In Vitro Xanthine Oxidase Inhibition of Ethanolic Crude Leaf Extract from *Leea aculeata* (Mali-mali)

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Abstract

Xanthine oxidase (XO) is the key enzyme responsible for the production of uric acid (UA). In humans and higher primates, UA does not undergo further metabolism. XO inhibition decreases serum uric acid levels which prevents hyperuricemia. Potential source of compounds that could inhibit enzyme activity could be obtained from plants. Using in vitro xanthine oxidase inhibition assay, the inhibition activity of *Leea aculeata* leaf extract at varying concentrations of 10ug/ml, 20ug/ml, 50ug/ml, and 70ug/ml was determined. The phytochemical components of *L. aculeata* were also tested which revealed the presence of [1]flavonoids, saponins, tannins, terpenoids and anthraquinones. Reducing sugars, alkaloids, and cardiac glycosides were not found. It was found out that all concentrations of *L. aculeata* leaf extract were able to inhibit XO with the highest activity (92.75%) observed at 50ug/ml concentration. When compared with allopurinol, only 50ug/ml concentration was found to have similar XO inhibition activity with a p-value of 0.270; however, allopurinol showed better xanthine oxidase inhibiting activity. Thus, *L. aculeata* leaf extract has inhibiting activity against xanthine oxidase, proving its potential of being an anti-hyperuricemic agent.

Keywords: Hyperuricemia, uric acid, xanthine oxidase inhibitor, *Leea aculeata*

INTRODUCTION

Xanthine oxidase (XO) is the key enzyme responsible for the production of uric acid (UA) via the conversion of hypoxanthine and xanthine from purine sources (Kostic et al., 2015). The purines are derived from endogenous and exogenous sources which pass through the liver and enter the bloodstream. Most of these purine nucleotides pass through the intestines and get

regulated back to their normal level (Kutzing & Firestein, 2007). However, excess of these purine nucleotides can lead to overactivity of the enzyme xanthine oxidase, resulting to increased production of uric acid in the body.

Uric acid, the purine catabolism end product, is present in humans, as well as in other mammals and higher primates (Fu, Luo, Ye, & Xiao, 2015). The enzyme uricase, which is present in most mammals, oxidizes UA into allantoin to be easily excreted via urine (Grassi et al., 2013). However, in humans and higher primates, UA does not undergo further metabolism; thus, they rely upon its excretion via the kidneys as urine to maintain homeostasis (Higgins, Dawson, & Walters, 2009). In some cases, overproduction and underexcretion of urate happens due to an underlying nephropathy, leading to hyperuricemia (Jin et al., 2012).

Hyperuricemia is defined as an excess of serum uric acid (UA) levels measuring to greater than 7.0 mg/dL, and is one of the common metabolic disorders in man (Kutzing & Firestein, 2007; Dincer, Dincer & Levinson, 2002). It is the chief precursor of gout, and has been found to be an important contributor to the rising number of chronic, degenerative, and preventable lifestyle diseases and disorders. It may either be an asymptomatic condition, with urate levels greater than 6.8 md/dl, or it may worsen to more complicated clinical manifestations as urate concentration elevates. Increase in uric acid levels happens regardless of gender.

The causes of hyperuricemia are narrowed down to three causes: increased urate production, decreased urate excretion, and a mixture of both. The first cause, increased uric acid production, includes high dietary intake of purines, and increased metabolism of purine. The second, decreased excretion of UA is due to an underlying nephropathy, intake of certain drugs, and competition for excretion between UA and other molecules. Third cause is the mixture of both increased intake and decreased excretion of UA, and is usually caused by alcoholism, starvation, or high levels of fructose in diet (Nagaya et al., 1999). In addition, factors leading to hyperuricemia vary. This includes genetics, hypertension, renal insufficiency, obesity, insulin resistance, diuretics use, hypothyroidism, and the consumption of alcoholic beverages. Of these, alcoholism appears to be the most significant influence.

In line with this, studies conducted on the prevalence of hyperuricemia showed that 37.8% of men, and 18% of women are affected of this condition (Calderon et al., 2015). At a symposium conducted by the American College of Rheumatology, it was reported that increase in serum uric acid was evident all throughout the regions of the world, including the

Philippines with a 25% prevalence in Southeast Asia (Smith & March, 2015). In addition, the Philippine Rheumatology Association (PRA) disclosed that the prevalence of hyperuricemia and gout among Filipinos has been increasing in the past two decades. In the PRA 2015 national report, 1.6 million Filipinos have been recorded to suffer from these conditions (Crisostomo, 2015). Also, Filipino men had a relatively higher mean blood uric acid than women; which could be accounted to diet, lifestyle, and/ or underlying renal genetic defects (Calderon et al., 2015).

Increase in the excretion and reduction of uric acid production helps reduce cases of hyperuricemia and gout (Kostic et al., 2015). Thus, the treatment of hyperuricemia and diseases linked to it such as gout, nephropathy and renal stones typically requires the use of therapeutic agents such as xanthine oxidase inhibitors (XOI) (Azmi, Jama, & Amid, 2012; Higgins et al., 2009). Xanthine oxidase inhibitors act by arresting the synthesis of urate from purine sources thus decreasing the chance of producing uric acid. By doing so, both circulating levels of uric acid and vascular oxidative stress are reduced (Kostic et al., 2015).

Among the various known XO inhibitors, allopurinol has been the most and commonly prescribed anti-hyperuricemic medication (Kumar, Kansal, Chaudhary, Sharma, & Sharma, 2015). Allopurinol, a purine analog of hypoxanthine, acts at the purine binding site by blocking the conversion xanthine and hypoxanthine into uric acid. This XO inhibition leads to the decrease in serum uric acid levels (Day et al., 2007). For several decades, allopurinol has been widely distributed in the market to lower the blood concentrations of urate. However, despite its availability, this drug is not devoid of its own set of complications and adverse effects (Sriram, Venkatesan, & George, 2017). Generally, allopurinol is well-tolerated, but it occasionally induces side effects ranging from simple cutaneous reactions like skin rashes to severe hypersensitivity reactions that occur after a few months of therapy (Kumar et al., 2015), affecting multiple systems such as Steven-Johnson Syndrome (Surnarni, Leviana, Fidrianny, Iwo, & Wirasutisna, 2015).

Considering this, a potential source of such compounds that could inhibit enzyme activity could be obtained from plants. Active compounds such as flavonoids (Hudaib et al., 2011), and polyphenolic constituents in plant extracts were reported to possess inhibitory action against XO (Kostic et al., 2015). With these findings, possibilities of isolation, characterization, and utilization of new natural compounds that have the targeted effects against xanthine oxidase (Sunarni et al., 2015), have been opened. This led to the gaining renewed interest in the

study of botanical plants for the treatment of various kinds of clinical disorders (Kostic et al., 2015; Unno, Sugimoto, & Kakuda, 2004).

Figure 1. *Leea aculeata* leaves and fruit

Leea aculeata or Mali-mali belongs to the family *Vitaceae*, and is a shrub usually found in thickets or forests, particularly along streams in low and medium altitudes. It is widely distributed in northern parts of Luzon to Palawan and Mindanao. *L. aculeata* leaves are traditionally used in the Philippines to purify blood (Quisumbing, 1979). In addition, it was reported that *L. aculeata* possesses antioxidant properties (Uy & Villazorda, 2015), which may be a significant factor for its folkloric use. Certain phytoconstituents such as cardiac glycosides, flavonoids, and terpenoids were found present in the leaves of *L. aculeata* (Lagunay & Uy, 2015), which may have contributed to its antioxidant activity.

Interestingly, however, there are no studies conducted to assess the anti-hyperuricemic capability of *L. aculeata* using an enzyme inhibition assay. Therefore, the aim and purpose of this research is to evaluate the anti-hyperuricemic property of *L. aculeata* using an in vitro inhibition assay of xanthine oxidase. This study was also performed to determine which concentration best inhibits xanthine oxidase, and determine whether it is dose dependent or not. All of these were done to recognize the plant's potential to be a natural substitute for commercial drugs used in the treatment and management of hyperuricemia, gout, and other inflammatory-related conditions.

MATERIALS AND METHODS

Plant Collection and Identification

Fresh leaves of *L. aculeata* were collected from Brgy. Dulungan, Baco, Oriental Mindoro, Philippines. The leaves were

then submitted to the Department of Agriculture - Bureau of Plant Industry in Manila for proper identification and authentication.

Extraction of *L. aculeata* Crude Leaf Extract

L. aculeata leaves (300 grams) were first washed with tap water followed by distilled water, then air-dried at room temperature (26˚C) for 2 weeks. The dried leaves were then homogenized using a blender, then weighed. *L. aculeata* extract was prepared by soaking 100 grams of the dry powdered leaves in 1L of ethanol at room temperature for 48 hours. The solution was then filtered through a cotton plug, and the resulting filtrate was filtered again using Whatman No. 42 filter paper (125mm). The filtrate was concentrated using a rotary evaporator with the water bath set at 40˚C (Zeb, Sadiq, Ullah, Ahmad, & Ayaz, 2014).

Phytochemical Screening

The ethanolic crude extract of *L. aculeata* leaves were subjected to phytochemical screening for alkaloids, anthraquinones, cardiac glycosides, flavonoids, reducing sugars, saponins, tannins, and terpenoids following the methods of Adesegun et al. (2008).

Test for Flavonoids

A solution containing a portion of the aqueous *L. aculeata* leaf extract added to 10 mL ethyl acetate was boiled, then filtered. Four milliliters of the filtrate were added to 1 mL dilute ammonia, then shaken. Formation of a yellow coloration or turbidity confirmed the presence of flavonoids.

Test for Reducing Sugars (Fehling's)

An aqueous ethanol extract, composed of 0.1 g *L. aculeata* crude leaf extract in 1 mL of water, was added to boiling Fehling's solution (A and B) in a test tube. A change from a deep blue to a copper red color of the solution indicated a positive test result.

Test for Saponins

In a test tube, 0.5 g of *L. aculeata* leaf extract and 5 ml of distilled water were shaken vigorously to form a stable persistent froth. Three drops of olive oil were added to the frothing and shaken vigorously. A positive test result was confirmed with the formation of an emulsion.

Test for Tannins

About 0.1 g of the *L. aculeata* leaf extract was mixed with 5 ml of water, boiled, and then filtered. Positive test was confirmed

upon addition of a few drops of 0.1% ferric chloride solution, resulting in brownish green or a blue-black color.

Test for Terpenoids (Salkowski)

About 0.5 g of the *L. aculeata* leaf extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulfuric acid, forming a layer. A reddish-brown coloration at the interface confirmed the presence of terpenoids.

Test for Alkaloids

About 0.1 g of the *L. aculeata* leaf extract was added to 5 ml acid alcohol, boiled and then filtered. In a 2.5 mL of filtrate,1 ml of dilute ammonia and 2.5 ml of chloroform were added, and the mixture was shaken gently to extricate the alkaloidal base. To the chloroform layer, 5 mL of acetic acid was added and then divided into two separate test tubes. Mayer's reagent was added drop by drop to the first tube until there was a formation of a cream or reddish-brown precipitate. Draggendorff's reagent was added to the second tube drop by drop until there was a formation of a cream or reddish-brown precipitate to indicate the presence of alkaloids.

Test for Cardiac Glycosides (Keller-Killiani)

L. aculeata leaf extract (0.5 g) was diluted with 5 mL of distilled water, and then mixed with 2 mL of glacial acetic acid, and a drop of ferric chloride solution. To this, 1 mL of concentrated sulfuric acid was added and slowly formed a layer. At the interface, a brown ring indicated deoxysugar which is characteristic of cardenolides. An acetic layer (greenish ring) may form on the top and gradually spread, while a violet ring may appear below the brown ring.

Test for Anthraquinones

From *L. aculeata* leaf extract, 0.1 g was added to 2mL of sulfuric acid, which was then filtered while still hot. After the addition of 1 ml of chloroform to the filtrate, the solution was shaken. The chloroform layer was extracted and transferred into separate test tube, and 0.2 mL of dilute ammonia was added. Presence of a pink to red color change indicated a positive result.

Preparation of Positive Control

The positive control was prepared by dissolving 5mg of allopurinol into 5mL of 0.15M phosphate buffer (pH 7.5) (Apaya & Chichioco-Hernandez, 2011).

Preparation of experimental samples

To prepare the experimental samples, an assay mixture containing 1ml of *L. aculeata* leaf extract of varying concentrations (10, 20, 50, and 70 ug/mL) was added to 2.9 mL phosphate buffer (pH 7.5) and 0.1 mL of xanthine oxidase enzyme solution (0.1 units/mL in phosphate buffer, pH 7.5). the fractions were then dissolved in dimethyl sulfoxide (DMSO). Proper controls with DMSO were executed. After the pre-incubation period at 25°C for 15 minutes, the reaction was initiated by adding 2mL of the substrate solution (150uM xanthine in the same
buffer). The assay mixture was then incubated at 25°C for 30 minutes, and the reaction was stopped by the addition of 1N hydrochloric acid (Apaya & Chichioco-Hernandez, 2011; Umamaheswari et al., 2009).

In Vitro Xanthine Oxidase Inhibition Assay

All the prepared concentrations of *L. aculeata* leaf extract were assayed for their activity in inhibiting the xanthine oxidase in vitro. The inhibitory activity was measured spectrophotometrically under aerobic conditions, with xanthine serving as the substrate (Owen & Johns, 1999), and the absorbance was measured at 290nm using a UV-Vis spectrophotometer. The measured values of each concentration were compared against the positive control (5mg of allopurinol added to 5mL of 0.15M phosphate buffer with a pH of 7.5) (Apaya et al., 2011). The xanthine oxidase inhibitory activity was expressed as the percentage inhibition of the extract against the enzyme xanthine oxidase. The percentage inhibition of XO in the above assay system was calculated as

$$\% \text{ XO inhibition} = (1 - \beta/\alpha) \times 100$$

where α is the activity of XO without the plant extract, and β is the activity of XO with the plant extract. The assay was done in triplicate (Umamaheswari et al., 2009; Nguyen et al., 2004).

Statistical Analysis

The In vitro xanthine oxidase inhibitory activity of *L. aculeata* leaf extract was analyzed by calculating the mean and percentage values of the assay mixture absorbance. The data obtained were statistically analyzed using one-way analysis of variance (ANOVA) to compare the experimental samples against the positive control. Tukey's multiple comparison post hoc tests were also utilized to compare the level of significance between control and experimental groups. The values of $p<0.05$ were considered as significant for each group determining if results

were significantly different from one another or if they were relatively the same. In order to determine the differences between the experimental and negative control group, an independent sample t-test was used. All computations were done using SPSS version 17.

RESULTS AND DISCUSSION

Extraction of Ethanolic Crude Leaf Extract

From the 300 grams of air-dried *L. aculeata* leaves, 15 grams of the ethanolic extract were obtained after using a rotary evaporator. The percentage yield was 15% (wt/wt). The extract produced was dark green in color with a mucoid consistency similar to the study of Reddy, Navanesan, Sinniah, Wahab, & Sim (2012).

Figure 2. *Leea aculeata* crude leaf extract

Phytochemical Testing

Table 1 shows the phytochemical screening results of *L. aculeata* leaf extract. Based on qualitative testing, it was found to contain flavonoids, saponins, tannins, terpenoids and anthraquinones. Reducing sugars, alkaloids, as well as cardiac glycosides were found to be absent.

Table 1
Phytochemical Screening Results

Phytochemical	Result
Flavonoids	+
Reducing sugars	-
Saponins	+
Tannins	+
Terpenoids	+
Alkaloids	-
Cardiac glycosides	-
Anthraquinones	+

Legend: (+) = presence, (-) = absence

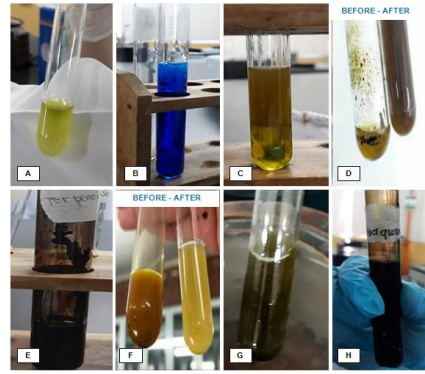

Figure 3. Phytochemical screening A. flavonoids B. reducing sugars C. saponins D. tannins E. terpenoids F. alkaloids G. cardiac glycosides H. anthraquinones

Flavonoids, as shown by a yellow color, are essential phytochemicals found in a variety of plant foods. They possess a wide variety of biochemical and pharmacological properties that include antiplatelet, antioxidant, antiradical, anticarcinogenic, antiviral, antimicrobial, antithrombotic as well as antimutagenic activities (Liu et al., 2014). They can reduce blood lipids, and help manage oxidative stress induced by diabetes (Yadav, Chatterji, Gupta, & Watal, 2014). They have also been reported to enhance human immunity. For this, flavonoids have been studied extensively as they show great potential value in human health care.

Saponins with the formation of an emulsion, have the unique ability to precipitate and coagulate red blood cells (Iloki-Assanga et al., 2015). In contrast to the study of Lagunay & Uy (2015) which they revealed saponins to be absent in *L. aculeata* leaf extract, the researchers found out the presence of saponins in *L. aculeata* leaf extract.

Tannins, terpenoids, and anthraquinone resulted to a brownish-green coloration, a reddish-brown coloration, and a reddish coloration, respectively. These findings correlate with a study previously conducted by Lagunay and Uy (2015).

Among these phytochemicals, previous studies have shown that flavonoids can be attributed to the enzyme inhibition, as they interact with xanthine oxidase competitively, resulting to XO inhibition (Jiao, Ge, Shi, & Tan, 2006). Isolated fractions of flavonoids like myricetin and luteonin were found to be potential inhibitors of xanthine oxidase (Li et al., 2011). In addition,

quercetin, rutin, and astilbin were also reported to be potent xanthine oxidase inhibitors (Kostic et al., 2015).

In Vitro Xanthine Oxidase (XO) Inhibition Assay

The activity of *L. aculeata* leaf extract was examined against XO. The inhibitory activity of varying concentrations of the extract (10, 20, 50, 70 ug/ml) was determined.

Table 2
Mean Percent Inhibition of
***L. aculeata* Leaf Extract**

Concentration (ug/ml)	Average (%)
Allopurinol	91.3
10	61.96
20	5.43
50	92.75
70	43.12

Table 2 shows the mean percent inhibition of the *L. aculeata* leaf extract against xanthine oxidase. This was performed in triplicate. Using allopurinol, the resulting percent inhibition was 91.3%. This clearly shows that allopurinol can inhibit xanthine oxidase. Using 10ug/ml *L. aculeata* leaf extract, it resulted to 61.96% inhibition. However, when 20ug/ml was used, only 5.43% XO was inhibited. Using 50ug/ml of *L. aculeata* leaf extract, the resulting percent inhibition is 92.75%. This corresponds to the most efficient percent inhibition among the different concentrations of *L. aculeata* leaf extract. In addition, when 70ug/ml of the *L. aculeata* leaf extract was used, 43.12% inhibition was obtained. It is noticeable that at increasing concentrations of *L. aculeata* leaf extract, no increasing percent inhibition of XO was observed, instead varying results were determined. This is more likely due to the phytochemical substances present such as flavonoids, saponins, and terpenoids. Flavonoids can act as xanthine oxidase inhibitors and as scavengers of superoxide radicals. They have an additional pro-oxidant effect (Cos et al., 1998). Saponins possess XO inhibitory activity as supported by Umamaheswari et al. (2009). The structure of saponins enables them to inhibit xanthine oxidase by non-competitive inhibition (Apaya & Chichioco-Hernandez, 2014; Xu, Zhao, Yang, Wang, & Zhao, 2014). Terpenoids can also inhibit xanthine oxidase as well as reduce reactive oxygen species (Lin et al., 2010). The structure of terpenoids enables them to cause XO inhibition (Afrianti, Sukandar, Ibrahim, & Adnyana, 2007). This type of xanthine oxidase inhibitor is similar to flavonoids that have an additional pro-oxidant effect (Cos et al., 1998).

Comparison of varying concentrations of *L. aculeata* leaf extract against allopurinol is presented in Table 3. Since allopurinol is the standard drug to treat gout and help prevent increased uric acid levels, the resulting inhibition of the different concentrations of *L. aculeata* leaf extract was compared against it. The table reveals that there were significant differences between 10, 20, and 70ug/ml concentrations of *L. aculeata* leaf extract and allopurinol as they reached p-values of 0.032, 0.000, and 0.041, respectively, which were less than 0.05. This implies that there was a low level of xanthine oxidase inhibitory activity seen when using 10, 20, and 70ug/ml concentrations of *L. aculeata* leaf extract when compared to the allopurinol. On the other hand, using 50ug/mL concentration of *L. aculeata* leaf extract generated a p-value of 0.270, which was greater than the test value, in which the resulting xanthine oxidase inhibitory activity was almost the same as the allopurinol. This clearly shows that among the four concentrations, 50ug/ml has the highest xanthine oxidase inhibitory effect. This is due to the structure-activity relationship of flavonoids as xanthine oxidase inhibitors. The greater the number of aromatic hydroxyl groups in a flavonoid, the stronger the inhibition of the flavonoid against xanthine oxidase (Iio, Moriyama, Matsumoto, Takaki, & Fukumoto, 2014). It can also be due to the inhibitory activity of tannins and anthraquinones. On a study by Hatano et al. (1990), it was suggested that the inhibition of xanthine oxidase by tannins is not based on non-specific binding to the protein, rather by non-competitive inhibition. The strength of the inhibition is affected by the molecular weight of tannin, which reflects the number of phenolic hydroxyl groups in the molecule. Anthraquinones were also found to inhibit xanthine oxidase in vitro via uncompetitive inhibition as supported by the study of Noro et al. (1987).

Table 3
Comparison of *L. aculeata* Leaf Extracts to Allopurinol with XO

Concen-tration (ug/ml)	t	p-value	Interpretation
10	5.473	0.032	Significant
20	68.416	0.000	Significant
50	-1.512	0.270	Not Significant
70	4.796	0.041	Significant

Legend: Significant at p-value <0.05; Test Value = 0.008

Table 4
Multiple Comparison of Concentrations of *L. aculeata* Leaf Extract on Percent Inhibition

Concentration (ug/ml)	Concentration (ug/ml)	p-value	Interpretation
Allopurinol	10	0.036	Significant
	20	0.000	Significant
	50	0.187	Not Significant
	70	0.004	Significant
10	Allopurinol	0.036	Significant
	20	0.001	Significant
	50	0.022	Significant
	70	0.173	Not Significant
20	Allopurinol	0.000	Significant
	10	0.001	Significant
	50	0.000	Significant
	70	0.007	Significant
50	Allopurinol	0.187	Not Significant
	10	0.022	Significant
	50	0.000	Significant
	70	0.001	Significant
70	Allopurinol	0.004	Significant
	10	0.173	Not Significant
	20	0.007	Significant
	50	0.001	Significant

Legend: The mean difference is significant at the 0.05 level

Table 4 shows multiple comparison of varying *L. aculeata* leaf extract concentrations based on their % inhibition. The data presents the different concentrations based on their % inhibition. The data presents the different concentrations of *L. aculeata* leaf extract and their corresponding p-values. When the allopurinol was compared to 10ug/ml, 20ug/ml, and 70ug/ml concentrations, the resulting p-values were 0.036, 0.000, and 0.004, respectively, which were all less than 0.05 level that showed significant differences. This means that the allopurinol exhibited greater level of xanthine oxidase inhibitory effect than 10ug/ml, 20ug/ml, and 70ug/ml concentrations. On the other hand, when it was compared to 50ug/ml concentration, the resulting p-value was 0.187 which is greater than 0.05 level. This shows no significant difference, which means that the xanthine oxidase inhibitory effect of the allopurinol is the same with the inhibitory effect of 50ug/ml concentration. When 10ug/ml concentration of *L. aculeata* leaf extract was compared to

allopurinol, 20 ug/ml, and 50ug/ml concentrations, the obtained p-values were 0.036, 0.001, and 0.022, respectively, which were less than 0.05 level; thus, showing a significant difference. This means that the % inhibition of 10ug/ml is significantly lower than the allopurinol and 50ug/ml concentration but greater than the 20ug/ml concentration. On the other hand, when 10ug/ml was compared to 70ug/ml, the resulting p-value was 0.173, which means that there is no significant difference. This implies that both 10ug/ml and 70ug/ml concentrations exhibit the same xanthine oxidase inhibitory activity. Consequently, when 20ug/ml was compared to allopurinol, 10ug/ml, 50ug/ml, and 70ug/ml, the resulting p-values (0.000, 0.001, 0.000 and 0.007, respectively) were less than 0.05 level; thus, there is a significant difference. This shows that the % inhibition of 20ug/ml concentration is significantly lower than that of allopurinol, 10, 50, and 70ug/ml concentrations. When 50ug/ml concentration was compared to 10ug/ml, 20ug/ml, and 70ug/ml, the obtained p-values were 0.022, 0.000, and 0.001, respectively. This shows significant differences which means 50ug/ml has a greater inhibitory activity than 10ug/ml, 20ug/ml, and 70ug/ml. In addition, when 50ug/ml was compared with allopurinol, the resulting p-value was 0.187; thus, showing an insignificant difference in their percent inhibition. This means the xanthine oxidase inhibitory activity of 50ug/ml is the same as the allopurinol. Lastly, when 70ug/ml was compared to allopurinol, 20ug/ml, and 50ug/ml, the resulting p-values were 0.004, 0.007and 0.001, respectively, showing a significant difference. This means that 70ug/ml has lower inhibitory activity than allopurinol and 50ug/ml concentration but greater inhibitory activity than 20ug/ml. Consequently, when it was compared to 10ug/ml, the p-value was 0.173, which is greater than 0.05 level, showing no significant difference. This means that there was an equal inhibitory activity between 70ug/ml and 10ug/ml concentrations. The result revealed that the inhibition of xanthine oxidase varies depending on the concentration of the extract showing mixed inhibition of the enzyme as supported by Sharma (2012). This is more likely due to the dual activity of flavonoids as both xanthine oxidase inhibitors and as superoxide radicals scavengers, wherein the prooxidant activity is relatively higher than enzyme inhibition supported by Cos et al. (1998). This explains why there is no linear pattern and dosage dependency in the inhibition of the enzyme xanthine oxidase.

Looking at the presented data from Tables 2, 3 and 4, all concentrations of *L. aculeata* leaf extract have xanthine oxidase inhibitory activity. This may be associated with the amount of different phytoconstituents, specifically flavonoid, present in the varying concentrations of the leaf extract samples. Although no

study regarding the use of varying concentrations of isolated fractions of flavonoids have been done, abundance of flavonoids in the plant subjects used in previous similar studies of Calderon et al. (2015), Azmi et al. (2009) and Nguyen et al. (2004) has been greatly regarded to be the cause of this xanthine oxidase inhibitory
activity.

CONCLUSION

Several concentrations (10ug/ml, 20ug/ml, 50ug/ml, and 70ug/ml) of *L. aculeata* leaf extract were evaluated for their xanthine oxidase inhibitory activity. It was observed that all the prepared concentrations exhibited various percentage inhibition when tested in vitro, with 50 ug/ml showing the best inhibitory activity against xanthine oxidase, yielding inhibition results at par with allopurinol. Thus, *L. aculeata* leaf extract has inhibiting activity against xanthine oxidase, proving its potential of being an anti-hyperuricemic agent.

RECOMMENDATIONS

Since *L. aculeata* leaf extract showed potential biologic activity against xanthine oxidase, the researchers recommend that this study be further explored through the use of an animal model. Fractionation and isolation of the specific phytochemical substances responsible for the XO inhibition can also be performed. Increasing the concentrations of the extract and inclusion of toxicity assays can also be done. Other components of the plant such as roots, bark, and fruiting bodies can also be studied for xanthine oxidase inhibition activity.

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