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Evaluation of inhibitory activity of aqueous, methanol and ethyl acetate extracts of *Citrus aurantifolia* leaf on crude xanthine oxidase isolated from cow milk

Waheed, S. A.*, Chioma, C. F., Ibraheem, Y. O., Sadiq, M. G.

Department of Biochemistry, Federal University of technology Minna, Niger State, Nigeria

* For correspondence: waheedsackson@gmail.com

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ABSTRACT

Lime (*Citrus aurantifolia*) is a citrus fruit from the Rutaceae family which is used in beverages, as food additives and in cosmetic industries. *Citrus aurantifolia* plant is used in the treatment of several complications including anemia, oxidative damages and inflammation. Xanthine oxidase is the enzyme that catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid. This study was aimed at determining the inhibitory activity of aqueous, methanol and ethyl acetate extract of *Citrus aurantifolia* leaves on xanthine oxidase isolated from cow milk. The inhibitory activities of the three extract at different concentrations (20, 40, 60, 80 and 100 µg/ml) was analyzed at 290 nm, pH 7.3 and at 25°C using spectrophotometric methods. Line Weaver-Burk plots were also used to determine enzyme kinetic constants. The aqueous extract of *Citrus aurantifolia* leaves has maximum velocity (Vmax); 0.05 U/ml and Michaelis-Menten constant (Km) value of 510.64 µM. The Vmax and Km value for the methanolic extract were 0.02 U/ml and 86.05 µM, while ethyl acetate extract has Vmax 0.03 U/ml and Km value of 87.84 µM. Compared to allopurinol (6.51±0.01 µg/mL), the IC_{50} of aqueous extract, methanol extract and ethyl acetate extracts were 14.68±0.13 µg/mL, 16.08±0.18 µg/mL and 19.13±0.25 µg/mL respectively. The study showed that the extracts can be used as a good source of natural xanthine oxidase inhibitors and this can also be used as alternative herbal drugs in the management and treatment of gout.

Keywords: *Citrus aurantifolia*, Xanthine oxidase, Uric acid, Cow milk

1. INTRODUCTION

Xanthine oxidase is an important enzyme catalyzing the oxidation of hypoxanthine to xanthine and xanthine to uric acid [1]. Over activity of xanthine oxidase results in the medical condition known as gout, which is characterized by hyperuricemia that leads to uric acid deposition in the joints resulting in painful inflammation [2]. Xanthine oxidase (XO) serves as an important biological source of oxygen-derived free radicals that contribute to the oxidative damage of living tissues [3]. Free radicals are extremely reactive and generally highly unstable chemical species [4]. They have been regarded as the fundamental cause of different diseases, including aging, coronary heart disease, inflammation, stroke,

Parkinson's disease, diabetes mellitus, rheumatism, liver disorders, renal failure, cancer, and possibly Alzheimer's disease In addition to exogenous sources of free radicals, such as ionizing radiation, tobacco smoke, pesticides, pollutants, and some medications, free radicals are produced continuously in all cells, as metabolic by-products, by a number of intracellular systems [5].

Hyperuricemia, which is present in 5–30% of the general population, seems to be increasing worldwide and is considered an important risk factor in serious disorders, e.g. renal failure [6]. Uricosuric drugs which increase the urinary excretion of uric acid, or xanthine oxidase inhibitors which block the terminal step in uric acid biosynthesis, can lower the plasma uric acid concentration, and are generally employed for the treatment of gout [7]. Allopurinol is the only clinically used XO inhibitor in the treatment of gout. However, this drug suffers from many side effects such as hepatitis, nephropathy, and allergic reactions [8]. Thus, the search for novel XO inhibitors with a higher therapeutic activity and fewer side effects are desired not only to treat gout but also to combat various other diseases associated with the XO activity [9].

Citrus aurantifolia belongs to family of plants known as Rutaceae, it is a poly-embryonic plant cultivated in several part of the world especially hot subtropical or tropical region such as India, USA, Nigeria, Mexico, and Egypt [10]. Citrus fruits are well known for their antioxidant actions due to high content of ascorbic acid and other secondary plant metabolites such as flavonoids, terpenoids [11]. The plant is used in traditional medicine for treatment of several diseases such as cold and stomach ailment. It can also be used as an antiseptic, mosquito repellant, antifungal, antibacterial and antiviral agent. The health benefits of *Citrus aurantifolia* plant are highly associated with the high amount of bioactive constituents such as phenols, flavonoids, carotenoid, vitamins and minerals [12]. These constituents are capable of functioning as antioxidant and thus can play an important role in prevention of degenerative diseases such as cancer, Alzheimer, and Parkinson's disease caused by oxidative stress.

However, many medicinal plants have been used for the prevention and treatment of hyperuricemia and related inflammatory disorders, but they lack sufficient scientific evidence. Because there is minimal evidence that citrus leaves can inhibit xanthine oxidase, this research will focus on determining the inhibitory activity of aqueous, methanol and ethyl acetate extracts of *Citrus aurantifolia* leaf on crude xanthine oxidase isolated from cow milk.

2. MATERIALS AND METHODS

2.1. Samples collection

Cow milk was collected from Maizube Farms, Minna, Niger State. The fresh cow milk collected was stored in refrigerator (preserved) at 4°C to avoid spoilage before further analysis. The *Citrus aurantifolia* leaves were obtained from Agwam-Biri Farm Area, Bosso Local Government Area, Minna, Niger State.

2.2. Samples Identification

The cow milk collected from Maizube farm was screened and identified at the Department of Agriculture, FUTMINNA for a safe use for extraction of xanthine oxidase enzyme. The fresh leaves collected were also identified at the Department of Biology, FUTMINNA and it was proofed to be safe for scientific analysis.

2.3. Preparation of Samples

The freshly collected *Citrus aurantifolia* leaves were air dried at room temperature for two weeks, powdered using electric blending machine, and the powdered leaves were further stored before extraction process [12].

2.4. Isolation of xanthine oxidase from cow milk

Procedure for isolation of xanthine oxidase from cow milk was adapted from Gandhi *et al.* (2020) with some modifications [12]. Fresh cow milk (1000 mL) was centrifuged at 5000 rpm for 30 minutes to separate the cream. The cream (50 g) was agitated for 90 minutes in 250 mL of 0.2 M sodium phosphate solution containing EDTA. The suspension mixture was further centrifuged for 15 minutes at 5000 rpm and the supernatant was collected into a clean test tube. The supernatant was mixed with 37 mL of 15 % cold butanol and 15 % ammonium sulfate was then slowly added into the mixture followed by stirring for 1 hour. The whole supernatant was then centrifuged at 5000 rpm for 15 minutes and the supernatant was also collected into a clean test tube. Furthermore, 20 % ammonium sulfate was added slowly to the supernatant followed stirring for 30 minutes and the mixture was allowed to stand overnight. After standing overnight, the mixture was then centrifuged at 2500 rpm for 30 minutes and the precipitate was collected. Therefore, the enzyme precipitate was obtained, weighed (28 g) and preserved for further analysis.

2.5. Determination of xanthine oxidase activity

Xanthine oxidase activity was determined by checking the conversion of xanthine to uric acid by monitoring the change in absorbance at 293 nm, using UV–Visible Spectrophotometer. One milliliter (1mL) of 0.15 mM xanthine solution was measured into a clean test tube and 1.9 mL of monobasic sodium buffer (pH 7.3) was pipette into the test tube. The assay was initiated by the addition of 0.1 mL of crude isolated enzyme solution and mixing by inversion. After every 10 minutes for 1 hour, the increase in absorbance of the mixture (the amount of uric acid liberated due to the enzyme catalyzed reaction) was measured using distilled water as blank [13]. One unit of enzyme activity was defined as the amount of enzyme that converts 1 mmol of xanthine to uric acid per min under defined conditions.

2.6. Preparation of aqueous, methanol and ethylacetate extract of C. aurantifolia Leaves

Procedure for preparation of the extracts was adapted from Omede, (2016) with some modification [14]. Fresh leaves of. *C. aurantifolia* were extracted using soxhlet apparatus. Fifty grams (50 g) of powdered *C. aurantifolia* was inserted into soxhlet apparatus with 1 L of 80% (v/v) methanol-water. The extraction was carried out for 72 hours and preserved for further analysis. Ethyl acetate and aqueous extract of powdered *C. aurantifolia* were prepared by placing 7.91 g of methanol extract in a beaker followed by the addition of 50 mL of distilled water and 600 mL ethyl acetate. The mixture was emptied into a separating funnel, allowed to stand for few hours into two distinct layers (ethyl acetate and aqueous). The layers were collected into separate beaker and it was further concentrated for 3 days using the water bath. The ethyl acetate extract and aqueous extract were weighed, 6.38 and 0.08 g respectively. The three extracts were used for further inhibitory studies.

2.7. Inhibitory activity of Aqueous, Methanol and Ethyl acetate extracts on crude xanthine oxidase

The assay mixture consisted of 1 mL of the fractions (20, 40, 60, 80 and 100 µg/mL), 2.9 mL of phosphate buffer (pH 7.5) and 0.1 mL of XO enzyme solution (0.1 unit/mL in phosphate buffer, pH 7.5), which was prepared immediately before use. After pre-incubation at 25°C for 15 mins, the reaction was initiated by addition of 2 mL of the substrate solution (150 mM xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 mins. The reaction was then stopped by the addition of 1 mL of 1N hydrochloric acid and the absorbance was measure at 290 nm using a UV-Spectrophotometer [16]. Proper controls with DMSO were carried out. Allopurinol

(20, 40, 60, 80 and 100 µg/ml), a known inhibitor of xanthine oxidase, was used as the standard.

Xanthine oxidase inhibitory activity was expressed as percentage inhibition of xanthine oxidase in the above assay system calculated as

$$\text{Inhibition (\%)} = \left(1 - \frac{A}{B} * 100\right) \quad \text{...eq. 1}$$

Where;
A= activity of enzyme without plant extract
B= activity of enzyme in the presence of plant extract

The enzymatic inhibitory mode of action of different extracts was determined by the Line Weaver Burk plot using varying concentration of substrate (xanthine). The assay mixture consisted of 1 mL (100 µg/mL) each of the extracts was mixed with 2.9 mL of phosphate buffer and 0.1 mL of XO enzyme at pH 7.5. The mixture was incubated at 25°C for 15 mins, and the reaction was initiated by addition of 2 mL of the substrate solution (0.1, 0.15, 0.2, 0.25 and 0.3 mM xanthine). The mixture was further incubated at 25°C for 30 mins, and 1 mL of 1N hydrochloric acid was added to stop the reaction followed by measuring the absorbance at 290 nm using a UV-Spectrophotometer [16]. The procedure above was carried out in the absence of the extract (negative control). Allopurinol (100 µg/mL) was used as positive control.

2.8. Data analysis

Data obtained were analysed using SPSS and Microsoft Excel and results were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was carried out as $p<0.05$ considered statistically significant. Duncan's multiple range test (DMRT) was used to compare means values of test groups and controls as well as differences within group mean of the various test groups.

3. RESULTS AND DISCUSSION

3.1. Percentage yield of C. aurantifolia fractions

The table 1 below shows the percentage yield of crude and fractionated extracts of powdered *Citrus aurantifolia* leaf.

3.2. Effects of reaction time on Xanthine oxidase activity

Figure 1. shows the effect of time on xanthine oxidase activity. As depicted in the graph, xanthine oxidase activity was observed to be time-dependent as there was increase in xanthine oxidase activity with increase in time. Gout and hyperuricemia are on the rise around the world, according to new research, potentially due to changes in dietary patterns, such as increased consumption of nucleic acid-rich foods. Xanthine oxidase inhibitors and uricosuric medicines are examples of hypouricemic pharmaceuticals that are routinely used to

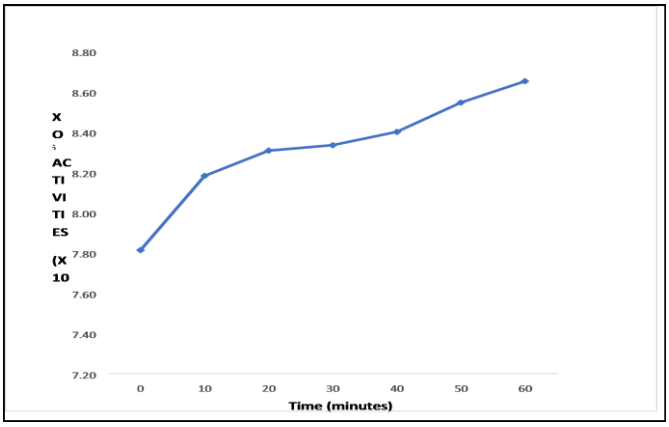

Figure 1. Effect of Time on the activity of crude xanthine oxidase isolated from cow milk

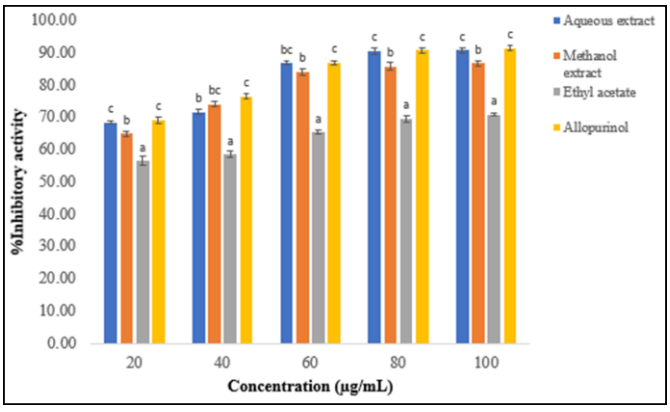

Figure 2. Percentage inhibitory activities of the aqueous, methanol and ethyl acetate extracts on xanthine oxidase

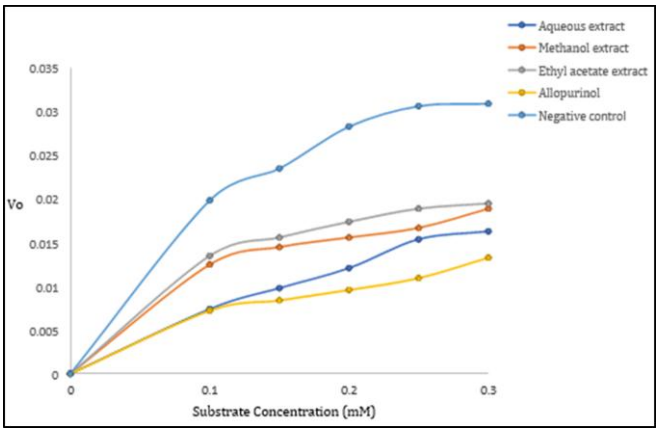

Figure 3. Michaelis-Menten plot of xanthine oxidase in the absence and presence of inhibitors

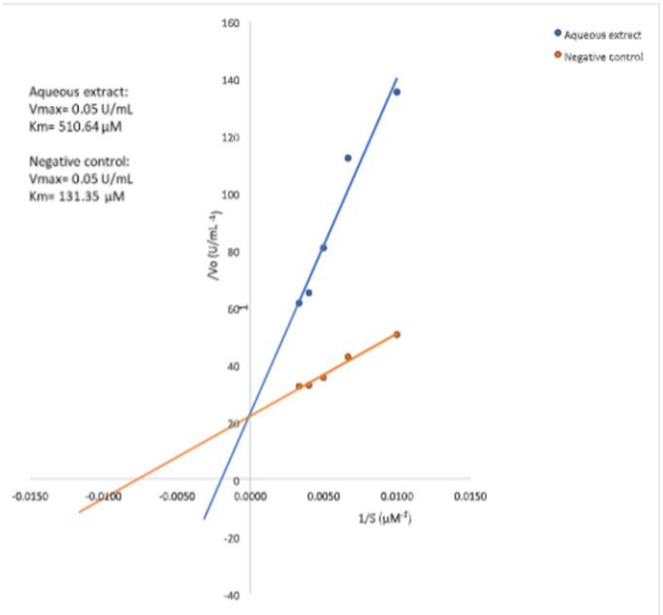

Figure 4. Line weaver-Burk plot of inhibition of xanthine oxidase by the aqueous extract

treat and prevent hyperuricemia and gout. Allopurinol is the medicine of choice in general; nonetheless, it has been noted that it has negative effects such as allergic responses amongst others. An alternative to allopurinol is the use of medicinal plants. The leaves of *C. aurantifolia* were selected based on ethno-pharmacological evidence [17]. The leaves are said to contain bioactive phytochemicals; like flavonoids and terpenoids which have anti-inflammatory, anti-oxidants, anti-diabetic, anti-parasitic and neuroprotective properties and have been used traditionally for treatment of cardiovascular, hepatic, and also for insecticide activity.

3.3. Percentage inhibitory activities of aqueous, methanol and ethyl acetate extracts of C. aurantifolia leaves on xanthine oxidase isolated from cow milk

As shown in Figure 2. the extracts exerted significant inhibitory effects on the xanthine oxidase in a concentration-dependent manner. The aqueous, methanol and ethyl acetate were found to have xanthine oxidase inhibitory activities highest at a concentration of 100µg/ml. The highest xanthine oxidase inhibitor activity was the aqueous extract with (90.41±1.3%) with an IC_{50} value of 14.68±0.13. The percentage inhibitory effects of methanol extract (83.32±2.46%) with an IC_{50} value of 16.08±0.18 µg/mL was significantly higher than that of ethyl acetate extract (65.38±1.89%), which had an IC_{50} value of 19.13±0.25 µg/mL. IC_{50} for allopurinol is 6.51±0.01 µg/mL.

3.4. Michaelis-Menten plot of xanthine oxidase inhibition of fractionated extracts

Figure 3.3 shows a Michaelis-Menten plot of inhibition of xanthine oxidase activity by the aqueous, methanol and ethyl acetate extracts. The two extracts as well as allopurinol significantly reduced both the Vmax and Km of the xanthine oxidase. However, the ethyl acetate extract exerted more inhibitory effects on the xanthine oxidase than the methanol extract. The flavonoids, which are group of polyphenolic compounds has been reported to possess xanthine oxidase inhibitory activity.

3.5. Double reciprocal plot of xanthine oxidase inhibition

Table 1. Range, Mean, P-value of Physicochemical Parameters of Surface water in Karshi reservoir

Fractions	Sample weigh (g)	Extract yeild %
Methanol	50	19.82
Ethyl acetate	7.91	80.66
Aquoeus	7.91	1.01

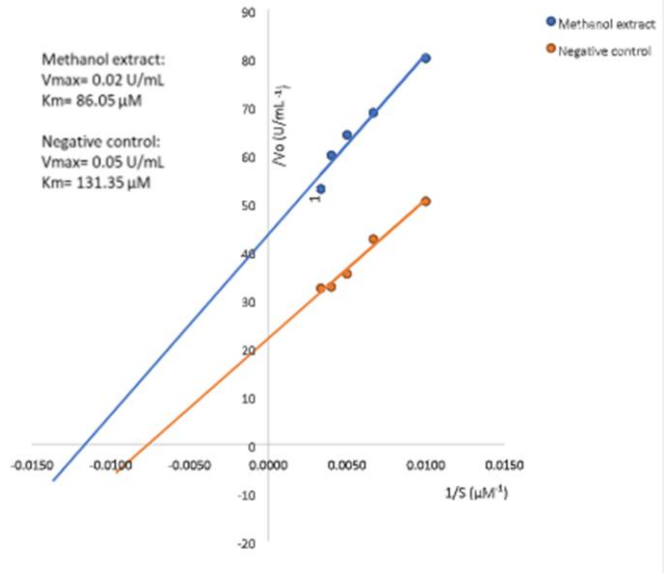

Figure 5. Line Weaver-Burk plot of inhibition of xanthine oxidase by the methanol extract

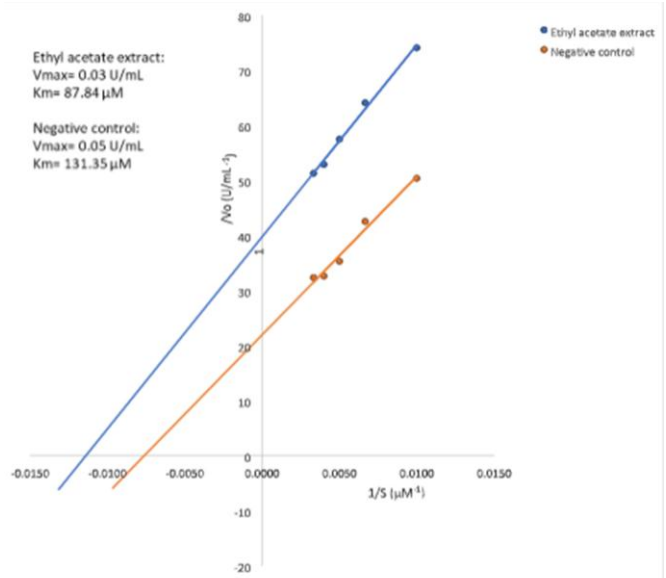

Figure 6. Line weaver-Burk plot of inhibition of xanthine oxidase by the ethyl acetate extract

by ethyl acetate extract

The double reciprocal plot of xanthine oxidase inhibition by aqueous extract is shown in Figure 3.4. The maximum velocity (Vmax) was unchanged while Km increased significantly.

3.6. Double reciprocal plot of xanthine oxidase inhibition by methanol extract

The double reciprocal plot of xanthine oxidase inhibition by methanol extract is shown in Figure 3.4. There in, the methanol extract reduced both Vmax and Km of the xanthine oxidase from 0.05 U/mL, and 131.35 µM to 0.02 U/mL and 86.05µM respectively.

3.7. Double reciprocal plot of xanthine oxidase inhibition by ethyl acetate extract

The inhibitory effect of the ethyl acetate extract on the xanthine oxidase was observed to be similar to that of methanol extract. The ethyl acetate extract reduced the Vmax and Km from 0.05 U/mL, and 131.35µM respectively to 0.03U/mL and 87.84µM respectively.

The crude xanthine oxidase was isolated from fresh cow milk. The xanthine oxidase inhibitory activity of aqueous, methanol, and ethyl acetate extracts of *C. aurantifolia* leaves were investigated in this work. The percentage yield was 19.82, 80.66 and 1.01 % from methanol, ethyl acetate and aqueous extract respectively as seen in table 1. The activity of xanthine oxidase as a function of reaction time is shown in figure 1. and shows an increase in enzyme activity with time this correlates with work done by Sainn *et al.,* (2019), who reported an increase in xanthine oxidase activity from 15 min due to an increase of product formation (uric acid) with incubation time [14].

Percentage inhibitory studies on the various extracts showed that the three extracts exhibited maximum inhibitory activities at 100 µg/ml concentration with no significant difference ($p>0.05$) between 60, 80 and 100 µg/ml concentration of each extracts as seen in figure 2. The IC_{50} of the three extracts showed variation from the standard allopurinol (6.51+0.01 µg/ml) with ethyl acetate extract having IC_{50} value of (19.13+0.25 µg/ml), methanol extracts having IC_{50} value (16.08+0.18 µg/ml) and aqueous extract having lowest IC_{50} value of (14.68+0.13 µg/ml). This is similar to work done by McMullen *et al.* (2018) who documented the IC_{50} of the

aqueous extract of *C. aurantifolia* to be (16.67 µg/ml) and it was found to be more potent than the other extracts [18].

The effect of substrate concentrate on reaction velocity is shown in figure 3., there was increase in velocity of reaction with increase in substrate concentrations until maximum velocity of enzyme is attained. Line-Weaver Burk plot of inhibition of xanthine oxidase shows increase and decrease in kinetic constant (Km) and Vmax respectively. The Michaelis-Menten constant (Km) in the absence of the extract (negative control) was 131.35 µM and maximum velocity (Vmax) of (0.05 U/mL). The aqueous extract with Km of 510.64 µM and Vmax of 0.005 U/mL showed a competitive mode of inhibition as shown in figure 4. with increased Km value, while the Vmax remained unchanged [19]. This happens when the inhibitor competes with the substrate for the active site of the enzyme. The methanol extract with Km of 86.05 µM; Vmax of 0.02 U/mL and ethyl acetate extract with Km of 87.84 µM; Vmax 0.03 U/mL showed an uncompetitive mode of inhibition on the enzyme as seen in figure 5. and 6. above with a decrease in Km and Vmax value. The inhibitor binds to the enzyme substrate complex at an allosteric site.

From these findings, the aqueous, methanol and ethyl acetate extract of *C. aurantifolia* leaves were found to be an alternative herbal drug at higher doses for inhibition of XO enzyme and the inhibitory properties could be due to the presence of flavonoids and other polyphenols.

4. CONCLUSION

From the results obtained from this research, it can be concluded that *C. aurantifolia* extracts (most especially the aqueous extract) have significant inhibitory activities on xanthine oxidase and can be useful for the treatment of hyperuricemia and gout. The inhibitory activity of the *C. aurantifolia* extracts can be attributable to the polyphenol contents and they can be used to combat other complications that are related to xanthine oxidase activity

5. RECOMMENDATION

To identify a possible chemical entity for clinical usage and therapeutic drug development, more research, phytochemical screening, isolation and extraction of active compounds present in the extracts is required. More so, in vivo studies is recommended.

6. ACKNOWLEDGEMENT

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7. CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

8. SOURCE/S OF FUNDING

NA

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