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Research Article

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In Vitro Effects of Annona Senegalensis Root Bark, Musa Sapientum L and Malus Pumila Peel Extracts On Xanthine Oxidase

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Abstract

Background: *Xanthine Oxidase activity may increase plasma urates, superoxide radicals and hydrogen peroxide leading to gout, arthritis and cancer. Allopurinol, a known Xanthine Oxidase inhibitor, is noted to have various adverse effects. Many laboratories are in research projects to find alternative inhibitors of XO including plant sources. Plants are known to contain therapeutically effective agents. A. senegalensis, M. sapientum L and M. Pumila are reported to contain phytochemicals with antioxidant, anti-inflammatory and enzyme inhibitory activities.*

Methods: *Aqueous extracts of Root bark of A. senegalensis, peels of M. sapientum L and M. pumila were assayed for their inhibitory effects on Xanthine oxidase in vitro*

Results: *All aqueous extracts exhibited the presence of flavonoids. A. senegalensis root bark and M sapientum L and M pumila peels were investigated for their effects on Xanthine Oxidase activity. A. senegalensis root bark, M. sapientum L and M. Pumila peel extracts inhibited Xanthine Oxidase activity by 83%, 90% and 61% respectively as which are significantly different ($p < 0.05$) from that of the positive control, allopurinol (65%)*

Conclusions: *The results obtained in this study suggest that the flavonoids found in A. senegalensis root bark and M. sapientum L and M. pumila peel extracts could be potential Xanthine Oxidase activity inhibitors.*

Keywords: Flavonoids, Inhibition, Gout, Phytochemicals, Extraction, Uric Acid.

Introduction

Plants have been known to be of medicinal use in many societies and cultures around the globe. They have served and still serve as alternatives for conventional medicine in homes as natural remedies for infections, inflammations and noncommunicable diseases such as diabetes mellitus, gout, and hypertension. In other circumstances induction of labour has been achieved by plants. Elsewhere, *Marantodes pumilum (Blume) Kuntez* is commonly used to treat parturition, flatulence, dysentery, dysmenorrhoea, gonorrhoea, and bone diseases [1].

Recently, there has been an increased interest in use of plant-based remedies either to find new drugs, employ cheaper sources of medicine, or even to take advantage of the claimed safety in plants [2, 3]. The use of plants as medicine has been done either through food, or special preparations such as infusions, smoothies, decoctions, or poultices. Therefore, many edible plants are part of the search for alternative medicines. However, there are still many plants whose mechanism of action is known [4].

Plants are also used as raw materials for pharmaceutical products. A major interest has been in the plant phytochemistry and their natural oils. Xanthine oxidase (XO) is a key enzyme in formation of uric acid from degradation of purine nucleotides in the last

step of in humans. XO is associated with inflammation through production of free radicals. During re-oxidation of XO, molecular oxygen acts as an electron acceptor, producing superoxide radical (O_2-) and hydrogen peroxide (H_2O_2). XO is part of an important biological source of superoxide radicals [5]. Under favourable conditions especially when XO is overproduced, uric acid can crystallise in arthrosis (joints) and kidneys and cause inflammation known as arthritis or gout and renal calculi respectively. Uric acid is a marker for gout and several haemodynamic abnormalities [1].

Xanthine oxidase is a therapeutic target for Allopurinol and Febuxostat, the commonly available xanthine oxidase inhibitors (XOI). Xanthine oxidase inhibitors are associated with side effects including Steven Johnson Syndrome, fever, skin rash, eosinophilia, hepatitis, and renal toxicity [6]. Both of these drugs are expensive, inaccessible to some developing countries. Such unmet medical needs and health hazards posed by these drugs require more effort in finding novel Xanthine oxidase inhibitors that are much effective and have a good safety profile. These findings indicate the necessity for the development and discovery of more precise Xanthine oxidase inhibitors aimed at improving the treatment of gout and a reduction of complications that arise due to hyperuricemia while realising fewer adverse effects profile [6]. The use of plant-based products may be very efficient as they are easily available and generally safe for biological systems [4]. *Musa sapientum L* is one of the species in the banana family, and is one of the common fruits in the world. Nearly all parts of a banana tree are commonly used as traditional medicine for treating diarrhoea, menorrhagia, diabetes, dysentery, and antiulcerogenic, hypoglycaemic, antilithic, hypolipidemic conditions, plus antioxidant actions, inflammation, pains and even snakebites [7].

Malus pumila is largely cultivated around the world in temperate regions. It is usually eaten as a fruit and flowers can be used as tea. Studies have demonstrated that the plant contains some medicinal properties which can be targeted against ageing, oxidative stress, cancers, and bacterial infections. The chemical constituents of *M. pumila* include flavonoids, terpenoids and organic acids. Its main chemical components are dihydrochalcone such as phlorizin, phloretin, and other flavonoids such as quercetin, kaempferol and rutin [8].

Annona senegalensis is commonly called wild custard apple, used as food or a food additive as all parts of the plant contain varying amounts of essential oils. According to some study, it contains major bioactive constituents including tannins, flavonoid, saponins, alkaloids, glycosides, steroids, volatile acids and anthocyanin [9]. It has also been reported in literature that the plant contains various minerals such as calcium, potassium, magnesium, zinc, copper, manganese as well as ascorbic acid and amino acids which makes it an important source of nutrients. The roots, root bark and leaves have been reported to have been used to treat malaria, tuberculosis [9, 10].

Annona senegalensis is used for both food and medicinal purposes. It has also been reported in literature that different parts of the plant are employed in traditional medicine and home remedies to cure some diseases such as tuberculosis, hernia, diabetes, gastritis, male sexual impotence, difficulty in swallowing, and snake bites [11]. *Annona senegalensis* has also been reported to have anti-cancer properties [12]. Again, some researchers reported on the potential of *A. senegalensis* in the treatment of a minimum of three COVID 19 symptoms such as cough, fever, myalgia, and the treatment of liver, breast, and colon cancers [11]. In this study, aqueous extracts of *Musa sapientum* and *Malus pumila peels*, and *A. senegalensis* root bark were investigated for the inhibitory potential on xanthine oxidase activity.

Materials and methods

Specimen collection, Identification, Authentication and preparation

The samples of *A. senegalensis* were collected from areas around Malamulo, Thyolo while *M. sapientum L* and *M. pumila* samples were bought at a local market in Makwasa, Thyolo and Limbe respectively. Samples were taken to Mulanje Mountain Conservation Trust for identification and National Herbarium and Botanical Gardens of Malawi where they were authenticated. *Annona senegalensis* roots were washed with clean tap water and shade dried for 2 weeks [13]. *Musa sapientum L* and *M. pumila* samples were washed under running tap water, the surfaces were sterilised with 70% ethanol, rinsed with distilled water, *A. Senegalensis* samples were refrigerated until needed. Peels of *M. pumila* were removed and shade dried for 2 weeks.

Plant material extractions

A. senegalensis root bark extractions

After drying, *A. senegalensis* root barks were pounded to a fine powder using a mortar and pestle, active ingredients were obtained by using the extraction method as described in literautre with slight modifications [14]. Where 40 g of the pounded sample was soaked in 350 ml of distilled water in a sterile conical flask and left to stand for 24 hours with periodic mixing and then it was filtered with a filter paper (Whatman No.1) after which the filtrate was stored in a refrigerator for further investigations.

M sapientum L peels were taken and added to distilled water after it had just boiled, left to cool. After sometime the contents were mixed and then filtered to remove large, non-homogenised particles in order to get clear aqueous extract. The extract was then kept at 4 0C until the time it was ready for use [15]. *M. pumila* peels extracts were obtained using the method as described elsewhere, where 40 g of the dried peels were soaked in distilled water for 24 hours at room temperature with periodic vortexing, after which the mixture was filtered using a filter paper (Whatman, No. 1) the filtrate was stored in a refrigerator for further investigations [14].

Phytochemical screening

Test for Flavonoids

A.Senegalensis, M. Sapientum L and *M. pumila* phytochemical analyses were done according to literature with slight modifications [14]. Extracts (1 ml) was added into 2 ml of sodium hydroxide (NaOH) solution. The resulting appearance of a yellow solution disappeared upon adding hydrochloric acid, which indicated the presence of Flavonoids.

Xanthine Oxidase activity assay

XO activity determination was performed according to the method described in literature, where the substrate and the enzyme solutions were prepared immediately before use (16). The reaction mixture contained sodium phosphate buffer (50mM pH 7.5, 300 µl), XO (100 µl, 0.1U/l), the reaction mixture was pre- incubated at 37 ^{0}C for 15 minutes. Then 100 µl of substrate solution (0.15mM of xanthine) was added into the mixture and incubated at 37 ^{0}C for 30 minutes. The reaction was stopped by adding HCl (0.5M, 20 µl).

The absorption was read at 295 nm against an assay blank, checking for uric acid formation at 37 ^{0}C using a UV spectrophotometer. Enzyme activity was determined using the formulae;

$$\text{Enzyme activity} = \mathbf{(\Delta abs\ .Vt)/\ (\varepsilon.\ t.\ Ve)} \qquad (1)$$

Where ***Δabs*** is the change in absorbance; Vt is the total reaction volume (800 µl); ε = the extinction coefficient of uric acid (12.56); t is the time in minutes; ***Ve*** is the volume of the extract which was added in the reaction mixture (100 µl). The calculated results were expressed in U.L^{-1}. One unit of enzyme activity was defined as the amount of enzyme that converts 1 µmol of xanthine to uric acid per min under defined conditions [17].

Xanthine Oxidase Inhibitory assay

The inhibitory effects of the extracts on XO activity was measured spectrophotometrically at 295 nm using a UV spectrophotometer, measuring the uric acid formation under aerobic conditions, with some modifications according to the method described elsewhere [16]. Prior to the assay, the enzyme and *A. senegalensis, M. sapientum L* and *M. pumila* extracts were mixed in a ratio of 1:1 v/v to obtain a final enzyme concentration of 0.1 U/L. The reaction mixture contained sodium phosphate buffer (50mM pH 7.5, 200 µl) and 200 µl of XO-extract pre-mixture, the reaction mixture was pre- incubated at 370C for 15 minutes. Then 100 µl of substrate solution (0.15mM of xanthine) was added into the mixture and incubated at 37 ºC for 30 minutes. The reaction was stopped by adding HCl (0.5M, 200 µl). The UV spectrophotometer was blanked with an inhibition assay blank prepared in the same way but the enzyme solution was replaced with a phosphate buffer. XO inhibitory activity was calculated and expressed as a percentage inhibition of XO in the above assay.

$$\text{Inhibition \% (I\%)} = 100 \times (ABS_{control} - ABS_{test} / ABS_{control}) \qquad (2)$$

Quality control

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 XO inhibitor (100 ug/ml) was used as a standard for the XO inhibitory studies. Negative control (blank: 0% XOI activity) was prepared containing only the assay mixture without extract.

Results

Plant extractions and phytochemical screening

Flavonoids were identified in all aqueous extracts as summarised in Table 1.

Table 1: Phytochemical Screening

Plant Name	Flavonoid Test Results
A. senegalensis	++
M. sapientum L	++
M. pumila	+

Key: (+) = low in abundance (++) = moderate in abundance

XO Inhibition Assay

The results of XO activity determination and XOI studies for *A. senegalensis* and *M. sapientum L* are summarised in table 2. XO had an activity of 20.9 U/L. The experimental data indicate that the extracts under study showed good to outstanding inhibitory effects towards XO. *A. senegalensis* reduced XO activity from 20.9 to 3.50U/L representing 83% activity inhibition. *M. sapientum L* exhibited a 91% inhibition by reducing XO activity to 3.50 U/L and *M. pumila* reduced XO activity to 5.8U/L representing 80% inhibition. Allopurinol, the positive control, reduced XO activity from 20.9U/L to 7.26U/L, representing 65% inhibitory effects, a summary is presented in table 2 with graphical representation in figures 1 and figure 2 respectively. Statistical analysis is as summarized in table 3.

Table 2: A summary of XO enzyme activity and in vitro inhibitory studies

Plant name	Avg Abs	Activity (U/L)	% inhibition
XO	0.324	0.206	0
A. senegalensis	0.055	0.035	83.0
M. sapentium	0.031	0.019	90.6
M. Pumila	0.092	0.058	84
Allopurinol	0.114	0.072	65.23

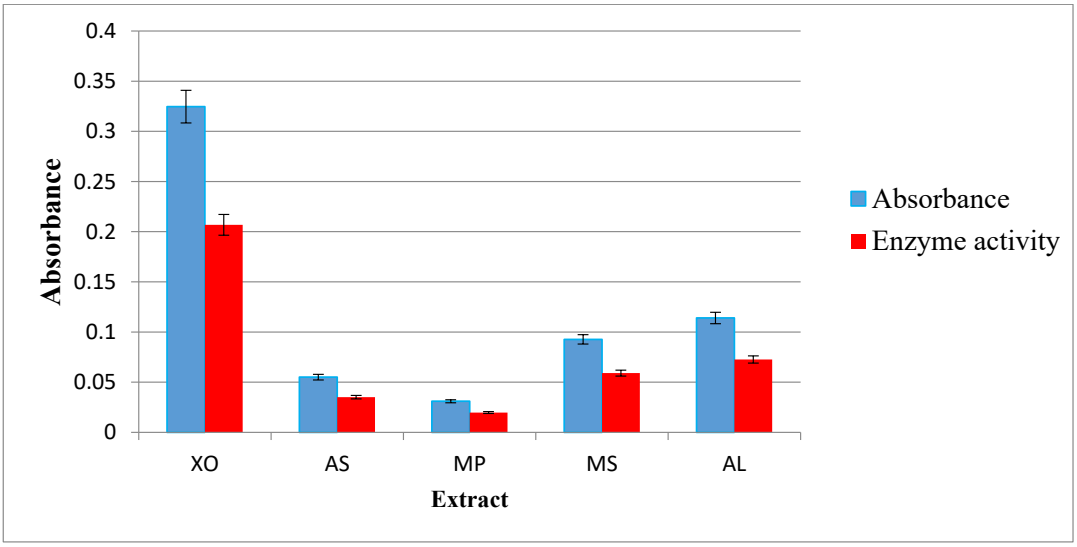

Figure 1: A graph of mean absorbance and enzyme activity against different extracts (XO = Xanthine oxidase, AS = A. senegalensis, MP = M. pumila, MS = M. sapientum, AL = allopurinol)

Table 3: The differences in mean absorbance between the positive control and the test sample; enzyme activity 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 enzyme activity		In relation to mean I%		Mean Inhibition difference (%)
	t-value	p-value	t-value	p-value	t-value	p-value	
A. senegalensis	29.4675	0.0000	28.5349	0.0000	-7.2259	0.0010	-18.229 (-17.77)
M. sapentium	26.2473	0.0000	25.2918	0.0000	-7.4072	0.0009	-25.569 (-25.37)
M. pumila	25.7729	0.0000	25.1366	0.0000	26.7655	0.0000	64.70 (-18.77)

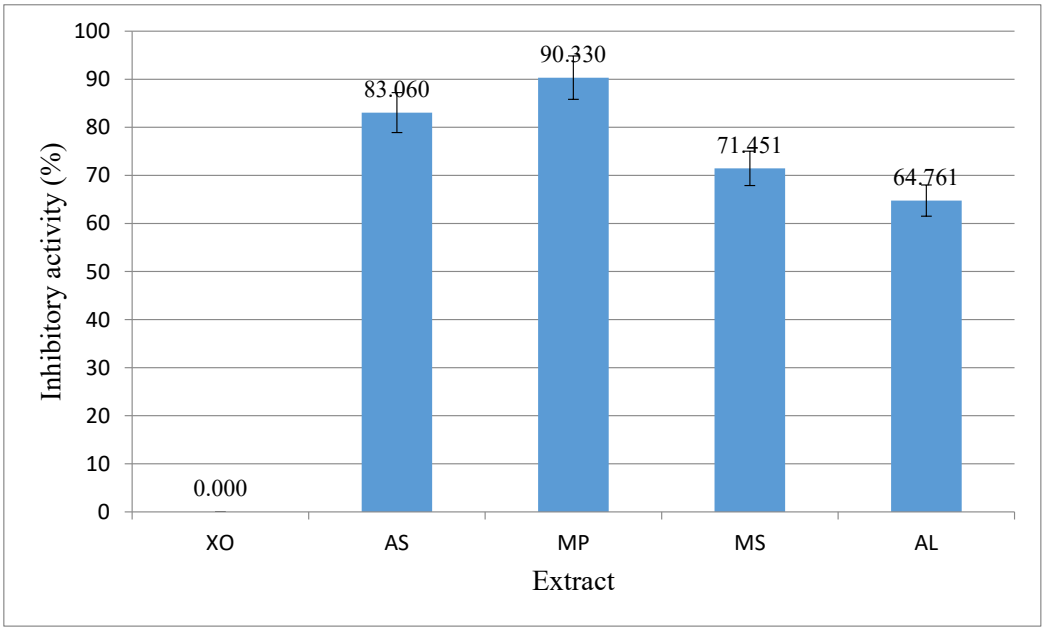

Figure 2: A graph of inhibitory activity against different extracts (XO = Xanthine oxidase, AS = *A. senegalensis*, MP = *M. pumila*, MS = *M. sapientum*, AL = allopurinol)

Discussion

In the quest to search for alternative drugs for the cure of disease, and as a step towards identifying a novel medicinal agent, this study assessed three plants for their effect against the activity of XO. This study found slightly lower concentrations of flavonoids, which may be attributed to the type of extraction medium employed. Some literature reported that there are observed variations of phytochemical presence in medicinal plants owing to solvents used for extraction and extraction procedure [18]. Water as a solvent for extraction is advantageous as it effectively extracts most polar compounds, cheap, nontoxic and nonflammable [19].

However it may affect the extraction efficiency and content and hydrolysis of compounds due to high heat requirements to concentrate extracts [20, 21]. According to literature, low to no evidence of alkaloids was reported upon using water as a solvent [19].

Therefore the use of aqueous solvents might have contributed to the observed flavonoids test results in the current study.

Flavonoids, a member of a group of naturally occurring active compounds in plants, have been reported to possess tremendous health benefits [22]. Medically important flavonoids are reported be very potent antioxidants and thus have attracted a significant amount of interest among researchers as possible potent therapeutic agents for illnesses whose aetiologies and pathogenesis are associated with free radicals [22]. Free radicals including hydroxyl radicals, superoxide anions, hydrogen peroxide, oxygen singlets, hypochlorite and nitric oxide are reported to play a key role in various inflammatory diseases; vis rheumatoid arthritis and gout [23, 24]. XO catalyzes the formation of uric acid and hydrogen peroxide from purine degradation which are responsible for oxidative damage that causes gout, hyperuricemia, arthritis, vascular endothelium damage and ageing [25, 26].

Various parts of *M. sapientium, A. senegalensis* and *M. pumila* have been reported to contain active secondary metabolites active on various enzymes that effectively inhibit various enzymes including Glutathione-s-transferase, Acetylcholinesterase, Carboxylesterase and Xanthine oxidase (XO) α-glucosidase and α-amylase, angiotensin 1 converting enzyme (ACE) [27-29]. The flavonoids observed XO inhibition as also reported by elsewhere, might be helpful in the prevention of slowing down the pathogenesis of gout [30].

Interestingly results obtained in the current research indicate that aqueous extracts of *M. Pumila* peels exhibited higher inhibitory effects as compared to those observed by some research fellows, whereby they reported that aqueous extracts of *M. pumila* exhibited no inhibition and methanolic extracts inhibited XO activity by 28% [31].

Annona senegalensis crude extracts are reported to inhibit several enzyme activities including XO, lower than observed in this study [29]. This study also found that *A. senegalensis* together with other species of *Annona* inhibited xanthine oxidase activity by 25% which is also lower than that obtained in this study. This variation was suggested to arise from some interaction of compounds between the species that led to retardation of the inhibition [29].

There is limited information pertaining to the interaction of *Musa sapentium L* and XO to support its inhibitory activity, however, some researchers found that other antioxidative *Musa species* decrease uric acid levels by inhibiting the xanthine oxidase enzyme [32, 33].

The antioxidative properties of *M. sapientum L peel, M. pumila peel* and *A. senegalensis* root bark extracts have potential to qualify that they are effective anti-gout agents due to their ability to inhibit XO enzyme activity.

Conclusion and Recommendations

The results of this study indicate that *A. senegalensis, M. sapentium L peel* and *M. pumila* aqueous extract possess significant inhibitory effects on xanthine oxidase activity. Further in *vitro* studies may be conducted on the effects of *A. senegalensis, M. sapentium* and *M. pumila* extracts obtained using various extraction solvents and methods. Further, purifications and identification of purified extract are considered to identify exact active phytochemical(s) that exhibit the inhibitory effects observed in the current study.

List of abbreviations

XO	Xanthine oxidase
H_2O_2	Hydrogen peroxide
O_2-	Superoxide radical
XOI	Xanthine oxidase inhibitors
NaOH	Sodium Hydroxide
HCL	Hydrochloric acid
Δabs	Change in absorbance
V_t	Total reaction volume
V_e	Extract volume
$U.L^{-1}$	Enzyme activity unit
UV	Ultraviolet
AS	*Anonna senegalensis*
MP	*Malus pumila*
MS	*Mussa sapientum*
AL	Allopurinol
ACE	Angiotensin converting enzyme

Declarations

Ethical Approval

This research was approved by the National Health Sciences Research Committee (NHSRC) and Malawi Adventist University Research Committee. *A. senegalesis, M. pumila* and *M. sapientum L* were identified and authenticated by a Botanist at the National Herbarium and Botanical Gardens of Malawi, under authentication deposition numbers of 15053 and 1729 respectively. All methods were carried out in relevant guidelines and regulations. National

Health Sciences Research Committee (NHSRC) and Malawi Adventist University Research Committee gave permission to collect samples of *A. senegalensis*

Consent to Participate
Not applicable

Consent for Publication
Not applicable

Availability of Data
The datasets used and/or analysed during the current study are available from the Corresponding author on reasonable request.

Competing Interests
The authors declare that they have no competing interests

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Authors' Contributions
MM, EB, ES, AMN, CM and AK: Data analysis and write up
MM, EB, JM, WT and MK: Literature review and write up
EB, MM, AMN, LL, PC, RC, ZK, CK and BK: Proof reading and discussion of results
MM, AMN, EB and ES: Data curation and editing.
All authors reviewed the manuscript.

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