

In Vitro Combined Effect of *Annona Senegalensis* and *Piliostigma Thonningii* Leaf Extracts on Alpha Amylase Activity

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Abstract

Background: Diabetes mellitus (DM), a group of metabolic disorders characterised by hyperglycaemia, has been attributed to increased activity of α -amylase, one of the key enzymes in carbohydrate metabolism. Alpha amylase inhibition can potentially control postprandial hyperglycaemia, hence significant in the management of diabetes mellitus, especially type 2. Acarbose, the proven α -amylase inhibitor, has been associated with several side effects. Plants used in traditional medicine represent a valuable source in the search for an alternative. The study aimed at determining the combined effect of *Piliostigma thonningii* and *Annona senegalensis* leaf extracts on alpha amylase activity.

Methodology: Mortar and pestle were used to crush the leaves into powder. The fine powder was dispensed in distilled water. Percolation was used to filter the crude extract, evaporated in a water bath at 65 °C to concentrate the extract then reconstituted. Spectrophotometric assay method was used for alpha amylase activity at 540 nm.

Results: The results of the study showed that *A. senegalensis* inhibited α -amylase by 27%, 32% and 34% at 15, 30 and 45 minutes respectively. *P. thonningii* exhibited inhibitory effects on α -amylase by 25%, 27% and 37% at 15, 30 and 45 minutes respectively. The combined inhibitory effects of *A. senegalensis* and *P. thonningii* on α -amylase were 28%, 37% and 62% at 15, 30 and 45 minutes respectively which are significantly different ($p < 0.05$) from that of the positive control, acarbose (71% at 45 minutes)

Conclusion: This study revealed the increased strength to inhibit alpha amylase enzyme by a combination of *A. senegalensis* and *P. thonningii* and a proportional increase in the inhibitory effect with increase in incubation time than their individual potential to inhibit alpha amylase.

Keywords: Type 2 Diabetes Mellitus (T2DM), Carbohydrate Metabolism, Hyperglycaemia, Acarbose, α -Amylase Inhibitor, Extraction, Traditional Medicine, Anti-Diabetics.

Introduction

According to Adjakpa, *et al.*, 80% of the whole world's population and 90% in the developing world use medicinal plants for primary health care. Common advantages of herbal drugs are effectiveness, safety, low costs and acceptability. Modern medicine fails to cure some diseases such that they are traditionally treated, making herbal medicine an inescapable science [1, 2].

Diabetes mellitus (DM) is a group of metabolic disorders characterised by increased blood glucose levels resulting from either

the pancreatic failure to produce enough insulin or the abnormal response of the body's cells to insulin [3, 4]. It is one of the worldwide rapidly growing health problems and one of the leading causes of mortality. About 422 million DM cases were reported in 2014 and 1.5 million deaths per year [5, 6], accounting for 76% of diabetes deaths in the African continent, more especially under the age of 60 years as of 2013 data [7].

Two main types of DM include type 1 (T1DM) and type 2 (T2DM) diabetes mellitus. The former is caused by absence of insulin pro-

duction resulting from auto-immune mediated destruction of the beta cells of Langerhans in the pancreas while the latter is caused by the deficiency in the action of insulin (8, 9). DM reduces life expectancy, and is associated with diabetic ketoacidosis and other nonketotic syndromes [8, 5]. The mechanisms of anti-diabetics include: reducing carbohydrate absorption, α -glucosidase, α -amylase, and aldose reductase activity; increasing glucose uptake in muscle and adipose tissues & insulin sensitivity; stimulating β -cell insulin secretion; and inhibiting hepatic gluconeogenesis or glycolysis [10-12].

Postprandial hyperglycaemia is known to increase the risk of development of several health disorders [13]. Inhibition of the enzymes responsible for carbohydrate degradation, α -glucosidase and α -amylase, can therefore treat T2DM. This delays carbohydrate digestion and prolong overall carbohydrate digestion time, hence reducing glucose absorption rate and consequently slowing the postprandial plasma glucose rise [10, 2].

Excess activity of alpha-amylase is one of the leading factors to development of chronic postprandial hyperglycaemia. Ishnava & Motisariya, indicated that there is a positive correlation between human pancreatic α -amylase activity and the increase in postprandial glucose levels, demonstrating the relevance of suppressing postprandial hyperglycaemia in the T2DM through reduction or inhibition of pancreatic α -amylase activity (10). Taha, *et al.*, considers alpha-amylase inhibitors as the most important tool to control T2DM (14). By inhibiting this enzyme, the rate of absorption of sugars will be slowed down, thereby maintaining homeostatic blood glucose levels due to the inhibited starch digestion [15].

Acarbose has been proven to be an α -amylase inhibitor. However, it has been associated with several side effects, of which bloating, abdominal discomfort, diarrhoea and flatulence have been reported as the specific adverse effects experienced by the patients using the α -amylase inhibitors [8, 16, 17]. Hence a need to look for alternatives, and plants used in traditional medicine as new medicinal compounds [18].

Many plants use alpha amylase inhibitors (AAIs) as a protective mechanism from insects by altering the digestive action of alpha amylases and proteinases in their gut and inhibit their normal feeding behaviour. Thus, AAIs have potential roles in controlling blood sugar levels, and plants are their good source [19, 20]. The phytochemicals found in different plants provide health benefits to mankind. They have antioxidant, anti-inflammatory, anti-cancer and anti-bacterial properties, anti-diarrheal, analgesic, and wound healing activity [21, 22].

Piliostigma thonningii is a leguminous plant belonging to the sub-family of Caesalpiniaceae in the legume family, *Leguminosae/Fabaceae*. It is also known as “Camel’s foot”, “Monkey bread”, “wild bauhinia”. Commonly known as “*Chitimbe*” in Malawi, the tree is perennial in nature and its petals are white to pinkish coloured, produced between November and April. It grows to

a height of 8 metres with branches. It has large bi-lobed simple leaves without thorns or spikes [23, 24]. Its edible and chewable leaves are believed to relieve thirst. It is used for the treatment of typhoid fever, ulcers, wounds, heart pain, arthritis, malaria, pyrexia, leprosy, sore throat, diarrhoea, toothache, gingivitis, cough, and bronchitis. Its roots and twigs are used in the treatment of dysentery, fever, wound infections, cough, and skin diseases. *P. thonningii* has also been reported to possess antilipidemic, antibacterial, antihelminthic, analgesic, antipyretic, antioxidant, anti-inflammatory, and antidiabetic activities, and being an anti-snake [23, 24].

Annona senegalensis is commonly known as African custard-apple, wild custard apple, and wild soursop, and in Malawi, commonly known as “*Mpoza*”. A species of flowering plant in the custard apple family, *Annonaceae*, its specific epithet, *senegalensis*, translates to mean “of Senegal,” where it was collected. At an early stage, *A. senegalensis* fruits look dark green, and yellow when ripe and finally turn orange during the later stage of life. The edible white pulp of the ripe fruit has a pleasant, pineapple-like aroma with the flavour of apricots. Its leaves, roots and root barks have been used on different ailments [1, 25, 26]. It is used as food, medicine, and a range of commodities for the local people. The shrub is widely found in semi-arid to sub-humid all over the parts of Africa, growing up to 7 metres, but it is not resilient in nature. It is cultivated for its leaves, fruits, flowers, bark, and stem for medicinal purposes. The species occurs along riverbanks, fallow land, and swamp forests, and at coasts. It commonly grows as a single plant among the savannah woodlands. It has a curved inner whorl around the stamens and ovary and with several stamens [25, 27]. *P. thonningii* and *A. senegalensis* have individually been locally used as potential anti-diabetic agents [23, 25]. Little is known however about their mechanism, and their combined potential effects on the activity of alpha amylase have not been researched.

Methods

Materials and reagents

In the current study, all reagents were of analytical grade procured from Sigma Aldrich (Germany).

Plant Collection and Extracts Preparation

In this study, the plants were obtained from Malamulo community, Thyolo district, Malawi, and the voucher specimens were taxonomically identified and authenticated by a botanist at the National Herbarium and Botanical Gardens of Malawi in Zomba. Fresh plant parts were thoroughly cleaned under running water to remove any contaminants and soil debris, rinsed three times under distilled water, and air-dried in the shade for three weeks to conserve light sensitive phytochemicals [28]. The leaves were then coarsely crushed into powder using a mortar and pestle, and using a kitchen sieve, fine powder was obtained, which was stored in separate, labelled airtight containers. For extraction and assay, 30 g of each plants’ powder was suspended in 300 ml of distilled water and left for 24 hours with three-hour interval shaking or swirling. The extract was then filtered using a clean cloth, followed by percolation with slight modification [21].

Phytochemical Screening

The plant extracts were qualitatively screened for the presence of Flavonoids, Saponins, Tannins, Terpenoids, Phenolics, Alkaloids, and Quinones using the crude and reconstituted extracts according to standard methods described by Chelladurai & Chinnachamy, and Kazeem, *et al.*, with slight modifications [29, 30].

Flavonoids were screened by adding 3 to 5 drops of 20% sodium hydroxide (NaOH) to 1ml of crude aqueous extract. The formation of intense yellow colour was observed. Disappearance of the yellow colour after adding a few drops of 32% hydrochloric acid (HCl) to the mixture, indicated the presence of flavonoids.

Saponins were screened by dispensing 0.5 g of each plant powder in 10ml of distilled water and boiled in a water bath for 5 minutes, and then filtered. 5 ml of the filtrate was then added with 2.5 ml of distilled water and shaken vigorously. For the reconstituted extracts, 5 ml extract was mixed with 2.5 ml of distilled water and shaken vigorously. Formation of persistent froth was observed in both. Terpenoids were screened by adding 1 ml of aqueous extract into 2 ml of chloroform, followed by gradual addition of 3 ml of 1% sulphuric acid. Red-brown colour was formed. However, the reconstituted extracts showed no red colour.

Phenolics were screened by adding 1 ml of the plant extract into 2 ml of distilled water followed by a few drops of 20% NaOH. Blue-green colour was formed. Alkaloids were screened by addition of 5 ml of 32% HCl to 1 ml of the plant extract. The mixture was then warmed in a water bath for 5 minutes. A red precipitate was formed.

Tannins were screened by dispensing 0.25 g of the powder into 10 ml of distilled water and boiled in a water bath for 5 minutes and filtered. A few drops of acetic acid were added to the filtrate. For the reconstituted extracts, 5 ml extract was boiled in a water bath for 5 minutes followed by the addition of a few drops of acetic acid. No red precipitate was formed.

Quinones were screened by addition of 1 ml of aqueous extract gradually to 1 ml of concentrated sulphuric acid. Red colour was formed to indicate their presence.

Alpha amylase activity assay

Alpha-amylase activity was determined following the standard procedure proposed by Simão, *et al.*, with slight modifications [31]. The tube containing 100 µl substrate solution (1% starch, buffered in 10 mM phosphate buffer containing 2.7 mM potassium chloride and 13.7 mM sodium chloride) was pre-incubated in a water bath for 20 minutes at 37°C. Then, 500 µl of 2.0 U/ml α-amylase enzyme was added to the substrate, followed by 600 µl of 10 mM phosphate buffer. The mixture was incubated for 15 minutes. Addition of 200 µl of 3.5 M Dinitrosalicylic acid (DNSA) stopped the reaction. Blanking was done by using the mixture of 200 µl of 1% starch, 500 µl phosphate buffer & 200 µl DNSA.

The product of the reaction was measured spectrophotometrically at 540 nm. The equation used to calculate the enzyme activity is outlined below:

$$\text{Enzyme activity} = (\Delta Abs \times V_t) / (\epsilon \times t \times V_e)$$

Where: ΔAbs is the mean change in absorbance; V_t is the total volume of the generated solution (1,400 µl); ϵ is molar extinction coefficient of starch (20.416); t is the time taken to read absorbance which is 1 sec; and V_e is the volume of enzyme used in the procedure (500 µl).

Alpha amylase inhibitory assay

The inhibitory effect of the extracts on alpha amylase activity was determined as proposed by Simão, *et al.* and Ogundele, *et al.*, with slight modifications [32, 30]. Thus, 500 µL of 10 mg/ml of plant extracts and 500 µL of 2.0 U/ml α-amylase enzyme and 100 µl of 0.1 M phosphate buffer of a pH of 7.0 was pre-incubated in a water bath at 37 °C for 20 min. 100 µl of 1% starch was added to the pre-incubated mixture of extract and enzyme. The mixture was incubated in a water bath for 15 minutes at 37 °C.

Three sets of experiments were run; test, blank and control. The reaction was allowed to cool at room temperature, and stopped by adding 200µl of 3.5 M DNSA. Blanked using the mixture of all of the above except the enzyme. Then measured absorbance of the reaction products spectrophotometrically at 540 nm. For the blank, the DNSA solution was added before the addition of the starch solution, but the rest of the method was the same as for the test [29].

The combined *A. senegalesis* and *P. thonningii* inhibitory activity was carried out by mixing their respective plant extracts in a 1:1 ratio. The procedures done for the individual plant extracts were then followed using the mixture as the test.

The tests were run in triplicates and replicated thrice and represented in terms of mean values and standard deviation values.

Then, the α-amylase inhibitory activity was calculated as follow:

$$\text{Inhibition Percentage (I\%)} = 100 \times (1 - \beta/\alpha)$$

or

$$\text{I\%} = 100 \times ((Abs \text{ Control} - Abs \text{ Test}) / Abs \text{ Control})$$

Where α is the activity of alpha amylase without extract and β is the activity of alpha amylase with extract.

Quality Control

A. senegalesis and *P. thonningii* were identified and authenticated by the National Herbarium and Botanical Gardens of Malawi. All assays were carried out in triplicates, an average absorbance was calculated and used for all enzyme activities and inhibition studies. Control assays were included, an assay blank and inhibition assay blank were used. A well-known alpha amylase inhibitor

(100 µg/ml acarbose) was used as a standard for the alpha amylase inhibitory studies. Negative control (blank: 0% AAI activity) was prepared containing only the assay mixture without extract. The inhibitory activities were compared to the positive control, acarbose, using Stata statistical software which showed a significant difference at 95% confidence interval.

Results

Phytochemical Screening

The qualitative phytochemical screening tests showed the presence and absence of different compounds as shown by colour change as per the procedures. Tables 1 and 2 below show the phytochemicals found before and after evaporation procedures respectively.

Table 1: Phytochemical composition before evaporation

Plant	Phytochemical Composition						
	Flavonoids	Saponins	Terpenoids	Phenolics	Alkaloids	Tannins	Quinones
<i>A. senegalensis</i>	+	+	+	+	+	-	+
<i>P. thonningii</i>	+	+	+	+	+	-	+

KEY: (+) means presence and (-) means absence

Table 2: Phytochemical composition after evaporation

Plant	Phytochemical Composition						
	Flavonoids	Saponins	Terpenoids	Phenolics	Alkaloids	Tannins	Quinones
<i>A. senegalensis</i>	+	+	-	+	+	-	+
<i>P. thonningii</i>	+	+	-	+	+	-	+

KEY: (+) means presence and (-) means absence

Yield extraction

The extracts yielded from 30 g leaf powder after evaporating the liquid from the aqueous extract were weighed and their yield were determined to be as follows:

Percentage yield = (Mass of the extract / Mass of the plant used for extraction) x 100

Extract percentage yield for

$$A. senegalensis = (1.3 \text{ g} / 30 \text{ g}) \times 100\% = 4.33\%$$

Extract percentage yield for

$$P. thonningii = (1.8 \text{ g} / 30 \text{ g}) \times 100\% = 6\%$$

Interaction studies

The potential of *A. senegalensis* and *P. thonningii* to inhibit alpha amylase enzymes were calculated from their respective mean

absorbance. Table 3 below shows that *A. senegalensis* inhibitory activity on alpha amylase increased with increase in incubation time as follows: 32%, 34% and 40% at 15, 30 and 45 minutes respectively as compared to *P. thonningii* which inhibited at 25%, 27% and 37% at 15, 30 and 45 minutes respectively. The combined inhibitory activity of *A. senegalensis* and *P. thonningii* on alpha amylase showed to be 28%, 37% and 62% at 15, 30 and 45 minutes respectively. A summary is presented in table 3 with graphical presentation in figures 1 and 2 respectively.

The mean differences of absorbance and the inhibitory activity as compared to the positive control are generally significant, at an average time, as shown by the calculated p-values which were less than 0.05 at 95% confidence interval as summarised in table 4 of statistical analysis.

Table 3: Inhibitory activity of 10 mg/ml plant extract on alpha amylase activity

Plant	Incubation Time (Minutes)	Mean Absorbance	Enzyme Activity (U/ml)	Inhibitory Activity (%)
<i>Enzyme without inhibitor (Neg Control)</i>	15	0.679	0.053	0
	30	0.696	0.055	0
	45	0.750	0.059	0
<i>100 µg/ml Acarbose (Pos control)</i>	15	0.266	0.021	61
	30	0.211	0.017	69
	45	0.200	0.016	71
<i>A. senegalensis</i>	15	0.461	0.036	32
	30	0.449	0.035	34
	45	0.407	0.032	40
<i>P. thonningii</i>	15	0.508	0.040	25
	30	0.495	0.039	27
	45	0.431	0.034	37
<i>Combined (Annona & Piliostigma)</i>	15	0.488	0.038	28
	30	0.425	0.033	37
	45	0.261	0.020	62

Table 4: The differences in mean absorbance between the positive control and the test sample, and the inhibitory activity between the positive control and the test samples and their t-values and p-values at 95% confidence interval

Test Sample	In relation to mean ABS		In relation to mean I%		Mean Inhibition difference (%)
	t-value	p-value	t-value	p-value	
<i>A. senegalensis</i>	-8.2310	0.0006	8.1460	0.0006	31.667
<i>P. thonningii</i>	-8.0968	0.0006	7.7655	0.0007	37.333
<i>Combined</i>	-2.3402	0.0397	2.3228	0.0404	24.667

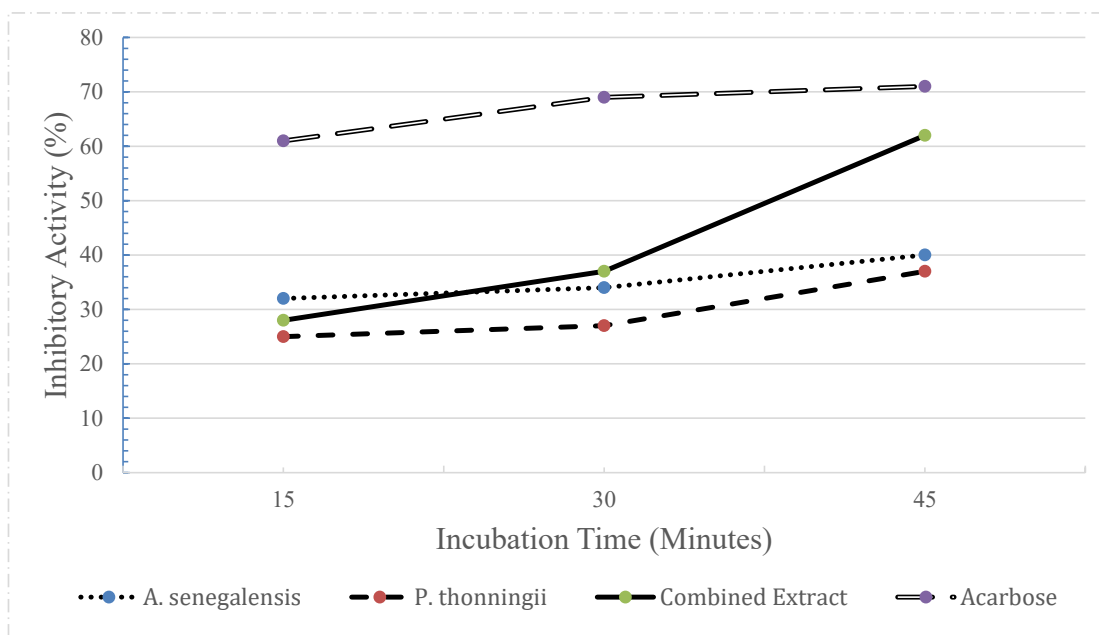


Figure 1: A linear graph of *A. senegalensis*, *P. thonningii* and the combined extracts' inhibitory activity on alpha amylase enzyme with reference to the positive control, acarbose against incubation time.

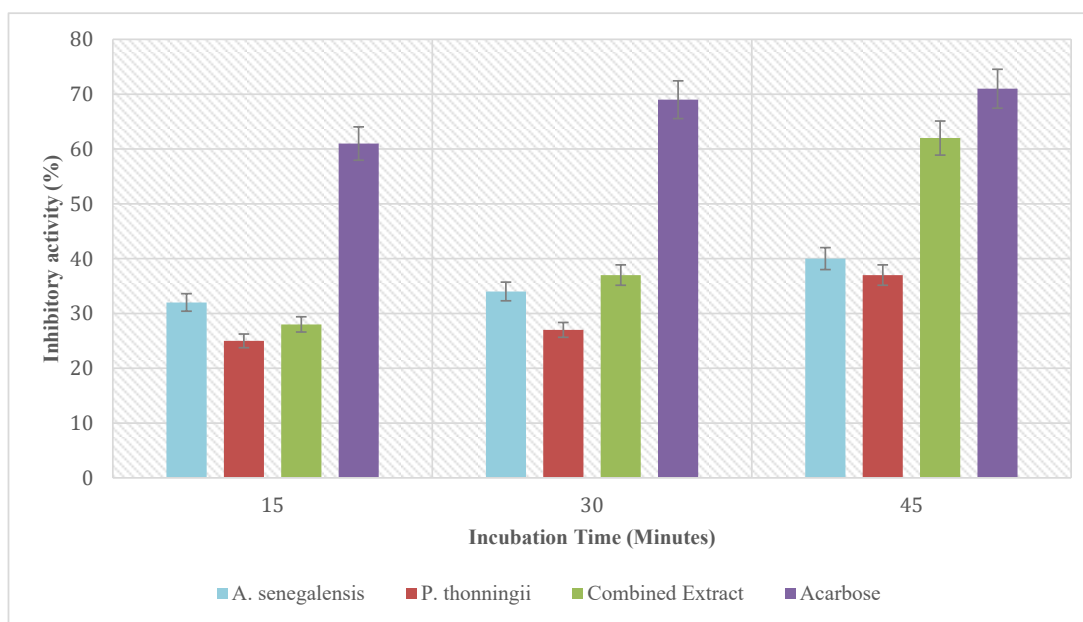


Figure 2: A bar graph of *A. senegalensis*, *P. thonningii* and the combined extracts' inhibitory activity on alpha amylase with reference to the positive control (acarbose) against incubation time.

Discussion

The presence of phytochemicals in *Annona senegalensis* and *Pilostigma thonningii* leaf extracts is thought to be responsible for the inhibitory activity of their extracts on alpha amylase enzyme, and also for their potency in the management of diabetes mellitus. The absence of terpenoids in the extracts after reconstitution of the yielded extracts, may be due to heat exposure, as some phytochemicals are heat sensitive [28].

The extract-yield showed to be higher in *P. thonningii* than *A. senegalensis*. Pharmaceutical industries consider the yield of extraction as a guide in drug manufacturing. Plants of higher yield are preferred than those of lower yield, however, lower yield plants are known to be richly potent [10]. *A. senegalensis*, therefore, may be considered to be more potent than *P. thonningii*, an observation occurring in the differences in effects on alpha amylase enzyme activity.

This study showed a significant inhibitory activity of *A. senegalensis* and *P. thonningii* on alpha amylase, individually, and is in agreement with Ibrahim, et al. and Aba & Asuzu, respectively [33, 34]. Such studies showed that *A. senegalensis* leaf extracts and *P. thonningii* bark extracts inhibited α -amylase due to the presence of their associated phytochemicals. The inhibitory activity of the combined *A. senegalensis* and *P. thonningii* leaf extracts drew close to that of positive control acarbose.

Conclusions and recommendations

This study has revealed the increased strength to inhibit alpha amylase enzymes by a combination of *A. senegalensis* and *P. thonningii* than their individual potential effect in slowing down carbohydrate metabolism, thereby reduction and prevention of

postprandial hyperglycaemia. This therefore, makes it scientifically evident of these two plants' robust combined effect in the management of diabetes mellitus. We therefore recommend for a need to determine the concentration of the plants capable of inhibiting the enzyme by half (IC50).

List of abbreviations

AAIs	Alpha amylase inhibitors
DM	Diabetes Mellitus
DNSA	Dinitrosalicylic acid
HCl	Hydrochloric acid
IC50	Concentration capable of inhibiting 50% enzyme activity
NaOH	Sodium hydroxide
NHSRC	National Health Sciences Research Committee
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
WHO	World Health Organisation

Declarations

Ethics approval and consent to conduct the study

This research was approved by the National Health Sciences Research Committee (NHSRC) and Malawi Adventist University Research Committee. *A. senegalensis* and *P. thonningii* were identified and authenticated by a Botanist at the National Herbarium and Botanical Gardens of Malawi. All procedures were conducted in accordance to the national guidelines. The deposition numbers were not assigned.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

AMN, MM, ES and EB: data analysis, write up and discussion of results

CK and PC: literature review and write up

JM and WT: data curation, editing and discussion of results

RC, MK, BK, JK and LL: proofreading and editing

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