

IN Vitro Xanthine Oxidase Inhibitory Activity of Spiny Pigweed (*Amaranthus spinosus* Linn.) Leaves Crude EXTRACT

Albert A. Malabarbas¹, Maureen Canda-Borcelas²

¹Instructor, San Pedro College, Davao City
albertmalabarbas@yahoo.com

²Faculty, University of the Immaculate Conception, Davao City, Philippines
mcanda@uic.edu.ph

ABSTRACT

Hyperuricemia (HUA), marked by elevated xanthine oxidase (XO) activity, is significantly linked to gout and various metabolic disorders. Since XO is a key enzyme in uric acid formation, it serves as a primary target for therapeutic interventions. This study aimed to evaluate the in vitro xanthine oxidase inhibitory activity of the spiny pigweed (*Amaranthus spinosus* Linn.) leaves crude extract. An experimental research design was employed where the XO inhibitory activity was assessed using a spectrophotometric method by measuring uric acid formation from a xanthine substrate. Ethanolic crude extracts at concentrations of 200, 100, 50, 25, 12.5, and 1 µg/mL were tested, with allopurinol serving as the standard reference at the same concentration levels. Qualitative phytochemical screening confirmed the presence of flavonoids, saponins, terpenoids and glycosides. *A. spinosus* leaves crude extract exhibits a high percentage of xanthine oxidase inhibitory activity—achieving 96.37% inhibition at 200 µg/mL—with an IC₅₀ value of 1.912 µg/mL determined using probit analysis, suggesting notable therapeutic potential as an XO inhibitor. Total flavonoid and total phenolic content results reveal an average amount of 446.258 mcg QE/g and 536.951 mcg GAE/g of crude extract, respectively. The diverse compounds present contribute significantly to the effective XO inhibitory activity of *A. spinosus*.

KEYWORDS: *Pharmacy, Amaranthus spinosus, xanthine oxidase inhibition, in vitro, Philippines*

INTRODUCTION

Elevation of uric acid levels, or hyperuricemia (HUA) is a complex metabolic and inflammatory disease that traditionally affected older adults but is now increasingly impacting younger populations. This shift underscores the

growing burden of hyperuricemia (Han et al., 2024), and its related comorbidities, which has been identified as the fourth largest chronic disease following hypertension, hyperglycemia, and hyperlipidemia (Feng et al., 2022). Furthermore, this disease has been associated with metabolic syndrome and other renal and cardiovascular diseases, as well as comorbidities such as diabetes, hypertension, hyperlipidemia, obesity, menopause, and post-operative complications (Wang et al., 2018; Singh & Gaffo, 2020). Additionally, increased medication management difficulty arises from adverse drug effects that correlate with poor adherence, all of which stem from the increased activity of xanthine oxidase, one of the primary free radical-producing enzymes responsible for oxidative tissue damage (Scavone et al., 2019; Hudaib et al., 2011).

The annual hyperuricemia (HUA) prevalence is rising globally with an earlier age of onset, specifically in China, the United States, and Japan, now affecting 15 to 39-year-olds (17.4%, 20.1%, 26.8% respectively) (Chen et al., 2022; Huang et al., 2022; Koo et al., 2021). In 2019, approximately 5.21 million individuals globally in this age group suffered from HUA and gout, with cases rising from 38.71 to 45.94 per 100,000 population, while the Philippines has been noted to have a rampant rate of the disease, with a prevalence of 25% (Zhang et al., 2023; Smith & March, 2015). It is projected that the number of these cases may rise by 55% by 2060, placing a large epidemiological burden worldwide, and this trend is unlikely to change very soon (Mattiuzzi et al., 2019).

Uric acid buildup in the body can cause hyperuricemia and gout; so reducing its production is key to treatment. Enzyme manipulation, particularly with xanthine oxidase inhibitors such as allopurinol, helps block the formation of excess uric acid (Manjuladevi et al., 2019). While effective, these drugs can cause side effects with long-term use, prompting interest and research in safer alternatives, focusing on natural, plant-based compounds with fewer adverse effects (Kostic et al., 2015). Plants are rich in phytochemicals, including flavonoids, phenolics, alkaloids, terpenoids, saponins, and tannins. Flavonoids and polyphenols have been found to inhibit xanthine oxidase, which may help prevent diseases linked to excess uric acid (Umamaheswari et al., 2013).

Interest in *Amaranthus* spp., or amaranth, has grown due to its impressive nutritional value and potential health benefits (Peter & Gandhi, 2017). One species, *Amaranthus spinosus* L., also known as spiny pigweed or kulitis, has been used traditionally for its health benefits in many cultures (Kumar et al., 2014). Often seen as a weed, this plant grows in clusters along sandbars and

stream edges and is common in the lowlands and waste areas of the Philippines. Studies have shown that it contains various phytochemicals, including alkaloids, flavonoids, saponins, and glycosides (Boro et al., 2023), which are linked to its antipyretic, anti-inflammatory, antioxidant, and liver-protective properties. These benefits support its traditional use for conditions such as arthritis, burns, bruises, and other inflammatory diseases (Baral et al., 2011), highlighting its potential in herbal medicine.

However, there are few studies supporting the efficacy of *Amaranthus spinosus* L. for hyperuricemia and gouty arthritis. Therefore, this study will address a specific research gap, as there is scarce scientific evidence on the in vitro xanthine oxidase-inhibitory effect of *A. spinosus*. Hence, the goal of all of this is to identify the phytochemical constituents present in the plant to explore its activity as an inhibitor of the xanthine oxidase enzyme and discover the potential of the plant as a natural alternative to pharmaceuticals used in the management and treatment of hyperuricemia, gout, and other disorders linked to hyperuricemia.

The findings of this study will be disseminated through publication in peer-reviewed journals and presentation at scientific conferences to contribute to the growing body of knowledge on medicinal plants. A plain-language summary will be made available via institutional platforms to reach various audiences. These efforts aim to support the informed, evidence-based use of *A. spinosus* in the management of hyperuricemia.

Statement of the Problem

1. What are the phytochemicals present in the ethanolic crude extract of *A. spinosus* leaves?
2. What is the xanthine oxidase inhibitory activity (in %XO inhibition) and IC₅₀ of the ethanolic crude extract of *A. spinosus* leaves?
3. What is the total flavonoid content (TFC) of the ethanolic crude extract of *A. spinosus* leaves?
4. What is the total phenolic content (TPC) of the ethanolic crude extract of *A. spinosus* leaves?

Theoretical Framework

Xanthine oxidase (XO) plays a pivotal role in purine metabolism by catalyzing the conversion of hypoxanthine to xanthine and subsequently to uric acid. The enzymatic activity is crucial for maintaining uric acid homeostasis;

however overactive XO leads to excessive uric acid production, resulting in hyperuricemia. This pathophysiology is further compounded by oxidative stress, a consequence of increased XO activity. XO generates reactive oxygen species (ROS) (Aziz & Jamil, 2023), during its catalytic process, which can damage cellular components and exacerbate inflammation, thereby promoting the progression of hyperuricemia and its associated ailments like gout, cardiovascular diseases, and systemic inflammatory disorders (Hernandez et al., 2022). Hence, the inhibition of the enzyme is the most common biochemical reaction sought after to treat hyperuricemia and its sequelae.

Under these circumstances, natural sources abundant in flavonoids and other phytochemicals serve a biological purpose and may have a potential XO inhibition and antioxidant activity. *Amaranthus spinosus* L. is a plant used for treating inflammation that is expected to have hypouricemic action to confirm this hypothesis (Kumar et al., 2014). Phytochemical constituents of the plant include quercetin, kaempferol, rutin, and D-glucuronic acid; all are said to be capable of regulating oxidative stress and the processes of inflammation (Abir & Ahmad, 2021; Stinzing et al., 2024; Swarnakumari et al., 2021).

This theoretical proposition has a mechanistic justification: flavonoids such as quercetin and kaempferol were reported to form hydrogen bonds and physisorption on XO, which would lead to more XO's activity being lost through the formation of a complex (Xue et al., 2023). The therapeutic potential of such compounds is plausible; however, given the strong inhibition they exhibit with IC₅₀ values (Zhang et al., 2018; Wang et al., 2015; Lin et al., 2015). Also, some secondary metabolites of *Amaranthus* spp., such as saponins and alkaloids, have been shown to affect uric acid metabolism (Jiang et al., 2020). The association between the xanthine oxidase (XO) inhibitory activity and phytochemical constituents of *Amaranthus spinosus* and its relatives, which comprise *A. hybridus*, *A. cruentus*, *A. gangeticus*, and *A. inamoenus*, appears to position *A. spinosus* within the category of hypouricemic agents. (Nana et al. 2012; Yang et al. 2020)

METHODOLOGY

Research Design. The researcher utilized a quantitative, descriptive, true-experimental research design to determine the in vitro xanthine oxidase enzyme-inhibition activity of the crude extract of *Amaranthus spinosus* leaves.

Research Locale. This study was conducted at four locations. Fresh

Amaranthus spinosus plants were harvested and collected at Brgy. Pamacaun, Municipality of Asuncion, Tagum, Davao del Norte, Philippines. The leaves were air-dried, then processed through maceration and rotary evaporation at the pharmacy-chemistry laboratory of San Pedro College, Davao City. The extracts were freeze-dried at the Science Resource Center of the University of the Immaculate Conception, Davao City. Preliminary phytochemical tests, as well as total flavonoid and phenolic content analysis, were performed at San Pedro College, Davao City. The in vitro xanthine oxidase inhibition assay was conducted at WVN Laboratories, Talomo, Davao City.

Research Procedure. After collection, the leaves, separated from the stems, were allowed to air-dry for 14 days at room temperature and stored well. After air-drying and storage, with a moisture content of less than ten percent, the dried leaves were ground and homogenized into small pieces. Eight hundred grams of *A. spinosus* leaves were soaked in 95% ethanol, which was then allowed to stand at room temperature with minimal shaking for 24 hours in a 1:10 (w/v) ratio. The mixture solution was then collected and filtered using Whatman No. 1 filter paper. The plant extract was concentrated using rotary evaporation at 40 °C under reduced pressure, then freeze-dried. The dried samples were kept in a desiccator, while the remaining crude extracts were stored at 4 °C.

The crude extract was subjected to phytochemical screening, and, prior to use, the required amount of dried crude extract was dissolved in dimethylsulfoxide (DMSO) to prepare the stock solution. Six apportioned concentrations (200, 100, 50, 25, 12.5, and 1 µg/mL) of the *Amaranthus spinosus* crude extract were subjected to an in vitro xanthine oxidase inhibition assay. Phytochemical screening was performed to detect the presence of flavonoids, saponins, tannins, terpenoids, alkaloids, reducing sugars, cardiac glycosides, and anthraquinones using standard procedures.

In Vitro Xanthine Oxidase Inhibition Assay. The various concentrations of *A. spinosus* extract were assayed for their in vitro xanthine oxidase inhibition using a UV-Vis microplate spectrophotometer, measuring absorbance at 405 nm. The measured absorbance of the varying sample concentrations was compared against the standard (Allopurinol) concentrations. A negative control (blank) was also performed. The experiment was performed in triplicate. The xanthine oxidase inhibitory activity was expressed as the percent inhibition of xanthine oxidase in the assayed samples. Subsequently, the IC₅₀ concentration was obtained from a probit analysis of the plot of concentration (in log X) against

probit-y values from the percent inhibition.

Determination of the Total Flavonoid Content (TFC). Quercetin standard solutions were prepared at concentrations of 20, 40, 60, 80, and 100 µg/mL in ethanol. Next, one mL of each standard solution was pipetted into separate test tubes, and then the following were added: 3 mL of ethanol, 0.20 mL of 10% aluminum chloride (AlCl₃·6H₂O) solution, and 0.20 mL of 1 M potassium acetate (KC₂H₃O₂) solution. Finally, the final volume was adjusted to 10 mL with ethanol and vortexed to mix well. The mixture was then incubated at room temperature, protected from light, for 30 minutes. The reaction mixture's absorbance was then measured at 415 nm using a UV-Vis spectrophotometer. The test was performed in triplicate. Additionally, a blank absorbance was also performed. A standard calibration curve was then plotted with absorbance values against quercetin concentration (µg/mL), and the linear equation $y = mx + c$ was then determined. Subsequently, a 25-mL plant extract solution at 1 mg/mL was prepared; one mL of the extract was then pipetted into a test tube and subjected to the same procedure as above.

Determination of the Total Phenolic Content (TPC). Gallic acid standard solutions were prepared at concentrations of 50, 100, 150, 200, and 250 µg/mL in distilled water. Next, five mL of each standard solution was pipetted into separate test tubes. Twenty-five (25) mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) was prepared next. Then, 20 mL of sodium carbonate solution (75 g/L) was added to each test tube. The solution was mixed well and allowed to stand for 30 minutes at room temperature in the dark. The absorbance was measured at 760 nm using a UV-Vis spectrophotometer. A calibration curve was plotted using the absorbance values against the gallic acid concentration (µg/mL), and the linear equation ($y = mx + c$) was determined. Additionally, a blank absorbance was also performed. Subsequently, a 50-mL plant extract solution (1 g/100 mL) was prepared. Then, 1 mL of the extract was pipetted into a test tube and subjected to the same procedure as above, with the amounts adjusted accordingly.

Data Analysis. The in vitro xanthine oxidase inhibitory activity of the crude extract of *Amaranthus spinosus* leaves was evaluated by analyzing the absorbance values of the assay mixture. The mean absorbance was determined by averaging the absorbance readings from triplicate samples at each sample concentration (200, 100, 50, 25, 12.5, and 1 µg/mL), along with similar concentrations for the positive control, Allopurinol. The percentage xanthine

oxidase inhibitory activity of the samples and standard was calculated using the formula:

$$\%XO \text{ inhibition} = \frac{\text{Absorbance of blank} - \text{Absorbance of sample (or standard)}}{\text{Absorbance of blank}} \times 100(1 - (A/B)) \times 100.$$

The half-maximal inhibitory concentration (IC₅₀) values, representing the concentration at which 50% of the enzyme activity is inhibited, were determined through probit analysis. This analysis will involve converting the percentage inhibition values obtained at different concentrations of the extract and the positive standard into probit units, then plotting them against the logarithm of the corresponding concentrations. From the resulting linear regression equation, the concentration corresponding to a probit value of 5.0 (equivalent to 50% inhibition) was calculated as the IC₅₀, assessing the potency of the treatments (*Amaranthus spinosus* leaves crude extract and a positive standard) in inhibiting the xanthine oxidase (XO) enzyme.

Ethical Considerations. The researcher applied for an Ethics Exemption from the REC. The University of the Immaculate Conception Research Ethics Committee (UIC-REC) assessed this to ensure that necessary elements (albeit exempt) met the required ethical features.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The *Amaranthus spinosus* leaves ethanolic crude extract was subjected to preliminary phytochemical screening using standard colorimetric reaction methods as shown in Table 1.

Table 1
Preliminary Phytochemical Screening Results

Phytochemical tests	<i>A. spinosus</i> leaves crude extract
Flavonoids	Positive
Saponins	Positive
Tannins	Negative
Terpenoids	Positive
Alkaloids	Negative
Reducing sugars	Negative
Cardiac glycosides	Positive
Anthraquinones	Negative

The preliminary phytochemical screening of the leaves' crude extract revealed the presence of various bioactive compounds, including flavonoids, saponins, terpenoids, and cardiac glycosides.

In Vitro Xanthine Oxidase Inhibitory Activity and IC₅₀ value

The various concentrations (200, 100, 50, 25, 12.5, and 1 µg/mL) of *A. spinosus* extracts were screened for their in vitro xanthine oxidase (XO) inhibition, spectrophotometrically. The degree of inhibition of the samples was compared with Allopurinol, the positive control. All experiments were performed in triplicate.

Table 2.1
Percentage Xanthine Oxidase Inhibitory Activity (%XO inhibition) of the Treatments

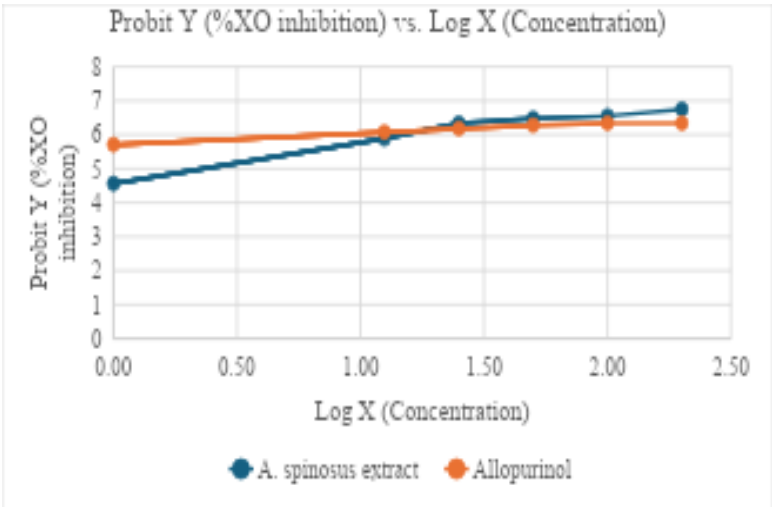
Treatments	Concentration (in µg/mL)	n	Mean	± SD
<i>Amaranthus spinosus</i> leaves crude extract	200	3	96.37	0.01
	100	3	93.59	0.02
	50	3	92.74	0.01
	25	3	91.02	0.00
	12.5	3	81.02	0.04
	1	3	32.52	0.10
Allopurinol	200	3	91.30	0.03
	100	3	91.18	0.05
	50	3	89.62	0.02
	25	3	88.20	0.05
	12.5	3	86.31	0.02
	1	3	75.61	0.06

Shown are the results of the percentage xanthine oxidase (XO) inhibitory activity of the prepared treatments (*Amaranthus spinosus* leaves crude extract and Allopurinol). The average %XO inhibition and its standard deviation are presented across different concentrations (µg/mL). The results of the assay revealed that *the crude extract of A. spinosus* leaves exhibited an increasing inhibition of xanthine oxidase (XO) activity.

Half-Maximal Inhibitory Concentration (IC₅₀) Determination

To further quantify the xanthine oxidase inhibitory activity of the *crude extract from Amaranthus spinosus* leaves, IC₅₀ values were determined using probit analysis. The concentrations were first converted to their log values and average percentage inhibition values obtained from the different treatments were

then converted into probit units and then plotted against the logarithm of the corresponding concentrations.



The graph above presents the comparative regression curves for the treatments used, *Amaranthus spinosus* leaves crude extract and Allopurinol, which show an increasing trend in inhibition with increasing concentration. Subsequently, the IC_{50} value, defined as the concentration required to inhibit 50% of xanthine oxidase activity, was then extrapolated from the resulting regression curve.

Table 2.2
IC50 values of the Amaranthus spinosus Extract and Allopurinol

Sample	IC ₅₀ (in µg/mL)	R ² value
Amaranthus spinosus leaves crude extract	1.912	0.9477
Allopurinol	0.002	0.9666

Shown are the IC_{50} values of the treatments used, determined using probit analysis. The *A. spinosus* leaves crude extract exhibited an IC_{50} value of 1.912 µg/mL, which is lower in potency than that of Allopurinol (IC_{50} = 0.002 µg/mL), the positive standard.

Total Flavonoid Content (TFC) in *Amaranthus spinosus* leaves crude extract

The total flavonoid content of the extract was determined using the aluminum chloride colorimetric method. The total flavonoid content was calculated from the calibration curve.

Table 3
*Total Flavonoid Content (TFC) in *Amaranthus spinosus* leaves crude extract*

Sample	Trial Number (mcg QE/g of crude extract)			Mean	Standard Deviation
	T ₁	T ₂	T ₃		
<i>Amaranthus spinosus</i> leaves crude extract	452.645	443.694	442.435	446.258	5.567

where: QE = quercetin equivalent

In the present study, the total flavonoid content (TFC) of the *Amaranthus spinosus* leaves ethanolic crude extract, expressed as mcg quercetin equivalent per gram of crude extract, was found to have a mean value of 446.258 mcg QE/g (SD = 5.567).

Total Phenolic Content (TPC) in *Amaranthus spinosus* leaves crude extract

The total phenolic content of the extract was determined using the Folin-Ciocalteu method. The total phenolic content was calculated from the calibration curve.

Table 4
*Estimation of Total Phenolic Content (TPC) in *Amaranthus spinosus* Leaves Crude Extract*

Sample	Trial Number (mcg GAE/g of crude extract)			Mean	Standard Deviation
	T ₁	T ₂	T ₃		
<i>Amaranthus spinosus</i> leaves crude extract	521.971	541.966	546.916	536.951	13.207

where: GAE = gallic acid equivalent

In the present study, the total phenolic content (TPC) of the *Amaranthus spinosus* leaves ethanolic crude extract expressed as mcg gallic acid equivalent per gram of crude extract, was found to have a mean value of 536.951 mcg GAE/g of crude plant extract (SD = 13.207). This is in close agreement with the findings of Swarnakumari et al. (2014) and Sarker and Oba (2019), who

reported comparable TPC values on *A. spinosus*. The variance of values, in existing literature, within *Amaranthus* genotypes is, in general, highly influenced by environmental factors (Nana et al., 2012), biological and methodological factors (solvent type used, extraction time and temperature, and analytical methods used).

Existing literature has supported that phenolic acids exert XO inhibitory activity through interaction with the enzyme's binding site, preventing substrate access (Mehmood et al., 2022) and through its antioxidant properties, reduces oxidative stress by scavenging reactive oxygen species, which is seen with increased XO enzyme activity (Ozyürek et al., 2009). Thus, the presence of phenolic compounds in plant extracts suggests the potential for XO enzyme inhibition. These findings are further confirmed with the results of the studies on the XO inhibitory activities of different *Amaranthus* species, which reveal that the relatively high detectable levels of total phenolics, along with other phytochemicals are attributed to the observed antioxidant and xanthine oxidase inhibitory activities, underscoring the potential of phenolic-rich plant extracts in managing conditions like hyperuricemia and gout.

The significant inhibition observed can be attributed to the presence of bioactive secondary metabolites such as flavonoids, phenolic acids (e.g., gallic acid), and terpenoids, which have previously been reported in *A. spinosus* and are known for their antioxidant and enzyme-inhibitory properties. The broad range of compounds in the crude extract may exert additive effects, enhancing overall activity. This shows that the *A. spinosus* shows favorable activity in XO inhibition, and thus, may present health benefits in the prevention of uric acid accumulation.

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