RESEARCH ARTICLE

Antihyperuricemic activity of polar fractions of Pili (*Canarium ovatum*) leaves

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ABSTRACT

Background: One of the causes of inflammatory arthritis is excessive production of uric acid or hyperuricemia. It is a painful disease that is treated with a commercial xanthine oxidase inhibitor to decrease uric acid synthesis. However, the treatment is associated with adverse side effects and thus, there is interest in medicinal plants that could have similar therapeutic effects with minimal side effects. There are many reported indigenous plants and trees in the Philippines that are reported to have therapeutic and bioactive compounds. One such plant is *Canarium ovatum* or locally called pili. This study aimed to determine the antihyperuricemic activity of the ethanolic extract of the leaves of C. ovatum.

Objective: Determine the antihyperuricemic activity of the crude ethanolic extract of *C. ovatum* leaves and its partially purified fractions through inhibition of xanthine oxidase and its effect on the blood uric acid level of oxonate-induced hyperuricemic mice.

Methodology: The crude ethanol extract from *C. ovatum* leaves and its partially purified fractions obtained through column chromatography were tested for their in vitro xanthine oxidase (XO) inhibitory activity by measuring spectrophotometrically the uric acid formation from xanthine as the substrate. The crude ethanol extract and the fraction with the most XO inhibitory activity were then tested for their *in vivo* XO inhibitory activity in oxonate-induced hyperuricemic mice by measuring their blood uric acid levels using uric acid test strips.

Results: The crude ethanolic extract of *C. ovatum* leaves at 100ppm showed 83.62±2.05% *in vitro* inhibition of XO while the most active fraction showed 80.30±4.00% inhibition. Both were comparable ($p>0.05$) to the positive control, allopurinol, which showed 91.47±5.64% inhibition. *In vivo*, the crude extract and the fraction that showed the highest XO inhibitory activity at 200 mg/kg significantly ($p<0.01$ and $p<0.05$) respectively reduced the serum uric acid levels of the hyperuricemic mice one hour after induction as compared to the negative control. Moreover, their antihyperuricemic activity were not statistically significant as compared to that of allopurinol ($p<0.0001$).

Conclusion: The crude ethanolic extract of *C. ovatum* leaves and its most active fraction showed statistically significant *in vitro* xanthine oxidase inhibition and *in vivo* antihyperuricemic activity. The activities shown by both crude and active fraction were not statistically different from that determined for allopurinol. Therefore, further studies can be conducted to isolate the most active compound and study its pharmacokinetic properties.

Keywords: *Canarium ovatum, antihyperuricemia, xanthine oxidase, uric acid, allopurinol*

Introduction

Hyperuricemia is a condition in which the body has high levels of uric acid. It is strongly associated with gout which is one of the common inflammatory arthritis in the world. It is caused by the overproduction and/or under excretion of uric acid [1,2]. The excess uric acid is deposited in the synovial fluid of the joints, usually in the feet, in the form of insoluble sodium urate crystals which consequently results in an inflammatory response [3,4]. It is characterized by chronic pain, joint damage, and tophi formation [5,6]. It also has risk factors such as genetics, alcohol use, obesity, lifestyle, and diet with high amounts of purine [7].

The prevalence of gout and hyperuricemia in the world has been reported to be increasing in recent epidemiological

studies [5]. In the Philippines, the prevalence of gout continues to increase for the past two decades and is currently affecting about 1.6 million Filipinos [8]. Filipinos who have gout are more prone to other cardiovascular and kidney conditions [9].

Xanthine oxidase (XO) is one of the enzymes responsible for the formation of uric acid from the purine catabolic pathway. Thus, one way of treating gout and hyperuricemia is the use of drugs that inhibit this enzyme. A commercially available XO inhibitor is allopurinol. However, its use has been associated with adverse effects such as hepatitis, vasculitis, hypertension, fever, and allergic reactions [10]. Hence, there is an interest in finding XO inhibitors that may have lesser or no adverse effects.

In recent studies, plants used in traditional medicine have been reported to have significant therapeutic effects against conditions such as diabetes, fever, kidney stones, gout, and inflammation. Their activities are attributed to phytochemicals such as flavonoids, triterpenoids, alkaloids, and phenols [11]. Some plants traditionally used in the Philippines against inflammation such as *Lagerstroemia speciosa* [12], *Blumea balsamifera*, and *Mimosa pudica* [13] have already been studied and reported to have significant inhibitory effects against xanthine oxidase.

Canarium ovatum, locally known as Pili, is considered the most important nut-producing tree in the country because of its various uses [14-17]. The leaves of *C. ovatum* have been reported to contain various bioactive compounds that may have medicinal properties. The extracts of its leaves consist of α-amyrin, β-amyrin, epi-β-amyrin, epi-α-amyrin, epi-lupeol, β-carotene, and lutein (18). These compounds are reported to have various biological activities and medicinal applications [19,20,21]. This study aimed to determine the antihyperuricemic activity of the ethanolic extract from the leaves of *C. ovatum.*

Methodology

Plant Material

C. ovatum leaves were obtained from Siba-o, Calabanga, Camarines Sur. The plant was authenticated by the University of the Philippines Los Baños where voucher specimens were also deposited.

Preparation of Crude Extract

Leaves were collected and air-dried. The dried leaves were ground to 60 mesh particle size then macerated in absolute ethanol as the extracting solvent. Extraction was repeated until the solvent was clear; the solvent was then removed by rotary evaporation (Stuart, USA) and the extract was dried in Speed vac (Labconco, USA). Prior to the tests, the ethanolic extract was dissolved in DMSO (Belman Laboratories) to give a final concentration of 100 ppm which was then used in the enzyme assay.

Fractional Purification of Crude Ethanolic Extract of C. ovatum

The crude ethanolic extract from the leaves of *C. ovatum* was fractionated by Silica gel 60 (Merck, particle size 0.063-0.2 mm) column chromatography using the following solvent system: hexane (100%), hexane:ethyl acetate (50:50), hexane:ethyl acetate (25:75), ethyl acetate (100%), ethyl acetate:methanol (80:20), ethyl acetate:methanol (50:50), methanol (100%), methanol:water:acetic acid (50:50:20), and water (100%).

Five grams of the crude ethanolic extract were loaded onto the column after packing it with 275g of silica gel. The resulting fractions were monitored by Thin Layer Chromatography (TLC) silica gel 60 F254 precoated plastic sheets (Merck) and visualized using UV light. Those that had the same TLC profiles were pooled and concentrated. The pooled fractions were then assayed for their XO inhibitory activity and the most active fraction was used for the *in vivo* tests.

Xanthine Oxidase Inhibitory Assay

The xanthine oxidase inhibitory assay was done under aerobic conditions based on the procedure reported by Noro *et. al* [22]. with modifications as implemented by previous studies [23,24]. The assay mixture composed of 100 ppm crude ethanolic extract, phosphate buffer (pH 7.5), and xanthine was prepared immediately prior to use. After incubating the mixture for 15 min at 37°C, the reaction was initiated by the addition of 0.01 units/mL xanthine oxidase to phosphate buffer (pH 7.5) and was incubated again at 37^{o}C for 30 min. The reaction was stopped by the addition of 50 µL of 1 M HCl. The absorbance of the mixture was measured at 290 nm in a 400 µL quartz cuvette using a UV/Vis spectrophotometer (Spectro 23, Labomed, Inc.). A blank was also made to eliminate the effect of the color of the plant extract itself to the absorbance measurement. It was prepared in the same way but the phosphate buffer was added in place of the enzyme solution. Commercial Allopurinol at 10 µg/mL purchased from Mercury Drug Store was used as a positive control. A negative control was also made by adding DMSO instead of the plant extract. The total DMSO concentration in the final test solutions was 2.0%.

The XO inhibitory activity is calculated as

$$\%\ inhibition = \frac{A-B}{A} \times 100$$

where A and B are the activities of the enzyme without and with the plant extract, respectively. The percent inhibition is reported as the mean of triplicate measurements.

The IC_{50} values of allopurinol, the crude ethanolic extract, and the most active fraction were determined using three replicates of different concentrations (5 ppm up to 400 ppm). The data were then used in the plot of % inhibition versus extract concentration.

Using the same methodology, enzyme kinetics was carried out using the fraction with the highest inhibitory activity with varying xanthine concentrations (400 µg/mL to 25 µg/mL) as the substrate. The data obtained were used in a Lineweaver-Burk plot analysis to determine the mode of inhibition by the most active fraction of the plant.

Phytochemical Screening

The qualitative tests for flavonoids, phenolic compounds, tannins, saponins, carbohydrates, proteins, glycosides, steroids, terpenoids, quinones, cyanins, coumarins, and alkaloids were performed both on the crude extract and the most active fraction based on phytochemical screening methods [25,26].

Experimental animals

The study was conducted after obtaining ethical clearance from the Institutional Animal Care and Use Committee of the National Institutes of Health, University of the Philippines Manila. Male BALB/c albino mice weighing between 20 and 30 g were used in this study. They were purchased from the Food and Drug Administration (Civic Drive, Filinvest Corporate City, Alabang, Muntinlupa City) and were housed individually in polypropylene cages in the animal house of the Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines Manila. The animals were acclimatized for a week in their respective cages. They were given drinking water ad libitum and fed with Vitality® lamb and beef feed pellets throughout the course of the study. A 12-hour light and a 12-hour dark cycle was also observed. Their respective cages and the laboratory room were cleaned regularly. The room was also maintained at a temperature of 25-28°C and a humidity between 30 to 70%. The animals were randomly divided into the experimental groups and marked individually for easy identification.

Acute Oral Toxicity Test

Only food was withheld for 3 to 4 hours prior to dosing. After fasting, the mice were weighed and the crude extract of the chosen plant was then administered. Three mice were used and each of them received a single oral dose of the highest acceptable dose level of 2000 mg/kg body weight. It was administered by gavage with a volume not higher than 1mL/100g body weight. Distilled water was used as a vehicle. Food was further withdrawn in the next 1 to 2 hours after dosing and the mice were observed at least once during the first 4 hours. Observation was continued periodically for the next 20 hours and daily thereafter for 14 days. Weekly weight changes of the animals were also recorded. Mortality was observed for two weeks. Since no mortality was observed at the highest dose level, a toxicity study at lower doses was not performed [27].

In vivo Antihyperuricemic Activity

The method used to assess the hypouricemic activity of the plant extracts was patterned after the methods used in previous studies [1,28] with some modifications. The mice, aged 6-8 weeks by the time of the *in vivo* study, were randomly divided into groups with six animals each.

The following treatment schemes were followed:

Negative Control: Normal untreated mice received the vehicle orally
Positive Control: Hyperuricemic mice received the vehicle orally
Treatment Group 1: Hyperuricemic mice received allopurinol, the reference drug, at 10 mg/kg body weight (positive control group)
Treatment Group 2: Hyperuricemic mice received the fraction at 20 mg/kg
Treatment Group 3: Hyperuricemic mice received the fraction at 200 mg/kg
Treatment Group 4: Hyperuricemic mice received the crude extract at 200 mg/kg

Food, but not water, was withdrawn from the animals two hours prior to their respective treatments. To optimize the induction of hyperuricemia in mice, different dosages of potassium oxonate (Chemline Scientific Corporation) ranging from 250 mg/kg to 1000 mg/kg were used. Hyperuricemia in all groups except the negative control was induced in the mice on the third day of the experiment by using the optimum concentration of potassium oxonate (1000 mg/kg body weight). It was dissolved in distilled water and was administered intraperitoneally to each mouse.

Groups 3 to 6 received their respective treatments orally for two consecutive days. On the third day, these were administered intraperitoneally right after the administration of potassium oxonate. Allopurinol, the most active fraction, and the crude extract were dissolved in distilled water. The body weight of the mice was recorded immediately prior to dosing and the volume of the solution administered did not exceed 1mL/100g body weight.

Evaluation of Uric Acid Levels

Whole blood samples from all the mice were collected through the venipuncture method in the tail on the third day. They were extracted every 30 minutes after the administration of potassium oxonate for two hours. The uric acid levels of the mice were then measured using uric acid test strips and uric acid meter (Easy Touch GCU). The mice were then injected intraperitoneally with 0.1 mL anesthesia and the 0.5 mL to 1 mL whole blood samples were collected through cardiac puncture. The blood samples were then sent to Medicus Diagnostic Center (Taft Avenue, Ermita, Manila) for a more accurate analysis of blood uric acid levels.

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 7.0 software. One way analysis of variance (ANOVA) was used, followed by Dunnett's test. Outliers were removed using Q-test at a 95% confidence level.

Results and Discussion

In vitro Xanthine Oxidase Inhibitory Activity of the Crude Ethanolic Extract and Partially Purified Fractions

The crude ethanolic extract from the leaves of *C. ovatum* at 100 ppm exhibited 83.62±2.05% XO inhibition. This activity showed no statistical difference (p>0.05) with the XO inhibitory activity of the control drug allopurinol (91.47±5.64%).

Eleven fractions were obtained from the first fractionation of the crude ethanol extract of *C. ovatum* and were assayed for their xanthine oxidase inhibitory activity (Fig 1). Among the different fractions, Fraction 7 showed the highest inhibition of 80.30±4.00% with no significant difference (p>0.05) as compared to the inhibition of allopurinol (81.82±3.00%). The concentration needed to inhibit an enzyme by 50% (IC_{50}) was then determined. The IC_{50} values of the crude extract and fraction 7 of *C. ovatum* are 62.50 ppm and 23.35 ppm, respectively. Allopurinol, on the other hand, has an IC_{50} of 3.20 ppm.

Lineweaver-Burke Analysis

The mechanism of inhibition of the fraction with the highest XO inhibitory activity was determined using a Lineweaver-Burke plot analysis as shown in Figure 2. The trend lines intersect at the third quadrant. The value of Vmax decreased from 0.422±0.015 µM/min to 0.229±0.024 in the presence of the most active fraction. On the other hand, the Km values increased from 715.5±72.13 µM to 1151±276 µM. These observations suggest that the phytochemicals present in fraction 7 of *C. ovatum* inhibit the enzyme through a mixed inhibition mechanism (31).

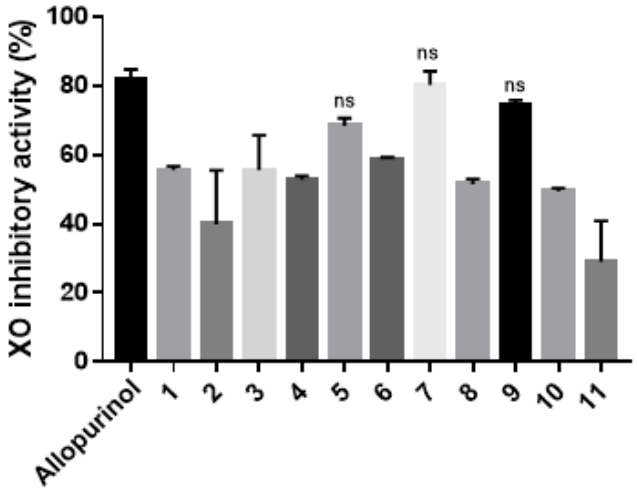

Figure 1. *Xanthine oxidase inhibitory activity of the 11 fractions obtained after the partial purification of C. ovatum. nsp > 0.05 compared with allopurinol.*

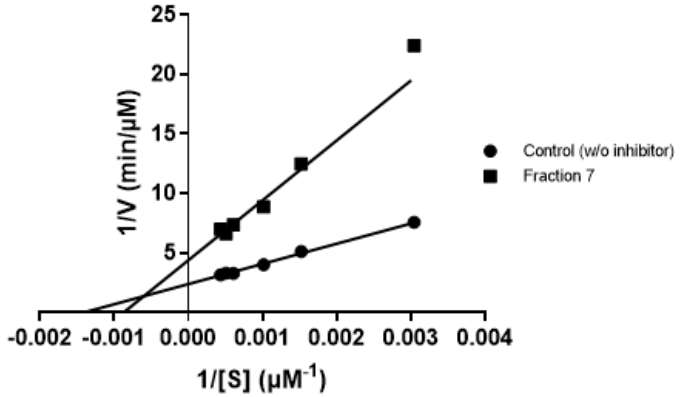

Figure 2. *The Lineweaver-Burke reciprocal plot for the inhibition of xanthine oxidase by the most active fraction from C. ovatum*

Phytochemical Analysis

Different qualitative phytochemical tests were done on both the crude extract and fraction 7 of *C. ovatum* (Table 1). The crude extract of *C. ovatum* contains steroids, terpenoids, quinones, flavonoids, phenols, saponins, and tannins. On the other hand, the flavonoids and saponins were more concentrated in fraction 7. Coumarins were present in fraction 7 but not in the crude extract.

Table 1. *Phytochemical profile of the crude extract and the most active fraction of C. ovatum.*

Phytochemical	Crude extract	Fraction 7
Carbohydrates and Reducing Sugars		
Molisch's test	-	-
Fehling's test	-	-
Proteins and Amino Acids		
Biuret test	-	-
Ninhydrin test	-	-
Alkaloids		
Hager's test	-	-
Wagner's test	-	-
Glycosides		
Keller-Killiani	-	-
Steroids		
Liebermann-Burchard test	+	+
Terpenoids		
Salkowski's test	++	+
Triterpenes		
Salkowski's test	-	-
Quinones		
H_2SO_4 test	+	+
Anthraquinones		
HCl test	-	-
Free anthraquinone	-	-
Cyanins		
Alkaline reagent test	-	-
Coumarin		
Alkaline reagent test	-	+
Flavonoids		
Alkaline reagent test	+	++
Saponins		
Froth test	+	++
Phenols		
FeCl3 test	+	+
Condensed Tannins		
FeCl3 test	+	+
Plant Acids	-	-

Acute Oral Toxicity

An acute oral toxicity was done to ensure that the plant extract itself is not toxic and harmful to mice which were used as the animal model of the study. The mice were given the highest acceptable dose level of 2000 mg/kg body weight and no mortality or any signs of toxicity was observed for 14 days. According to the guidelines set by the OECD (2001a) [27], the *C. ovatum* belongs to the hazard category 5 which means that it has a relatively low acute toxicity hazard and could be considered safe for consumption of the test animals.

In vivo Antihyperuricemic Activity

The *in vivo* study of the antihyperuricemic activity of *C. ovatum* was also conducted to determine if it would have parallel results with its *in vitro* study. The induction of hyperuricemia in mice was optimized using various concentrations of potassium oxonate (Figure 3). An intraperitoneal injection of 1000 mg/kg body weight of the mice was able to induce hyperuricemia with an increase in serum uric acid levels of up to 2.5 hours. The low toxicity of potassium oxonate allows its administration at large doses [40].

In order to assess the antihyperuricemic activity of *C. ovatum in vivo*, the crude extract, fraction 7, and allopurinol were given to the mice two days prior to the induction of hyperuricemia. On the third day, the serum uric acid levels of the mice were observed (Figure 4). The uric acid levels of the hyperuricemic control started to increase one hour after the induction and returned to its normal levels after 2.5 hours. Meanwhile, the mice treated with allopurinol did not exhibit any increase after induction. Those treated with the crude extract

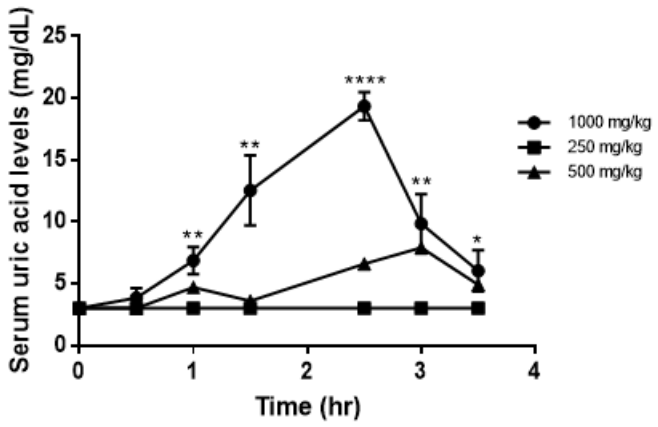

Figure 3. *Time-course effect of potassium oxonate on the serum uric acid levels in mice.*

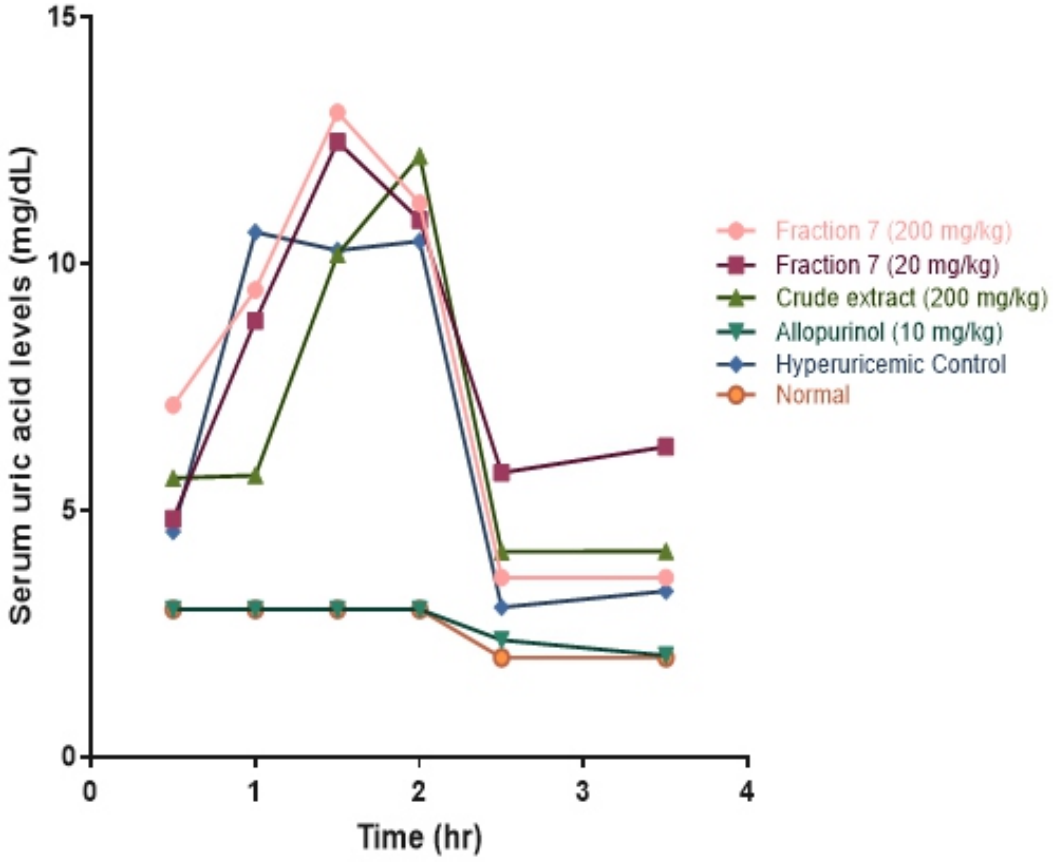

Figure 4. *Time-course effects of allopurinol, C. ovatum crude extract, and fraction 7 on serum uric acid levels in mice with potassium oxonate-induced hyperuricemia.*

and fraction 7 had an increase in uric acid that was less than that of the hyperuricemic control during the first hour; it also showed greater increase in uric acid than that of the hyperuricemic control during the second hour. The uric acid levels of all treatment groups decreased thereinafter due to the natural decrease of hyperuricemia induction of potassium oxonate.

The *in vivo* study conducted indicates that *C. ovatum* has an antihyperuricemic activity as shown in Figure 5. The administration of potassium oxonate (1000 mg/kg body weight) was able to significantly ($p<0.0001$) induce hyperuricemia (11.96±3.54 mg/dL) when compared to the normal group (2.03±0.52 mg/dL). The pre-treatment of the most active fraction of *C. ovarium* at 200 mg/kg and 20 mg/kg reduced the serum uric acid levels up to 6.85±2.84 mg/dL and 11.53±3.26 mg/dL, respectively. A significant ($p<0.05$) reduction compared to the hyperuricemic group was only observed when it was administered at the higher dose of 200 mg/kg. On the other hand, the crude extract of the plant exhibited a more significant ($p<0.01$) decrease in serum uric acid levels up to 5.72±1.83 mg/dL. However, there is no significant difference ($p>0.05$) between the serum uric acid levels of the mice treated with fraction 7 and crude extract. Allopurinol significantly ($p<0.0001$) reduced the uric acid levels up to 3 mg/dL at a smaller dose of 10 mg/kg.

Discussion

Hyperuricemia, which is the leading risk factor of gout, currently has an increasing prevalence in the Philippines for the past years [8]. However, the available commercial therapeutic agents such as allopurinol, a xanthine oxidase inhibitor, have associated adverse side effects [10]. Potential sources for novel and effective antihyperuricemic agents are natural medicinal plants and trees [45]. One of the endemic and important trees in the country is the Pili tree, also known as *Canarium ovatum*. Previous studies have shown that its leaves, nuts, and pulp have different bioactive compounds (18). Some studies also state that some parts of the plant species under the *Canarium genus* such as the fruit mesocarp of *Canarium schweinfurthii* [29] and the fruit of *Canarium album* [30] have XO inhibitory activities.

The ethanolic crude extract from the leaves of *C. ovatum* showed in vitro XO inhibitory activity with no significant difference ($p>0.05$) as compared to that of allopurinol, the control drug. Eleven fractions were obtained after partial purification of the crude extract. Fraction 7 showed the highest in vitro XO inhibitory activity with no significant difference ($p>0.05$) with that of allopurinol.

The phytochemicals detected from both the crude extract and fraction 7 of *C. ovatum* were also found in previous studies [31,32] such as terpenoids, phenols, and flavonoids which are found not only on the leaves of *C. ovatum* but also in the pulp and nut as well. There are other phytochemicals not detected in this study that are present in other parts of the *C. ovatum* tree such as alkaloids, glycosides [32] from the pulp and nut, andoxalates [33] in the pulp. These phytochemicals were reported by previous studies to have XO inhibitory activity. Flavonoids such as kaempferol [34] and glycitein [35] have been reported to be competitive inhibitors of XO [36]. Tannins, on the other hand, are reported to inhibit the enzyme through non-specific binding [37]. Coumarins such as esculetin [38] and umbelliferone are reported to inhibit the enzyme through competitive, uncompetitive, and mixed types of inhibition respectively. Other reported inhibitors of the enzyme include polyphenols [39], methoxylated hydroquinones [40], and saponins such as ilexsaponin and prosapogenin [41]. However, terpenoids and steroids have not been reported to be inhibitors of this enzyme [13].

Enzymatic and inhibition kinetic analysis of the most active fraction showed the pattern of mixed inhibition. This may either suggest that the fraction has a singular component that can both bind either the free enzyme or the enzyme-substrate complex; or the fraction has several components that exhibit different types of XO inhibitory activity (i.e. one competitive and one non-competitive). Ideally, the fraction should have been purified down to its singular components to have a more accurate enzyme kinetic and inhibition kinetic analysis.

Potassium oxonate is one of the common and most effective methods in inducing hyperuricemia in mice, however, its hyperuricemic effect is time dependent [42]. Theoretically, the serum uric acid levels will eventually return to its normal levels after 2.5 hours [43]. Thus, the treatment must be done within that time limit to ensure that the resulting decrease in serum uric acid levels is due to an inhibitor of xanthine oxidase and not because of the diminishing effects of the induction of potassium oxonate. This is the reason why beginning day three onwards, allopurinol, fraction 7, and the crude extract were given intraperitoneally to the mice right after the administration of PO to hasten delivery and absorption. The results shown in Figure 4 support the time-dependent hyperuricemia of PO as shown by the decrease of uric acid levels of all treatment groups after 2.5 hours from induction. Thus, analysis of the antihyperuricemic activities was done on the treatment groups one hour after induction because it is the only time period wherein the hyperuricemic control has the highest uric acid level compared to the other treatment groups.

The *in vivo* antihyperuricemic activity assay revealed that the most active fraction exhibited antihyperuricemic activity with a significant ($p<0.05$) decrease in plasma uric acid level observed at 200 mg/kg but not at 20 mg/kg as compared to the hyperuricemic group. The crude extract, on the other hand, showed a more significant ($p<0.01$) antihyperuricemic activity at the same dosage which may be due to the synergistic and/or additive effects of the different phytochemicals present in the crude extract [47]. However, both crude extract and the most active fraction were not as potent as allopurinol as evidenced by its significant hyperuricemic effect ($p<0.0001$) at a smaller dose. These results suggest that even though *C. ovatum* has antihyperuricemic activity, it does not exhibit the same therapeutic potency as allopurinol. Furthermore, the discrepancy between the in vitro XO inhibitory activity and *in vivo* antihyperuricemic activity of both the crude extract and the most active fraction of *C. ovatum* leaves may be attributed to their bioavailability and pharmacokinetics or the effects the body has on the drug (absorption, distribution, metabolism, and excretion) which can dampen their effects.

Conclusion

The ethanolic extract from the leaves of *C. ovatum* showed *in vitro* xanthine oxidase inhibitory activity, however, it only has a lower *in vivo* antihyperuricemic activity as compared to allopurinol, the current drug of choice for hyperuricemia. It may have the potential to be used as a source of bioactive compounds for the treatment of gout and hyperuricemia but further purification studies and in vivo studies must be done. Since the study used only the crude extract and semi-purified fractions, the IC_{50} of 23.35 ppm for fraction 7 showed potential, hence, future purification studies and further testing is recommended. Further purification of fraction 7 of *C. ovatum* can lead to the isolation of its active components that could subsequently have comparable activity with allopurinol. The pure xanthine oxidase inhibitor can further undergo structure elucidation, ADMETOX determination, and pharmacokinetics. It is also recommended to use a hyperuricemic animal model where the effects of the plant

can be measured through multiple time points to accurately determine the *in vivo* antihyperuricemic activity of the plant, fractions from the extracts of the plant, or isolates.

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