

# Comparative Study of Xanthine Oxidase Inhibitory Activity Of Methanolic Leaf Extracts Of *Calophyllum Blancoi* (Bitaog), *Diospyros pilosanthera* (Bolongeta) and *Syzygium Cumini* (Duhat)

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## ABSTRACT

Gout is characterized as an inflammation and warmth in the joints. It is associated with hyperuricemia wherein an upregulation of xanthine oxidase in purine degradation leads to increased levels of uric acid in the blood. Gout is not fatal. However, it affects one's quality of life. Thus, this research primarily focuses in determining the inhibitory activity of xanthine oxidase in the methanolic leaf extract of bitaog (*Calophyllum blancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygium cumini*) in gout. A quantitative-experimental research method was used in the study and the data were obtained by measuring the percent inhibition of the samples using UV-Vis spectrophotometer at 290 nm. The results showed that the methanolic leaf extract of above stated plants exhibited exemplary inhibition in comparison with the standard drug, allopurinol. The IC<sub>50</sub> value determines the ability of the inhibitor to decrease the biotransformation of a substrate. The principle behind IC<sub>50</sub> is, the lower the value the higher the inhibition. The bitaog (*Calophyllum blancoi*) trials have the lowest IC<sub>50</sub> value with an average of 124.3 after the standard drug, followed by bolongeta (*Diospyros pilosanthera*) have an average of 155.3 IC<sub>50</sub> value. Then duhat (*Syzygium cumini*) showed the highest IC<sub>50</sub> an average of 208.8. The bitaog (*Calophyllum blancoi*), next to allopurinol, showed the highest inhibition among all the extracts followed by the bolongeta (*Diospyros pilosanthera*). The least inhibitory activity was observed in duhat (*Syzygium cumini*). Hence, it can be concluded that bitaog (*Calophyllum blancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygium cumini*) can inhibit xanthine oxidase using *in vitro* analysis.

## Keywords

Gout; Antioxidant; Anti-inflammatory; *Calophyllum blancoi*; *Diospyros pilosanthera*; *Syzygium cumini*; Xanthine Oxidase; Uric acid; Allopurinol; Xanthine Oxidase Inhibition, IC<sub>50</sub>, *in vitro* assay.

## 1. INTRODUCTION

Gout is manifested by intense pain and inflammation of joints occurs when the soft tissue accumulate monosodium urate crystals [1]. It has been estimated that in 2016, it affects about 1.6 million of Filipinos as stated by Philippine Rheumatism Association President, Eric

Amante [2] and 4.3% in the US. Gouty arthritis can be caused by an increase of the urate serum levels, also known as uric acid in the blood.

Uric acid chemical is formed when there is a breakdown of substances called purines [3]. This uric-acid is created by the conversion of xanthine to hypoxanthine by the reactive oxygen species called xanthine oxidase. Xanthine oxidase (XO) is a significant biological source of free radicals that is oxygen-derived which contributes to the oxidative damages in tissues. It is widely distributed in liver and intestine of different species. It is the rate-limiting enzyme in the biosynthesis of uric acid and catalyzes the formation of uric acid. Thus, any up-regulation of the purine degradation pathway may result in the increase of uric acid known as hyperuricemia that eventually leads to gout [4].

Allopurinol is commonly used for the treatment of gout. However, it has induced severe cutaneous adverse reactions including Steven-Johnson's syndrome, renal toxicity and even the fatal liver necrosis [5-7]. In contrast, the use of natural therapy is being introduced to attain fewer side effects for an inexpensive price.

As stated by Jothy, Zuraini and Sasidharan [8], major compounds that act as primary antioxidants is the plant's phenolic constituent. They are capable of scavenging reactive oxygen species, such as xanthine oxidase, due to its electron donating properties. Furthermore, according to Bakar et al. [9], *Momordica charantia*, *Chrysanthemum indicum*, *Cinnamomum cassia*, *Kaempferia galanga*, *Artemisia vulgaris*, and *Morinda elliptica* had higher percentages of xanthine oxidase inhibitory activity (>85%) because of their diverse natural bioactive compounds, which include flavonoids, phenolics, tannin, coumarins, luteolin, and apigenin. Beside these plants, Alvionita et al. [10] reported that a flavonoid compound found in *Annona squamosa* L., has higher binding affinity to the xanthine oxidase than allopurinol which indicated that this plant may be a promising herbal drug for gout treatment. In a review done by Kapor et al. [11] on over 130 medicinal plants, they concluded that these plants exhibit anti-gout effect by different mechanisms, such as XO inhibition, uricosuric activity, anti-inflammatory activity, and antioxidant activity. Moreover, plant extracts and isolated constituents which showed promising XO inhibition are also considered and most of the isolated elements are found to be phenolic glycosides and flavonoids.

The researchers found potential plants of interest that contain phenolic compounds and flavonoids to further study. Consequently, the researchers chose bitaog (*Calophyllum blancoi*), bolongeta (*Diospyros pilosanthera*) and duhat (*Syzygium cumini*) as potential xanthine oxidase inhibitor in the study. In addition, limited studies regarding xanthine oxidase inhibition of these plants have been carried out. This study was conducted to evaluate and determine the potential xanthine oxidase inhibitory activity of the three plants namely using in vitro assay.

## 2. METHODOLOGY

### 2.1 Research Design

The study used quantitative-experimental method of research in the determination of the inhibition activity of xanthine oxidase of the leaf methanolic tract from the leaves of (a) bitaog (*Calophyllum blancoi*), (b) bolongeta (*Diospyros pilosanthera*), and (c) duhat (*Syzygium cumini*) which permits the measurement of variables within the desired collection resulting in numerical data which then subjected to statistical analysis. Besides, it confirms the presence phenolic and flavonoid. The methanolic extract of the plants and the standard drug, allopurinol, were manipulated to determine the effect of the material under the study. The dependent variable of the study was of xanthine oxidase inhibition of the methanolic

extract of each plant. The analysis is between the positive control of the standard drug allopurinol, and sample test group composed of (a) bitaog (*Calophyllumblancoi*), (b) bolongeta (*Diospyros pilosanthera*), and (c) duhat (*Syzygiumcumini*) methanolic leaf extracts

## 2.2 Collection and Preparation of the Plant and Plant Extract

The leaves of bitaog (*Calophyllumblancoi*) and bolong-eta (*Diospyros pilosanthera*) were collected at Dasmariñas City, Cavite and duhat (*Syzygiumcumini*) was collected from Indang, Cavite, Philippines in the month of January 2018.

The dried leaf samples were crushed using blender until it turned into powdered form. Every leaf samples were weighed for three times having 10 g each. Each sample was placed in of 90% methanol for 72 hours and stored in a tightly closed container wrapped with aluminum foil.

## 2.3 Phytochemical Analysis

### 2.3.1 Total Phenolic Content

The phenolic content was determined using the Folin-Ciocalteu assay. An exactly 100  $\mu\text{L}$  of each methanolic extract sample was diluted with 0.50 mL with distilled water in a test tube. Folin-Ciocalteu Phenol reagent (0.25 mL, 10x diluted) was added to each sample and were incubated. Afterwards, 1.50 mL of 7% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) After the incubation, each sample was subjected to vortex mixer to mix thoroughly and some amounts were transferred to cuvettes. The absorbance of each sample was read using UV-Vis spectrophotometer. Dilutions were carried out as needed. Gallic acid was used as standard in different concentrations (0.5, 1.0, 2.5, and 5  $\mu\text{g}/\text{mL}$ ). The total phenolic content (TPC) was expressed as  $\mu\text{g}$  of gallic acid equivalent per gram of sample ( $\mu\text{g}$  GAE/g).

### 2.3.2 Total Flavonoid Content

The total flavonoids content determination of the methanolic extract was done using the aluminum chloride colorimetric method following procedures outlined by Baba and Malik [12]. A 50  $\mu\text{L}$  of methanolic extract (1mg/mL ethanol) was diluted with 1mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% sodium nitrite ( $\text{NaNO}_2$ ) solution; 0.3 mL of 10% aluminum chloride ( $\text{AlCl}_3$ ) solution was added after five minutes of incubation at dark at room temperature, and the mixture was allowed to stand for six minutes. The mixtures were mixed thoroughly using vortex mixer and transferred to the cuvettes. Then, absorbance was read immediately at 510 nm versus blank sample (methanol) using UV-Vis spectrophotometer. Dilutions were carried out as necessary. Calibration curve was constructed using quercetin as standard with varying concentrations (0.10, 0.20, 0.50, 1 and 2  $\mu\text{g}/\text{mL}$ ). The total flavonoids content was expressed as  $\mu\text{g}$  of quercetin equivalent per gram of samples ( $\mu\text{g}$  of QE/g).

## 2.4 Thin Layer Chromatography

Each solvent extract was subjected to thin layer chromatography (TLC) as per conventional one-dimensional ascending method using CAMAG TLC Silica Gel 60 F254 Aluminum sheet. The starting point to finish line measures about 7 centimeters. The solvent system used was (1:4) acetone-chloroform. The TLC was set up by placing the sheet in a slanting position inside of 500 mL beaker with a #1 Whatman filter paper and 50 mL of the solvent system and cover by aluminum foil. Then, retention factor ( $R_f$ ) was measured where retention factor is distance travelled by the solute divided by distance travelled by the solvent as shown in Equation 1.

$$R_f = \frac{\text{distance travelled by the solute}}{\text{distance travelled by the solvent}} \quad (1)$$

## 2.5 *In vitro* Analysis

### 2.5.1 Chemicals

Xanthine oxidase powder from bovine's milk and xanthine powder were purchased from Sigma-Aldrich Co. (United States). Phosphate buffer solution (pH 7.4) and sodium hydroxide pellets analytical grade were available in De La Salle University Chemistry Research laboratory. The allopurinol (Llanol) 300 mg was purchased from Mercury Drugstore located in Dasmariñas City.

### 2.5.2 Preparation of Solutions for the Assay

#### 2.5.2.1 Xanthine Oxidase Buffer (Substrate)

Xanthine (1.52 g) was initially dissolved in 1 M Sodium Hydroxide (30 mL) and then diluted with 0.2 M phosphate buffer pH 7.4 to made up to 50 mL solution [13].

#### 2.5.2.2 Xanthine Oxidase (Enzyme)

The xanthine oxidase solution was prepared by dissolving 0.003 g of xanthine oxidase from bovine's milk in 5 mL of buffer pH 7.4 cold 0.2 M phosphate [13].

## 2.6 Test Solution and Test Sample

Three tablets of 300 mg allopurinol (Llanol) weighing 1.683 mg was mixed in 90 ml distilled water and produced a concentration of 18700 parts per million (ppm), which was then diluted with 0.2 M phosphate buffer (pH 7.5) to obtain a 400, 200, 100, 50, 10, and 5 ppm solution.

Methanolic leaf extract of bitaog (*Calophyllumblancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygiumcumini*) were diluted in 0.2 M of phosphate buffer to make concentration of 25,50,100, and 300 µg extracts/mL phosphate buffer [13].

The first control sample was made by adding a 25 µL of xanthine oxidase solution to 0.2 M phosphate buffer (1 mL) and the mixture was incubated .Then, 0.3 mL xanthine oxidase buffer solution was added to the mixture. The second control sample was prepared by diluting 1.025 mL of 0.2 M phosphate buffer (pH 7.4) to 0.3 mL of xanthine oxidase buffer solution and each control sample was made for four replicates. The blank sample contains only 0.2 M phosphate buffer solution [13].

## 2.7 Xanthine Oxidase Inhibition Assay

The modified method of Sivashanmugam and Chatterjee [14] was used for the determination of xanthine oxidase inhibitory activity of plant extracts. Exactly 0.3 mL of test solution (25, 50, 100 and 300 µg extracts/mL phosphate buffer, 0.8 mL of 0.2 M phosphate buffer (pH 7.4) and 25 µL of xanthine oxidase were combined. The mixtures were incubated in dark at room temperature for 15 minutes and the reaction was initiated by addition of 0.3 mL of xanthine oxidase buffer. Each sample was transferred to quartz cuvette and the absorbance of each was measured using JASCO V-530 UV-Vis spectrophotometer at 290 nm. The percentage of inhibition was calculated using equation 2.

$$\text{Percentage of inhibition} = \left( \frac{A-B}{A} \right) \times 100 \quad (2)$$

where A is equivalent to the change in absorbance of the assay without test samples, and B is equal to the change in absorbance of the test assay with test sample. The IC<sub>50</sub> was determined by linear regression and compared to the value of the standard drug allopurinol.

## 2.8 Statistical Analysis

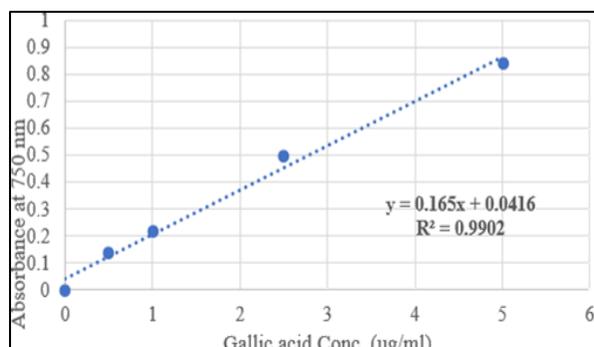
All data were statistically treated by one-way analysis of variance (ANOVA), T-Test, and F-Test followed by post-hoc studies using Tukey's Honestly Significant Difference to assess significant differences of mean between groups. The calculations and all values were expressed as mean ± S.E.M (standard error of mean) at 5% significant level. Values with P<0.05 is considered statistically significant.

### 3. RESULTS AND DISCUSSION

#### 3.1 Phytochemical Analysis

##### 3.1.1 Total Phenolic Content

The Folin-Ciocalteu assay confirms the presence of the phenolic compounds of the extracts by having an absorbance  $\leq 1$ . The TPC test used Gallic acid as the reference standard. Figure 1 demonstrates the calibration curve obtained from the absorbance of Gallic acid under 750 nm.



**Figure 1.** Calibration curve obtained from the absorbance of Gallic acid

On the other hand, Table 1 collates result of the absorbance of the samples from bitaog (*Calophyllum blancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygium cumini*) methanolic leaf extract. The results displayed above showed that the phenolic content primarily contributes to the antioxidant activities of the above-mentioned plants, therefore could play a significant role in inhibiting xanthine oxidase. The results shows that the Bitagog (*Calophyllum blancoi*) has the greatest amount of phenolic contents with an average of 944.8  $\mu\text{g GAE/g}$  sample, followed by bolongeta (*Diospyros pilosanthera*) having a 851.1  $\mu\text{g GAE/g}$  sample. The least amount of phenolic content was found in duhat (*Syzygium cumini*) with 647.5  $\mu\text{g GAE/g}$  sample.

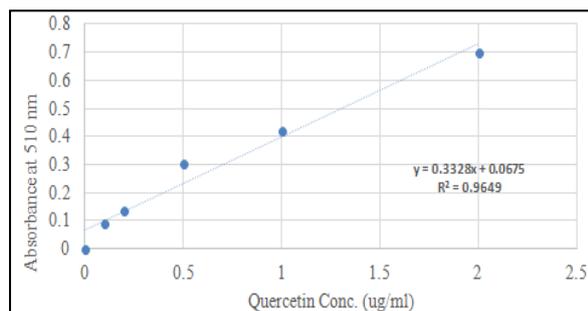
**Table 1.** Result of total phenolic content of plant samples

Trial	Bitagog ( <i>Calophyllum blancoi</i> )	Bolongeta ( <i>Diospyros pilosanthera</i> )	Duhat ( <i>Syzygium cumini</i> )
1	544.8 $\mu\text{g GAE/g}$	976.2 $\mu\text{g GAE/g}$	805.2 $\mu\text{g GAE/g}$
2	846.3 $\mu\text{g GAE/g}$	919.7 $\mu\text{g GAE/g}$	866.8 $\mu\text{g GAE/g}$
3	551.5 $\mu\text{g GAE/g}$	938.6 $\mu\text{g GAE/g}$	881.3 $\mu\text{g GAE/g}$
<b>AVERAGE</b> $\pm$ SD	<b>647.5 <math>\pm</math> 140.572</b> $\mu\text{g GAE/g}$	<b>944.8 <math>\pm</math> 23.483</b> $\mu\text{g GAE/g}$	<b>851.1 <math>\pm</math> 33.021</b> $\mu\text{g GAE/g}$

Legend:  $\mu\text{g GAE/g}$  = microgram of gallic acid equivalent per gram sample; SD = standard deviation; Values are expressed as  $\pm$  SD

##### 3.1.2 Total Flavonoid Content

Aluminum chloride colorimetric method affirms the presence of flavonoids compounds of the extracts by having an absorbance of  $\leq 1$ . Quercetin was used as the reference standard for the test. Figure 2 draws the calibration curve quercetin after subjected to the JASCO V-530 UV-Vis spectrophotometer.



**Figure 2.** Calibration cuve of quercetin

Table 2 shows the calculated total flavonoid content of bitaog (*Calophyllumblancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygiumcumini*).As seen in Table 2, the total flavonoid content of the plants has an average that ranges from 345.9 to 593.8 µg QE/ g sample. Bolongeta (*Diospyros pilosanthera*) showed highest the flavonoid content (593.8 µg QE/ g sample), followed by bitaog (*Calophyllumblancoi*) (389.1 µg QE/ g sample), whereas duhat (*Syzygiumcumini*) has the lowest phenolic content (345.9 µg QE/ g sample). This result supported the literature that the flavonoid content predominantly contributes to the antioxidant effect of the said plants, and therefore, the flavonoid content of the said plants could play a significant role in blocking xanthine oxidase.

**Table 2.**Result of total flavonoid content of plant samples

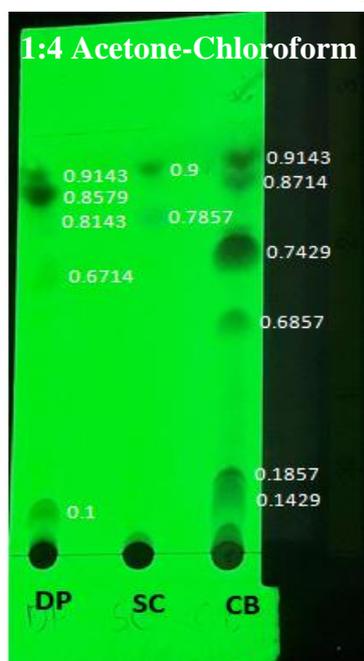
Trial	Bitagog ( <i>Calophyllum blancoi</i> )	Bolongeta ( <i>Diospyros pilosanthera</i> )	Duhat ( <i>Syzygium cumini</i> )
1	442.3 µg QE/g	470.9 µg QE/g	375.8 µg QE/g
2	338.5 µg QE/g	816.8 µg QE/g	348.1 µg QE/g
3	386.6 µg QE/g	493.6 µg QE/g	313.9 µg QE/g
AVERAGE ±SD	389.1 ± 42.417 µg QE/g	593.8 ± 158.013 µg QE/g	345.9 ± 25.337 µg QE/g

Legend: µg QE/g = microgram of quercetin equivalent per gram sample; SD= standard deviation; Values are expressed as means ± SD

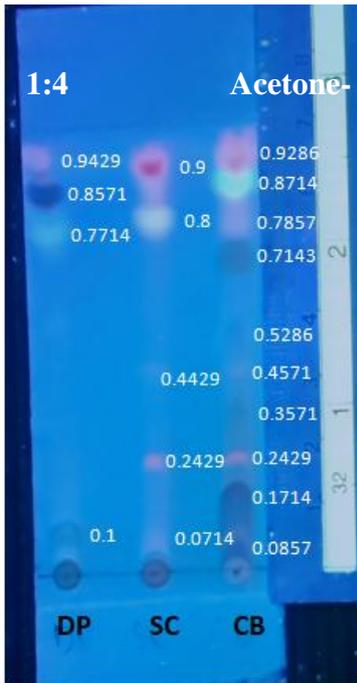
### 3.2 Thin Layer Chromatography (TLC)

Thin layer chromatography was performed for fingerprint identification of bitaog (*Calophyllumblancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygiumcumini*). The result of TLC attests the result of total phenolic content and flavonoids determination.

As shown in Figures 3 and 4, the functional group that has more affinity to the stationary phase adhered and travelled slower than others. The pinkish color, as seen in Figure 4, represents the phenolic groups that include flavonoids. Figures also shows that the plate is best viewed under the UV light long and short wave.



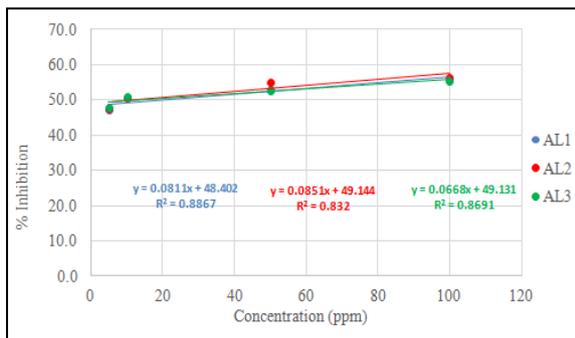
**Figure 3.** Thin Layer Chromatography Plate under 254 nm



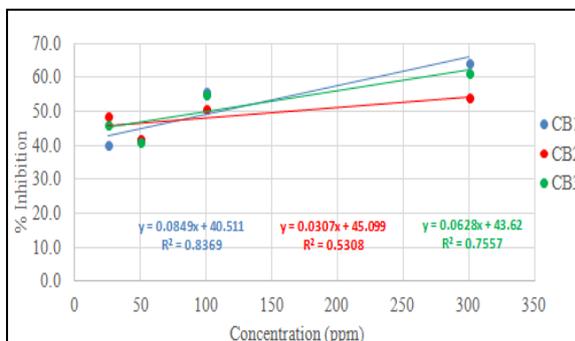
**Figure 4.**Thin Layer Chromatography Plate under 366 nm

### 3.3 Xanthine Oxidase Inhibition Assay

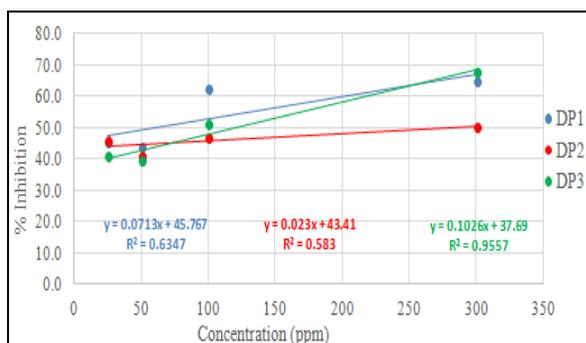
The xanthine oxidase inhibition assay used allopurinol as the positive control. Figures 5, 6, 7, and 8 illustrate the xanthine oxidase inhibition of allopurinol, bitaog (*Calophyllumblancoi*), Bolongeta (*Diospyros pilosanthera*) and duhat (*Syzygiumcumini*) respectively.



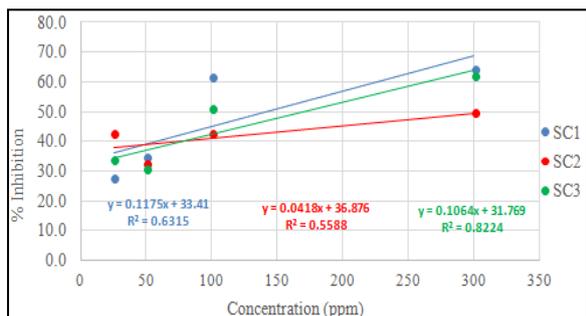
**Figure 5.**Xanthine oxidase inhibition of allopurinol



**Figure 6.** Xanthine oxidase inhibition of bitaog (*Calophyllumblancoi*)



**Figure 7.** Xanthine oxidase inhibition of bolongeta (*Diospyros pilosanthera*)



**Figure 8.** Xanthine oxidase inhibition of duhat (*Syzygiumcumini*)

Table 3 summarizes the inhibitory concentration (IC<sub>50</sub>) value of the samples in comparison with the positive control, allopurinol. The IC<sub>50</sub> value determines the ability of the inhibitor to decrease the biotransformation of a substrate. The principle behind IC<sub>50</sub> is, the lower the value the higher the inhibition. The bitaog (*Calophyllumblancoi*) trials have the lowest IC<sub>50</sub> value with an average of 124.3 after the standard drug, followed by bolongeta (*Diospyros pilosanthera*) have an average of 155.3 IC<sub>50</sub> value and duhat (*Syzygiumcumini*) showed the highest IC<sub>50</sub> an average of 208.8.

**Table 3.** Summary of IC<sub>50</sub> value of plant samples and allopurinol

Trial	Bitaog ( <i>Calophyllum blancoi</i> )	Bolongeta ( <i>Diospyros pilosanthera</i> )	Duhat ( <i>Syzygium cumini</i> )	Allopurinol
1	111.8	59.4	141.2	19.7
2	159.6	286.5	314.0	10.1
3	101.6	120.0	171.3	13.0
AVERAGE	124.3	155.3	208.8	14.26

As per the interpretation in Table 4, the methanolic leaf extract of bitaog (*Calophyllumblancoi*), bolongeta (*Diospyros pilosanthera*) and duhat (*Syzygiumcumini*) has a significant inhibitory effect in xanthine oxidase. The table also lead to the conclusion that the bitaog (*Calophyllumblancoi*) have the highest inhibitory activity after the standard drug, allopurinol, followed by duhat (*Syzygiumcumini*), whereas the bolongeta (*Diospyros pilosanthera*) have the lowest xanthine oxidase inhibition.

**Table 4.** Comparison of highest inhibitory activity and difference between plant samples and allopurinol

Samples	F-test value	P-value	Interpretation
Bitaog ( <i>Calophyllum blancoi</i> )	15.317	0.001	Significant
Bolong-eta ( <i>Diospyros pilosanthera</i> )	5.757	0.021	Significant
Duhat ( <i>Syzygium cumini</i> )	8.887	0.006	Significant

Level of Significance = P < 0.05 Alpha

#### 4. CONCLUSION

In conclusion, based on the study done on xanthine oxidase inhibitory activity of methanolic leaf extracts of the three plants, it was proven that these plants exhibited exemplary inhibition in comparison with the standard drug, allopurinol. Among the three plants, bitaog (*Calophyllumblancoi*) had the highest inhibition, followed by bolongeta (*Diospyros pilosanthera*), and lastly duhat (*Syzygiumcumini*). In terms of IC<sub>50</sub> value, the average for these plants were 124.3, 155.3 and 208.8 for bitaog (*Calophyllumblancoi*), bolongeta (*Diospyros pilosanthera*), and duhat (*Syzygiumcumini*) respectively.

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