ISSN: 2476-2377

Research Article

International Journal of Cancer Research & Therapy

Assessment of the In *Vitro Effects* of Six Selected Plant Extracts on Xanthine Oxidase

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Submitted:2023, Oct 06: **Accepted**: 2023, Oct 31: **Published**: 2023, Nov 14

Citation: Mlozen, M. M., Nyanda, A. M., Bonya, E., Siyano, E., Khunju, E., et al. (2023). Assessment of the In *Vitro Effects* of Six Selected Plant Extracts on Xanthine Oxidase. *Int J Cancer Res Ther, 8*(4), 172-180.

Abstract

Background: *Gouty arthritis is one of the most prevalent forms of arthritis caused by the deposition of uric acids mostly in joints, due to hyperuricemia. Xanthine oxidase (XO) is a key enzyme of the purine degradation pathway, catalysing the oxidation of hypoxanthine to xanthine and further to uric acid; increased activity leads to hyperuricemia hence a need to control XO activity. Commonly used therapeutic approach is the use of XO inhibitors. This study aimed to investigate if Vitis vinifera, Malus sylvestris fruit peels, Cymbopogon citratum leaves, Mucuna Pruriens leaves, Zanha africana roots and leaves, Mimosa pudica roots extracts have a Xanthine Oxidase inhibitory potential in vitro.*

Methodology: *Air dried samples were crushed into powder using a mortar and pestle. Maceration and percolation extraction methods were used to obtain active secondary metabolites from plant materials at 10%w/v. Spectrophotometric assays methods were used for Xanthine Oxidase activity and inhibition studies.*

Results: *The results of the current study showed that all aqueous plant extracts showed the presence of flavonoids. The experimental data showed significant inhibitory effects on XO activity. C. citratum inhibited XO activity by an average of 71%. M. sylvestris exhibited 68%, 61% and 64% at 15, 30 and 45 minutes respectively. V. vinifera exhibited 75%, 66% and 77% XO inhibitory activity at 15, 30 and 45 minutes respectively. Z. Africana exhibited 73%, 65% and 74% at 15, 30 and 45 minutes respectively. M. pudica exhibited 74%, 77% and 78% XO inhibitory activity at 15, 30 and 45 minutes respectively. M. pruriens exhibited 65%, 69% and 71% XO inhibitory activity at 15, 30 and 45 minutes respectively.*

Conclusion: *The results obtained from the current study indicates that the flavonoids present in all plant extracts under study could be potential anti-gout agents by their inhibitory activities on Xanthine Oxidase activity.*

Keywords: Xanthine Oxidase, Gout, Allopurinol, Extracts, Inhibition, Maceration

List of Abbreviations

$FeCl_3$	Ferric chloride
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
MAU-MCHS	Malawi Adventist University-Malawi college of Health Sciences
NAOH	Sodium Hydroxide
UV	Ultraviolet
U/l	Units per litre
XO	Xanthine Oxidase
XOI	Xanthine Oxidase Inhibitor

1. Introduction

Gout, an inflammatory arthritis caused by crystallisation of uric acid urates in the joints, is one of the most painful forms of arthritis associated with severe joint pain, swelling, and erythema. Xanthine oxidase (XO) is an important enzyme involved in catalysing the hydroxylation of hypoxanthine to xanthine, and xanthine to uric acid which is excreted by the kidneys [1]. Overactivity of

this enzyme has shown a positive correlation with gout which is characterised by an excessive concentration of uric acid in the blood (hyperuricemia). Hyperuricemia leads to the accumulation of monosodium urate crystals in joints and kidneys, leading to acute or chronic gouty arthritis, and uric acid nephrolithiasis [1,2].

The inhibition of XO serves as one of the effective strategies in treating and managing gout and complications that may occur due to the overactivity of the enzyme. Chemical agents such as allopurinol and febuxostat are used clinically to inhibit the activity of xanthine oxidase [4].

XO has shown to be associated with the production of free radicals, like superoxide radical (O_2-) and hydrogen peroxide (H_2O_2), which lead to inflammation [5]. The overproduction of XO results in crystallisation of uric acid in arthrosis and kidneys which may cause gouty arthritis and renal calculi respectively. Uric acid is a marker for gout and several haemodynamic abnormalities [6,7].

Despite the authorised use of Allopurinol and Febuxostat as xanthine oxidase inhibitors, their associated side effects; such as Steven Johnson Syndrome, eosinophilia, skin rash, fever, renal toxicity, alopecia and fatal liver necrosis, have been a threat and a great concern. In addition to that, both drugs are expensive especially febuxostat which makes its accessibility limited to developing countries, Malawi inclusive [8,9]. Such unmet medical needs and health hazards posed by these drugs require more effort in finding novel xanthine oxidase inhibitors that are much more effective and have a good safety profile. These findings indicate the necessity for the development and discovery of more precise xanthine oxidase inhibitors (XOI) aimed at improving the treatment of gout, realising fewer adverse effects profile [10]. Besides, the use of plant-based products may be very efficient as they are easily available and generally safe for biological systems [11].

Malus sylvestris, commonly known as apples, are among the most consumed fruits globally. Its peels have been known to have functional bioactive substances such as phenolic acids, vitamins, tannins, flavonoids, and essential minerals. They have also served as a good source of carbohydrates, minerals, dietary fibre, and antioxidants. Health benefits attributed to the consumption of *M. sylvestris* peels fruit juice include anti-proliferative, anti-inflammatory, anti-carcinogenic, and anti-viral effects [12].

Vitis vinifera, commonly known as grapes, is another widely consumed and industrialized fruit in the world. The peels of V. vinifera peels are a good source of polyphenolic compounds such as anthocyanins, flavonoids, phenolics, and resveratrol. Scientific evidence has shown that the compounds in *V. vinifera* peels have antiviral and anti-tumor activities. Other health benefits include the prevention of cardiovascular diseases, diabetes, and oxidative stress skin damage [13].

Cymbopogon citratum, also known as lemon grass, is a perennial plant with long thin leaves and is one of the cultivated medicinal plants in tropical and subtropical regions of Asia, Africa, and America [14]. *C. citratum* is extensively consumed for its medicinal, cosmetic, and nutritional benefits. Therapeutically, *C. citratum* is reported to have antifungal, antibacterial, anti-carcinogenic, anti-inflammatory and anti-oxidant, and diuretic effects. Phytochemical studies on *C. citratum* have shown the presence of tannins, saponins, flavonoids, phenols, anthraquinones, alkaloids, deoxy-sugars, and various essential oils [15].

Mucuna Pruriens, known in English as buffalo-bean, has been used as an antioxidant, antitumor, antibacterial, antivenom, antidiabetic and aphrodite. *M. Pruriens* has been shown to contain phytochemicals like alkaloids, saponins, phenolics, tannins, flavonoids, quinone and terpenoids [16,17].

Zanha africana, velvet-fruited zanha, which in Malawi is known as *kangaluche* or *Mzakaka*, has been used to treat painful arthritis, and other ailments like diabetes, abdominal pains, constipation, diarrhoea and dysentery, rheumatoid arthritis and rheumatism, fever, typhoid fever, malaria, many fungal, bacterial and viral infections [18]. It has also been used as a remedy for haematuria, haemorrhoids, hernia, heart and hypertension problems, nausea, nose bleeding, oedema, purgative and skin problems e.g. abscesses and scabies [19].

Mimosa. pudica has been used in gout treatment has also been proven to show to be an anti-ulcer, anti- inflammatory, anti-microbial, anti-toxin against Naja and Bangarus caerulus venoms, wound healing, anti-convulsant, anti-oxidant, anti-helminthic, aphrodisiac and hepatoprotector [20]. The underlying mechanism of antidiabetic and antioxidants activity of *M. pudica* is totally the presence of the phytoconstituents.

A rising interest in the use of plant-based remedies in search for new therapeutic agents and employing cheaper and safer sources of medicine is the basis for choosing plants as an alternative. Medicinal plants may be used as food or special preparations [6,21].

2. Materials and Methods

2.1 Chemicals and Reagents

The chemicals and reagents that were used in the study were purchased from SIGMA-Aldrich corporation, Germany. Other materials were procured from local trusted suppliers. Other materials were sourced from the MAU-MCHS training laboratory.

2.2 Specimen Collection, Identification, Authentication

The leaves of *C. Citratum* and *M. pudica* were locally collected from areas around Malawi Adventist University and Malamulo Campus (MAU). *M. sylvestris* and *V. vinifera* fruit peels were obtained from fruits purchased from Makwasa market near Malawi Adventist University, Malamulo Campus. *Z. Africana* and *M. pruriens* were collected from Dedza district in the central areas of Malawi and Chiradzulu district in the southern region of Malawi respectively. All plant materials were identified and

authenticated by the National Herbarium and Botanical Gardens, Zomba, Malawi.

2.3 Pre-Extraction Processing and Extractions

2.3.1 Pre-Extraction Processing

The collected plant fruit peels, leaves and roots were immediately cleaned with running water. The peels from *M. sylvestris* and *V. vinifera* peels were separated manually from the fruits. The prepared plant parts except for *V. vinifera* fruit peels which were dried at 30°C in an oven-dried due to persistent humid weather, the rest of the plants were dried under a shade for at least 1 month at room temperature to prevent loss of volatile and heat-sensitive phytoconstituents from the samples [22].

After drying, the samples of *C. citratum, Z. africana, M. pruiens* leaves were ground to powder, and the parts of *V. vinifera* and *M. sylvestris* fruit peels were shredded into small pieces using sterile scissors. All processed plants were kept in an airtight container.

2.4 Extractions

Active ingredients of the plant materials were obtained by using the extraction methods described in literature with slight modifications [23]. In this procedure each of the powdered plant materials were mixed with distilled water at 10% W/V. The mixture was left for 24 hours at room temperature with intermittent agitation. After the extraction period, the mixtures were filtered with a sterile filter paper (Whatman no. 1 filter paper), to remove unhomogenized and larger particles to obtain clear aqueous extracts, the filtered extracts were refrigerated for further investigations [21].

2.5 Qualitative Screening of Phytochemicals

The plant extracts were qualitatively screened for the presence of Flavonoids, Saponins, Tannins, Terpenoids, Phenolics, Alkaloids, and Quinones using the powdered and reconstituted extracts according to standard methods described in literature, with slight modifications [24,23].

Flavonoids were screened by the addition of 3 to 5 drops of 20% Sodium Chloride (NaOH) to 1ml of each of the aqueous extracts. The formation of intense yellow colour was observed. Disappearance of the yellow colour after adding 3 to 5 drops of 32% hydrochloric acid (HCl) to the mixture, indicated the presence of flavonoids.

Saponins were screened by adding 5 mls of each of the reconstituted plant aqueous extracts to 2.5 ml of distilled water and shaken vigorously. Formation of persistent froth was observed indicating presence of saponins.

Tannins were screened by adding 0.25 g of the powder into 10 ml of distilled water and boiled in a water bath for 5 minutes and filtered. Few drops of 0.1 % ferric chloride ($FeCl_3$) were added to the filtrate and observed for brownish green or a blue-black coloration indicating presence of tannins.

Terpenoids were screened by dissolving 1 ml of each of the aqueous extracts into 2 ml of chloroform, followed by gradual addition of 3 ml of 1% sulphuric acid. The appearance of Red-brown colour coloration on the interface of the two liquids indicated the presence of terpenoides.

Phenolics were screened by adding 1 ml of each of the plant extract into 2 ml of distilled water followed by a few drops of 20% NaOH. Formation of blue green colour indicated the presence of phenols. In order to screen for alkaloids, 5 ml of 32% HCl was added to 1 ml of each of the reconstituted plant extracts. The mixture was then warmed in a water bath for 5 minutes. Formation of a red precipitate indicated the presence of alkaloids.

The presence of quinones in the extracts were screened by adding 1 ml of the reconstituted aqueous extracts gradually to 1 ml of concentrated Sulphuric acid. Formation of red colour indicated the presence of quinones.

2.6 Assay for Xanthine Oxidase Activity

Determination of XO activity was performed according to the method described in literature, where the substrate and the enzyme solutions were prepared immediately before use [6] with slight modifications. 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 °C for 15 minutes. Then 100 µl of substrate solution (0.15mM of xanthine) was added into the mixture and incubated at 37 °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 °C using a UV spectrophotometer. Enzyme activity was determined using the formulae according to the literature [22].

Enzyme activity = $(\Delta abs .V_t)/ (\varepsilon. t. Ve)$

Where Δ Abs 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 [6].

2.7 Xanthine Oxidase Interaction Assay

The inhibitory effects of the reconstituted aqueous extracts on XO activity was assessed spectrophotometrically at 295 nm using a UV spectrophotometer under aerobic conditions, measuring the uric acid formation under aerobic conditions. Prior to the assay, the enzyme and *V. Vinifera, M. sylvestris, C. citratum, Z. Africana, M. pudica* and *M. pruriens* reconstituted 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-extracts pre-mixture, the reaction mixture was pre- incubated at 37 °C 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 terminated 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 activity using the formula below.

Where: β = Activity of enzyme with extract and α = Activity of enzyme without extract.

$$XOI\ \% = \left(1 - \frac{\beta}{\alpha}\right) \times 100$$

3. Results

3.1 Phytochemical Screening

The qualitative phytochemical screening tests showed the presence and absence of different compounds as per the interpretations of various screening tests explained in section 2.3.4 above as summarised in table 1 below.

Plant	Alkaloids	Flavonoids	Phenolics	Saponins	Tannins	Terpenoids
C. citratum	+	+	+	-	-	+
M. sylvestris	+	+	+	-	-	+
V. vinifera	+	+	-	+	+	+
M. Pruriens	+	+	+	+	-	+
Z. Africana	+	+	-	+	ND	+
M. pudica	-	+	-	+	+	+

Key: (+) = presence of phytochemicals; (-) = absence of phytochemicals; and ND = Not done

Table 1: Phytochemical Screening Results

3.2 Enzymatic Activity

XO activity assay carried out as described in section 2.3.5 at three different time intervals as a negative (base line) control exhibited enzyme activity of 0.431U/ml at 15 min, 0.461U/ml at 30 min and 0.500 U/ml at 45 min respectively. These obtained results were used as baseline values for the calculations of positive control (allopurinol) and enzyme inhibition studies.

3.2 Interaction Studies

The experimental data showed that allopurinol increasingly inhibited XO activity as incubation time also increased from 0.431U/ml to 0.194 U/ml at 15 min representing 60% enzyme inhibition, from 0.461 U/ml to 0.148 U/ml at 30 min representing 70% inhibition and from 0.500 U/ml to 0.102 U/ml at 45 min representing 80% inhibition respectively. XO enzyme interaction studies showed significant inhibitory effects on XO activity *C. citratum* exhibited an inhibitory effect on XO activity by about 70% at 15, 30 and 45 minutes. *M. sylvestris* reduced XO enzyme activity by 68% at 15 min, 61% at 30 min and 64% at 45 min. *V. vinifera* exhibited inhibitory effects on XO activity by 75% at 15 min, 66% at 30 min and 77% at 45 min respectively, *Z. Africana* inhibited XO activity by 73% at 15 min, 65% at 30 min and 74% at 45 min respectively. *M. pudica* exhibited an inhibitory effect on XO activity by 74% at 15 min, 77% at 30 and 45 min respectively and *M. pruriens* inhibited XO activity by 65% at 15 min, 69% at 30 min and 71% at 45 min respectively. A summary is as presented in table 2 below with graphical representation in figure 1 and figure 2 respectively. The statistical analysis of the results obtained are as summarised in table 3.

Plant	Time (Min)	Absorbance	Enzyme Activity (U/mL)	Inhibition %
Enzyme without inhibitor (Neg control)	15	0.607	0.431	0
	30	0.652	0.461	0
	45	0.705	0.500	0
Allopurinol: Pos control	15	0.224	0.194	59.92
	30	0.186	0.148	70.16
	45	0.128	0.102	80.62
C. citratum leaf	15	0.16	0.127	70.5
	30	0.168	0.134	70.9
	45	0.187	0.149	70.2
M. sylvestris peels	15	0.17	0.136	68.4
	30	0.225	0.179	61.2
	45	0.224	0.178	64.4

V. vinifera peels	15	0.135	0.107	75.2
	30	0.192	0.153	66.8
	45	0.145	0. 116	76.8
Z. africana leaf	15	0.243	0.174	59.6
	30	0.253	0.181	60.7
	45	0.327	0.234	53.2
Z. africana root	15	0.157	0.113	73.8
	30	0.225	0.161	65.1
	35	0.203	0.13	74.0
M. pudica	15	0.154	0.110	74.4
	30	0.148	0.106	77.0
	45	0.157	0.113	77.5
M. pruriens	15	0.212	0.152	64.7
	30	0.203	0.145	68.5
	45	0.199	0.143	71.4

Table 2: Inhibitory Activity of 10 mg/ml Plant Extract on Xanthine Oxidase Activity

Test sample	In relation to mean Abs		In relation to mean I%		Mean Inhibition difference (%)
	t-value	p-value	t-value	p-value	
C. citratum fruit	6.3138	0.3443	6.3138	0.2726	0.5
M. sylvestris fruit	6.3138	0.1272	6.3138	0.08935	-5.5
V. vinifera leaf	6.3138	0.1420	6.3138	0.0204	2.7
Z Africana leaf	6.3138	0.1466	6.3138	0.1443	-12.3
Z Africana roots	6.3138	0.0974	6.3138	0.0423	0.8
M. pudica leaf	6.3138	0.0628	6.3138	0.3862	6.1
M. pruriens roots	6.3138	0.1752	6.3138	0.1933	-2.0

Table 3: 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.

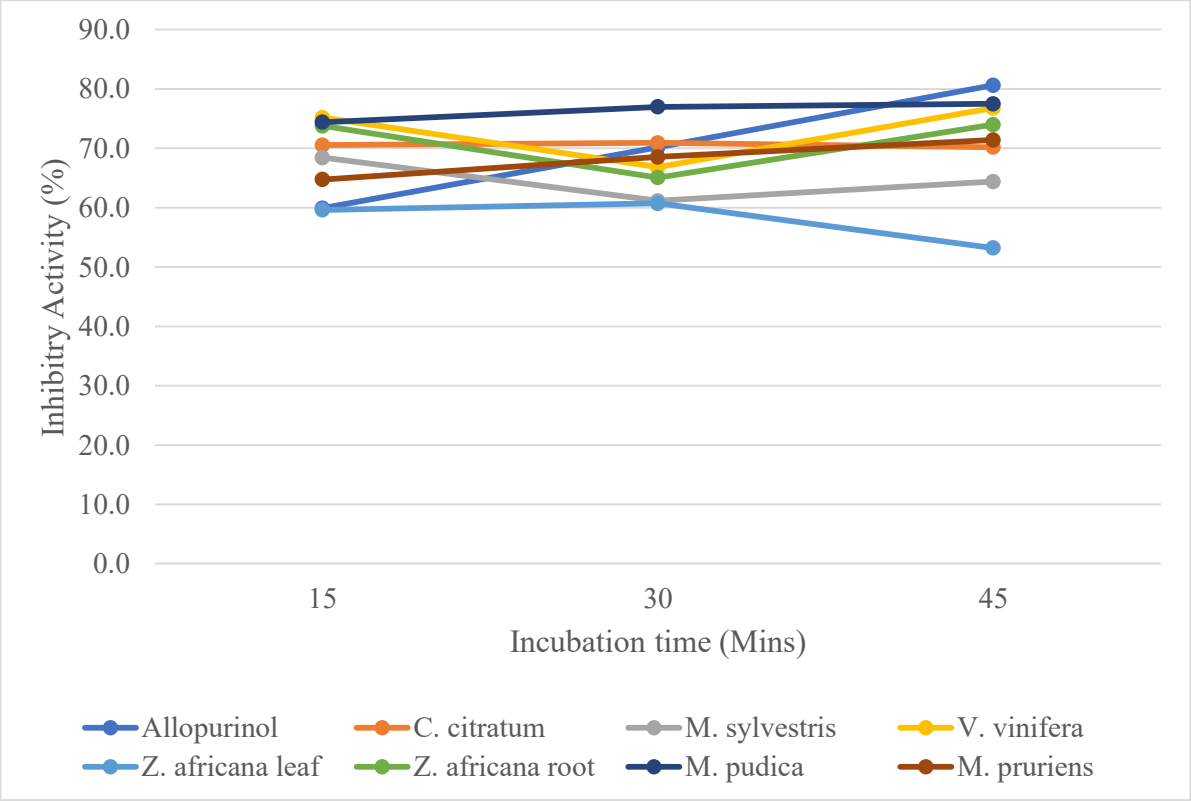

Figure 1: Linear graph of Inhibitory activity against Incubation Time

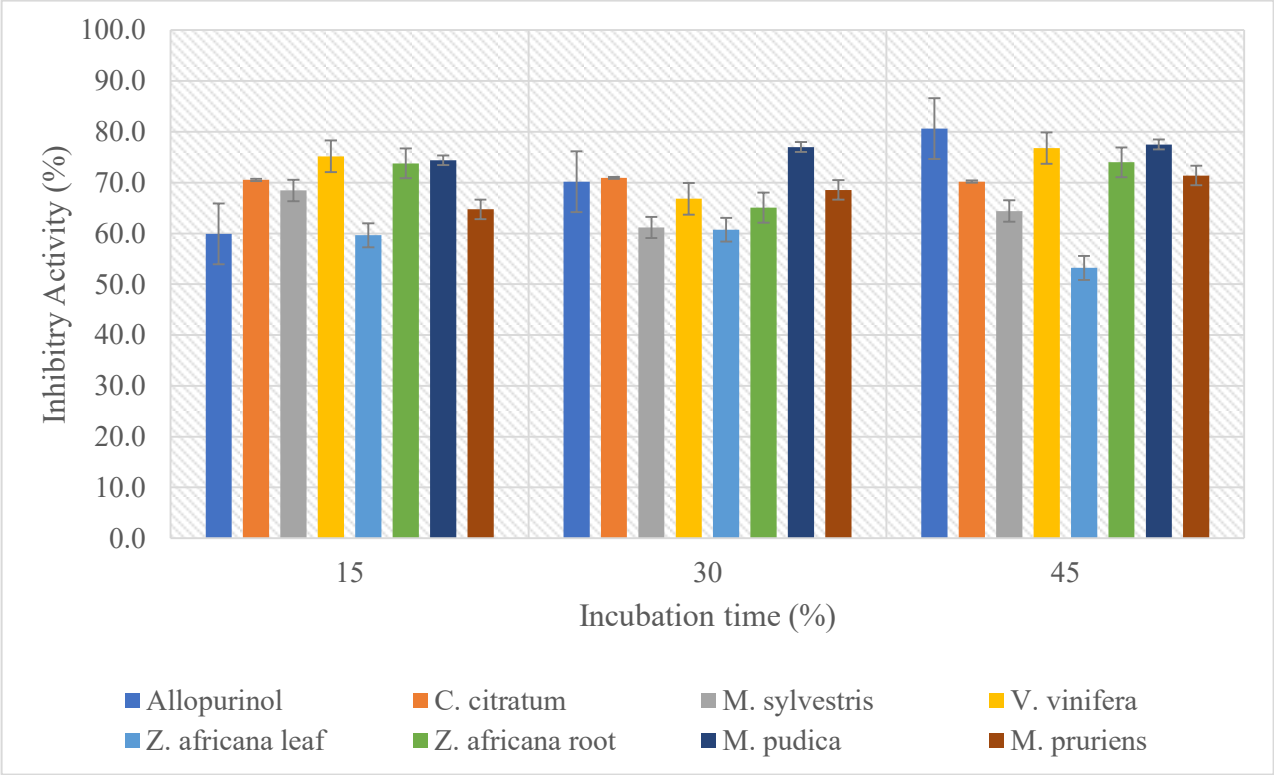

Figure 2: A bar graph of Inhibitory activity against Incubation Time

4. Discussion

Increased gouty and sometimes recurrent arthritis has been associated with overproduction of uric acid. Several approaches in the treatment of gout have been reported, one of them being the utilisation of Xanthine Oxidase inhibitors such as allopurinol and febuxostat. These substances inhibit the enzyme (XO) thereby reducing the production of uric acid. A constant and systematic search for XOI which includes testing of plant extracts as potential sources [25].

M. citratum leaf extracts revealed the presence of flavonoids, alkaloids, phenols, tannins, glycosides, and absence of terpenoids, quinones and saponins. The results from the present study have shown to differ from those reported in literature. as it showed that tannins and terpenoides which were present in their study were absent in the present study and terpenoids were present. It may be explained by differences in the sources of the plants and time of specimen collection [26].

M. sylvestris peel extracts revealed the presence of alkaloids, flavonoids, saponins, terpenoids and tannins; and the absence of phenolics which is in disagrees with the results from Zulkifli, et al., 2012 who found the absence of tannins in M. sylvestris [27].

V. vinifera peel extracts revealed the presence of alkaloids, flavonoids, saponins, terpenoids and tannins; and the absence of phenolics which is in agreement with the results from those reported in literature [28].

M. pudica revealed the presence of tannins only but not saponins, flavonoids and quinones. On the contrary, there are reports that flavonoids, saponins, tannins and terpenoids were present and the absence of alkaloids. However, there is an agreement between this study and that of Ranjan and colleagues, where they both report the absence of alkaloids [29].

M. pruriens revealed, as in agreement with those reported elsewhere, the presence of alkaloids, flavonoids, phenolics, saponins and terpenoids, but an absence of tannins. However, tannins were not screened for in the study [30].

It is apparent from experimental data from this current study that aqueous extracts of a *C. citratu*m showed an inhibitory effect on Xanthine oxidase activity. Remindful of the biological properties of secondary metabolites present in plants. The obtained inhibition of *C. citratu*m is in agreement with XO inhibition reported in literature [31]. In this study it showed that oil extracts from C. citratum stalk showed promising anti gout properties by inhibiting XO was as high as 81.34%, as compared to XOI observed with oils extracted from the leaves. This observed inhibition may explain the observations made by [32] where there was a decrease in blood uric acid levels in male Wilstar rats fed with lemongrass herbal steeping.

Excitingly, results obtained in this research indicate that the aqueous extracts of *M. sylvestris* fruit peels exhibited a significant inhibitory effect on XO as compared to those obtained within our laboratory where an inhibitory effect of 90% was observed with *M. Pumil*a, as reported by [6], is higher than 28% observed elsewhere [33].

Effects of *V. vinife*ra assessed in the current study revealed that the aqueous extracts of the fruit peels exhibited comparable inhibitions to those reported by various researchers [34,35]. In these studies, it is reported that *V. vinifera* contains secondary metabolites that exhibit XO inhibition properties.

There is scarcity of information with regards to the inhibitory effect of Z. africana. However, its use as a treatment for painful arthritis as reported by other researchers may be associated with the inhibition of XO enzyme activity revealed by the present study [19].

The results obtained in this study of *M. pudica* is comparable with those reported in literature [36] where *M. pudica* exhibited an inhibitory effect of 33.98%, 62.26% and 89.87% at 50 µg/mL, 100 µg/mL and 200 µg/mL concentration respectively after 30 minutes incubation time. The variation between the two studies may be subjected to differences in the extraction solvents used [36].

No study has shown the inhibitory effects of *M. pruriens* on XO, however reports indicates that the use of *M. pruriens* in gout treatment may be attributed to the observed XO inhibition observed in this study [16].

5. Conclusion and Recommendation

The results obtained in this study indicate that aqueous extracts of the secondary metabolites extracted from *Vitis vinifera, Malus sylvestris* fruit peels, *Cymbopogon citratum* leaves, *Mucuna Pruriens* leaves, *Zanha africana* roots and leaves, *Momodica* pudica roots extracts possess notable inhibitory effects on Xanthine Oxidase activity. The significant inhibitory effects observed from the plant extracts may be helpful in the development of unique antigout agents.

Declarations

Ethical Approval

The current research was approved by the National Health Sciences Research Committee (NHRSC) and Malawi Adventist University Research Committee. *C. citratum, M. Sylvestris V. vinifera, M. pruriens, Z. africana and M. pudica* were identified by a botanist at the National Herbarium and Botanical Gardens of Malawi. All methods were carried out using relevant guidelines and regulations.

The NHSRC and Malawi Adventist University Research Committee gave permission to collect samples of C. citratum, M. sylvestris, V. vinifera, M. pruriens, Z. africana and M. pudica.

Consent to Participate

Not applicable

Consent for Publication
Not applicable

Data Availability
All data sets used and/or analysed during this study are available from the corresponding
author on reasonable request

Competing Interests
The authors declare that they have no competing interests

Funding
The authors received no financial assistance from any other body, instead the project was self funded.

Acknowledgements
The authors would like to acknowledge National Herbarium and Botanical gardens of Malawi for their expertise in the identification and authentication of the plants.

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