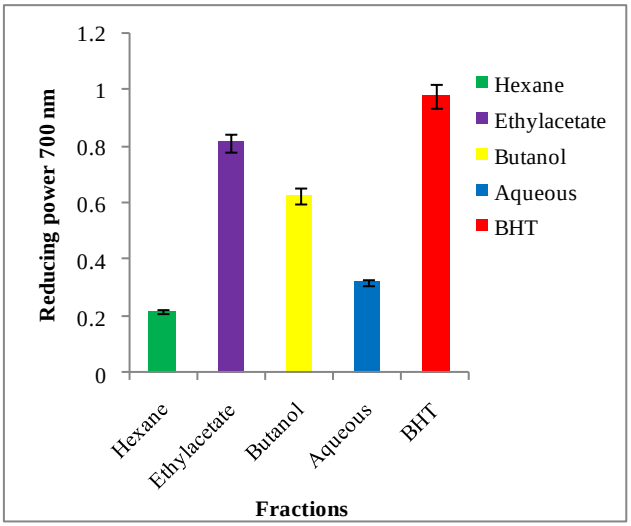

Figure 6: Reducing power of *S. warnekei* fractions (1000 µg/mL).

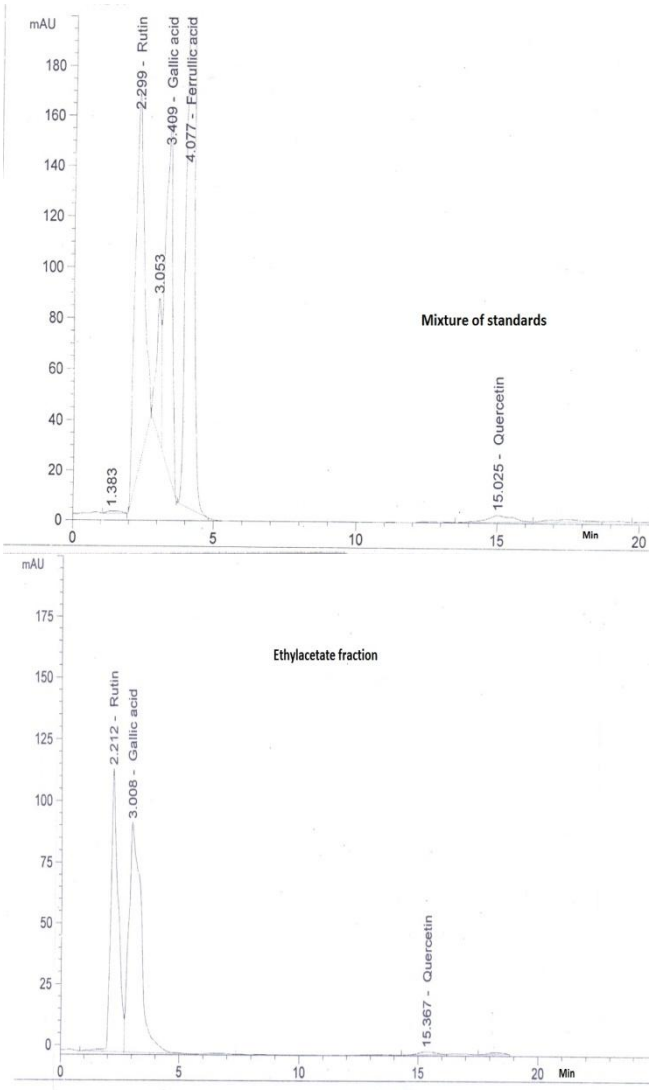

Figure 7: HPLC chromatograms of mixture of standards and ethyl acetate fraction of *S. warneckei* leaf.

Conclusion

In this study, the ethanol extract of *S. warneckei* was found to possess potent amylase inhibitors and antioxidant activity which may be due to phenolic compounds that localize mainly in the ethyl acetate fraction. The findings thus confirm that the leaves of *S. warneckei* can mitigate postprandial hyperglycemia and ameliorate oxidative stress and therefore assist in combating diabetic complications. However, further research on the extract and fractions could be carried out in order to confirm these results in vivo and to isolate, identify, and characterize the active compounds from the plant.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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