In *vitro* Xanthine Oxidase inhibitory effects of *Ficus nota* (Moraceae) flavonoid-rich extracts

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Abstract

Gout is a form of inflammatory arthritis which usually occurs in a single joint often found in the hallux or big toe. The production of tophi crystals from increasing levels of uric acid is its most common characteristic and this crystalline formation causes the inflammation leading to tissue damage. *Ficus nota* (Moraceae) also known as "tibig" is an endemic plant from the Philippines. Leaves and fruits of the plants were studied and proved to have antioxidant, antibacterial, and antimicrobial activities. The primary aim of this research was to ascertain the potential Xanthine oxidase (XO) inhibitory nature of *Ficus nota* by subjecting its methanolic leaf extracts to various tests. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were determined, and the highest concentration was found to be 145.6515 mg/g Gallic Acid Equivalent (GAE) of ethyl acetate extract and 279.685 mg/g Quercetin Equivalent (QE) of chloroform extract. The extract that yielded the highest concentration from TPC and TFC underwent column chromatography and thin layer chromatography respectively. A total of 15 pooled fractions (PF) were further assessed for TFC -- PF4 (266.022), PF5 (262.505), PF7 (268.659) resulted as the three highest concentrations. An *in vitro* assay was performed to test the XO inhibitory activity of the three PF with an IC50 of 341.3 µg/mL, 328.4 µg/mL, 334.5 µg/mL, respectively and allopurinol with an IC50 of 195.6 µg/mL. Upon the assessment of the results, the extracts showed comparable XO inhibitory activity to the standard drug Allopurinol.

Keywords: *Ficus nota, Xanthine oxidase inhibitor, flavonoids, phenolics*

INTRODUCTION

Inflammation is the mechanism of the body when it reacts to certain infection or injury. The release of enzymes and proteases that are bactericidal in nature are the cause of tissue damage and inflammation (Kumar, Bhat, Kumar, Bohra, & Sheela, 2011). Impairment of it can help to stabilize the lysosomal membrane which is needed to decrease the response of inflammation (Kumari, Yasmin, Hussain, & Babuselvam, 2015). Arthritis is a term used to describe the inflammatory conditions affecting the joints and surrounding tissues, the symptoms of which joint pain and stiffness, which may deteriorate with aging (Bykerk, 2015). Gout is one of the most common example of inflammatory arthritis or metabolic arthritis (Kuo, Grainge, Mallen, Zhang, & Doherty, 2015). It usually occurs in a single joint at a time and is often found in the hallux or big toe. Abnormally peaking levels of uric acid is the common characteristic of gout (Schauer et al., 2014). It will eventually result to the formation and deposition of urate crystals, also termed as tophi crystals that accumulate in the body particularly in the joints and tendons (Abd El-Rahman, 2015). The wide-ranging prevalence of gout accounts for 1-4% of the general population. It experienced in western countries by 3-6% of men and 1-2% in women. Prevalence in some countries may rise to 10%. Per 1000 individuals, the annual incidence of gout is marked at 2.68 (Kuo et al., 2015). In 2015 about 1.6 million Filipinos were affected with gout and the figure is expected to increase in the coming years due to unhealthy lifestyle (Philippine Rheumatology Association, 2015). Gout is more prevalent to happen among men than in women, adults suffering from obesity can also acquire the condition (Maiuolo, Oppedisano, Gratteri, Muscoli, & Mollace, 2016). It is not fatal, but it could gravely affect a person's quality of life. The formation of monosodium urate (MSU) crystals may result from an unhealthy lifestyle, family history, consumption of large amount of purinerich foods, alcoholic beverages, and medications like diuretics (Zhang et al., 2007). Normal uric acid level in men ranges from 3.4-7.0 mg/dL while those in women occurs at ranges of 2.4-6.0 mg/dL, formation of MSU crystals happens when the blood uric acid level rise above 7 mg/dL (Bykerk, 2015). The interactions of MSU crystals and the local tissues cause the clinical manifestations of gout (Dalbeth, Merriman, & Stamp, 2016). Uric acid is the final product of purine metabolism and the enzyme XO stimulates its overproduction from purine compounds (Busso & So, 2010).

Inhibitors of XO are used to manage and treat gout. It acts primarily by inhibiting the synthesis of uric acid from purine (Keenan & Schlesinger, 2016). Increased uric acid excretion or

the decreased production of uric acid is both helpful for the risk reduction of gout (Khanna et al., 2012). Current treatment for gout includes allopurinol, febuxostat, colchicine, Nonsteroidal anti-inflammatory drugs, and corticosteroids (Stamp et al., 2012). XO inhibitors that come from natural sources can be used as an alternative to allopurinol because of the fewer potential adverse effects (Martinon, Pétrilli, Mayor, Tardivel, & Tschopp, 2006). It has also been reported that polyphenols and flavonoids are potential inhibitors of XO that are plant-based (Robinson & Dalbeth, 2015).

Figure 1. *Ficus nota* tree, leaves, and fruits

Ficus nota (Blanco) Merr., locally known as "tibig" belongs to the Moraceae family. It is an endemic plant from the Philippines and is often found in Luzon and Visayas where it originated (Arquion, Nuñeza, & Uy, 2015). Amongst the *Ficus* species, the bioactivity of *Ficus nota* is the least explored (Ragasa, Tsai, & Shen, 2009). The *Ficus* family consistsmprimarily of flavonoids, alkaloids, and phenolic acids. Saponins, tannins, and coumarins can also be found (Sirisha, Sreenivasulu, Sangeeta, & Madhusudhana Chetty, 2010). The researchers' initial objective was to isolate flavonoids obtained from the methanolic extract of *Ficus nota* leaves. This was followed by attaining the percent yield of flavonoids from the extract and quantifying the flavonoids present in the isolate. Finally, to ascertain the XO inhibitory activity of *Ficus nota* by percent inhibition and by determining the IC50 of the flavonoid extract.

MATERIALS AND METHODS

Collection and Authentication of Plant Material

The leaves of Ficus nota were obtained from Manghinao Proper Bauan, Batangas and were authenticated at the University of Santo Tomas ResearchmCenter for the Natural and

AppliedmSciences – UST herbarium located in Sampaloc, Manila.

Chemicals and Equipment

Reagents used for the assessment of in vitro XO inhibition assay like xanthine oxidase, xanthine, allopurinol were purchased form Sigma- Aldrich. Analytical grade methanol, ethyl acetate, chloroform, dimethyl sulfoxide,nand n-butanol were purchased from RCI Labscan. Folin-ciocalteu phenol reagent (FCP) and Silica gel (100-200 Mesh size) were both purchased from LOBA Chemie Laboratory Reagents & Fine Chemicals. A microplate reader (HumaReader HS) was employed for the measurement of absorbance throughout the study.

Preparation for Extraction

The collected leaves were air dried at room temperature for at least aweek or until completely dried. A total of 700 grams of dried leaves were reduced into coarse particles using a blender and were stored in an air-tight container.

Defatting and Extraction process

(Sharma & Janmeda, 2017)

The extraction method by Sharma & Janmeda was adopted with some modifications on the apparatus and reagent used. The defatting and extraction process were done using a percolator. A total of 600 grams of leaves were defatted using hexane. The residue was dried and extracted exhaustively with 70% methanol. The methanolic extract was concentrated and then partitioned using different solvents: chloroform, ethyl acetate, n-butanol, aqueous, and methanol. The fractions collected were concentrated using a 6- well digital controlled-temperature tank at 60°C.

Percent yield was computed using the formula:

$$\frac{Weight\ of\ Concentrated\ Extract}{Weight\ of\ Dried\ Leaves} x100$$

Total Phenolic Content

(Thingbaijam, Dutta, & Paul, 2012)

Stock solutions with a concentration of 0.1g/mL were prepared from the crude, chloroform, n-butanol, aqueous, and methanol partitioned extracts and ethyl acetate extract with a concentration of 0.01g/mL. An aliquot of each stock solution was used to obtain 1000 µg/mL concentration. For the determination

of TPC, FCP reagent was employed and gallic acid served as the reference standard. An aliquot of 200 µL for the reaction mixture was transferred to the microwell plate and 800 µL of FCP reagent was added. After five minutes, 600 µL of 20% sodium carbonate was also added. The mixture was kept in a dark room for two hours and the absorbance was read using a microplate reader (HumaReader HS).

Total Flavonoid Content

(Hassan, Aqil, & Attimarad, 2013)

A concentration of 500 µg/mL of the stock solutions from crude, chloroform, n-butanol, aqueous, ethyl acetate, and methanol partitioned extracts were tested using the aluminum chloride method. Quercetin was used as the reference standard and was prepared into a series of concentrations using methanol as a solvent: 1000, 500, 250, 125, 62.5 µg/mL. A volume of 100 µL obtained from the admixed solution (stock:methanol) and quercetin was added with 100 µL of 1% aluminum chloride. After mixing, the solution underwent incubation for 1 hour in a dark room. The absorbance was read using a microplate reader (HumaReader HS).

Column Chromatography

(Erecta, Devika, & Koilpillai, 2015)

The solvent system for column chromatography was ascertained by subjecting the determined highest flavonoid and phenolic content extract using thin layer chromatography (TLC). It was tested with a solvent concentration of methanol and ethyl acetate (1:1). The fractions were spotted on a silica gel 60 F254 (Merck) and was viewed under long wavelength ultraviolet (UV) light.

The method for column chromatography was used with several modifications on the solvent system. Admixture was packed on a silica gel (60-120 mesh) and the elution started with a 1:1 ratio of methanol and ethyl acetate, followed by 100% methanol, 100 % chloroform, and lastly with 100% hexane.

Thin-layer Chromatography

(Erecta et al., 2015)

Each fraction collected from column chromatography was spotted on a silica gel plate. The solvent system used in the TLC chamber was ethyl acetate and methanol with a ratio of 1:1. The pooled fractions were determined by viewing them under UV light and were subjected for the determination of TFC.

***In vitro* Xanthine Oxidase Inhibitory Assay**

(Hendriani, Sukandar, Anggadiredja, & Sukrasno, 2016)

The fractions with high flavonoid content were subjected to XO inhibitory assay. The activity test for XO inhibition was conducted *in vitro* by measuring the enzyme's activity using a microplate reader as described in previous studies with several modifications. Xanthine substrate was prepared by diluting it with phosphate buffer (7.8 pH) it was also added with 5 drops of 1.0 M sodium hydroxide to increase solubility. The plant extracts were dissolved in 1% dimethyl sulfoxide (DMSO) and was made into a series of dilution to obtain final concentrations of 1000, 500, 250, 125 µg/mL. Allopurinol, the reference drug was used as a positive control, it was diluted using 0.15M phosphate buffer with a pH of 7.8. The total volume of the assay mixture using the microplate format was 190µl. It was consisted of 50 µL of plant extract, 50 µL of 0.15 M phosphate buffer (pH 7.8), 20 µL of XO enzyme. After preincubation of the test solution at 37°C for 15 min, the reaction was initiated by addition of 20 µL xanthine substrate solution, and was incubated at 37°C for 30 min. The reaction was stopped by adding 50 µL of 1N HCl. The absorbance was measured at 405 nm to indicate the formation of uric acid. The percent of XO inhibitory activity of the assayed samples were determined by measuring absorbance of uric acid from assay mixture without test extract (blank sample) and with test extract. IC50 values was obtained using GraphPad prism 8.1.1 (330). Percent inhibition was computed using the formula:

$$\frac{(A - B) - (C - D)}{A - B} \text{ X } 100$$

"A" represents the enzyme without the addition of a sample, "B" is the control of "A" without the samples and enzymes, "C" and "D" are the sample enzyme activity with and without enzyme respectively.

RESULTS

Extraction

The Percent yield of the crude extract was 9.825%.

$$\frac{58.95\ grams}{600\ grams}\ X\ 100 = 9.825\ \%$$

Total Phenolic Content

The TPC of the extract was quantified based on the linear regression equation derived from the gallic acid (y = 0.0066x + 0.2487, R^2 = 0.9965), expressed as gallic acid equivalent (GAE)

(Figure 2). Table 1 shows the absorbance and GAE of the different fractions of methanolic leaf extract of Ficus nota, ethyl acetate contained the highest value with 144.1364 mg/g of GAE.

Table 1. GAE of Ficus nota leaf extracts

Name	Absorbance	Gallic Acid Equivalent (y = 0.0066x + 0.2487, R^2 = 0.9965)
Crude Extract	0.742333	60.80303
Chloroform	0.403	24.43939
Ethyl Acetate	**1.21**	**144.1364**
N-butanol	0.676	59.59091
Distilled water	0.444333	47.16667
Methanol	0.262333	3.075758

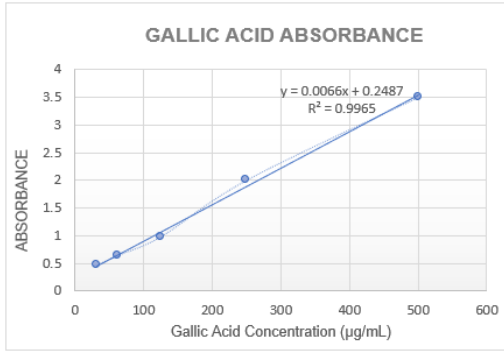

Figure 2. Standard Calibration Curve of Gallic Acid

Total Flavonoid Content

The highest flavonoid content was found to be 287.7802 mg/g of chloroform extract using the quercetin calibration curve (y=0.0091x+ 0.1952, R^2 = 0.9892) (Figure 3). Table 2 shows the absorbance and QE of the different fraction of methanolic leaf extract of *Ficus nota*, and the result indicate chloroform obtained the highest value.

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Table 2: QE of *Ficus nota* leaf extracts

Name	Absorbance	Quercetin Equivalent (y=0.0091x+ 0.1952, R^2 = 0.9892)
Crude Extract	0.949	74.04396
Chloroform	**2.74**	**287.7802**
Ethyl Acetate	1.062	81.18681319
N-butanol	1.119	83.93407
Distilled water	0.486	26.24176
Methanol	0.337	13.38462

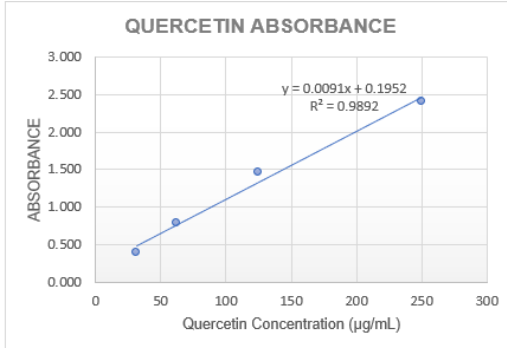

Figure 3. Standard Calibration Curve of Quercetin

Column Chromatography

Ethyl acetate and chloroform extracts were chromatographed yielding 55 fractions F1-F55, respectively. The fractions were subjected to TLC and were viewed under an ultraviolet light, pooling 15 fractions (PF1 – PF15) and were dried in an oven in 37°C. Table 3 shows that PF4, PF5 and PF7 have the highest TFC among the PFs.

Table 3: Pooled Fractions

Pooled Fractions	Absorbance	Quercetin Equivalent
PF1	1.206	111.1135531
PF2	1.290	120.3076923
PF3	1.353	127.1941392
PF4	**2.616**	**266.021978**
PF5	**2.584**	**262.5054945**
PF6	2.130	212.6153846
PF7	**2.640**	**268.6593407**
PF8	0.862	73.31135531
PF9	2.515	254.9230769
PF10	0.350	17.01098901
PF11	2.035	202.2124542
PF12	0.138	-6.249084249
PF13	1.853	182.1758242
PF14	2.013	199.7216117
PF15	0.358	17.89010989

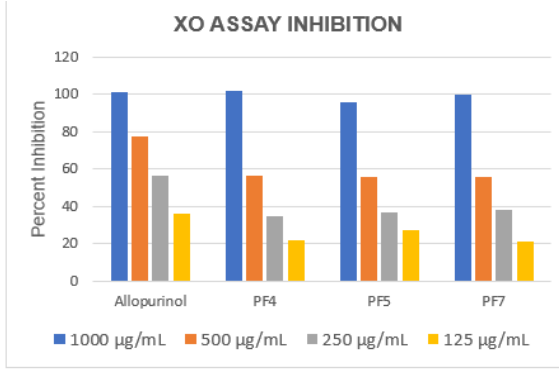

Figure 4. XO Inhibitory Activity of *Ficus nota*

***In vitro* XO Inhibitory Assay**

Allopurinol, the standard drug obtained an IC50 of 195.6 µg/mL whereas PF4, PF5 and PF7 obtained IC50 of 341.3 µg/mL, 328.4 µg/mL, 334.5 µg/mL, respectively. Although the highest percent inhibition was achieved in PF4 at the concentration of 1,000 µg/mL, Figure 4 shows that allopurinol inhibits more effectively in different concentrations.

DISCUSSION

Amongst the *Ficus* species, the bioactivity of *Ficus nota* is the least explored making studies concerning XO inhibition limited (Ragasa et al., 2009). Hence this study reported that the *in vitro* XO inhibitory assay of *Ficus nota* could produce a promising result. The extracts of *Ficus nota* exhibited potential XO inhibition in high concentrations. XO inhibitors that come from natural source can be used as an alternative treatment to gout because of the fewer potential adverse effects (Abd El-Rahman, 2015). Phytochemical investigation on *Ficus nota* revealed that its leaves contain flavonoids, alkaloids, phenolic acids, steroids, saponins, coumarins, tannins and triterpenoids (Sirisha et al., 2010). A considerable amount of studies had been made on *Ficus nota* which proved different pharmacologic activities of its leaves like antibacterial, antioxidant and antimicrobial (Latayada & Uy, 2016) Certain flavonoids such as Quercetin are potent inhibitors of the production of prostaglandins, a group of strong pro-inflammatory signaling molecules. Studies have shown that this effect is due to flavonoid inhibition of key enzymes involved in prostaglandin biosynthesis like lipoxygenase, and Quercetin were used as the standard for quantifying TPC and TFC, respectively. Different

fractions that underwent TPC were quantified and resulted to ethyl acetate which had the highest value of 144.1364 mg/g GAE. The TFC of chloroform extract was found to have the highest value of 287.7802 mg/g QE. The reaction of TFC was based on the formation of stable complex between aluminum chloride, keto and hydroxyl groups of flavonoids (Hassan et al., 2013). In support to the initial findings, both extracts were subjected to column chromatography and was eluted with a solvent system of increasing polarity. This technique has been used to purify compounds and works on the principle that different compounds will have diverse solubilities and adsorption to the two phases between which they are to be partitioned (Bajpai, Majumder, & Park, 2016). Subsequently, TLC was performed for pooling the resulting 55 fractions which yielded a total of 15 PFs that were again quantified using TFC. PF4, PF5, and PF7 obtained the highest QE and were used for XO inhibition assay. The inhibitory activity of PF was based on the IC50 results. Figure 4 shows that PF4 yielded the highest inhibition which is 341.3 µg/mL. Although the highest percent inhibition was achieved in PF4 at the concentration of 1,000 µg/mL the graph shows that allopurinol inhibits more effectively in different concentrations.

Flavonoids are plant compounds that naturally occur with XO inhibitory properties (Abdullahi et al., 2012). Because of this, the fraction which contained the highest flavonoid content was used for XO inhibitory assay. The results revealed that PF4 contributed an activity towards XO inhibition with an IC50 of 341.3 µg/mL whereas that of allopurinol was 195.6 µg/mL.

CONCLUSION

Upon subjecting the methanolic leaf extract to quantitative analysis, researchers concluded that it has a positive result for flavonoids. Standard calibration curve of Quercetin for Total Flavonoid Content and Gallic acid for Total Phenolic Content proves that the extracts contain flavonoid and phenolic compounds. Moreover, the Thin-layer chromatography result supports the presence of flavonoids in the leaf extracts of *Ficus nota*. The results obtained from IC50 and percent inhibition revealed that *Ficus nota* possesses XO inhibitory activity.

RECOMMENDATIONS

Studies concerning flavonoids have already proved that they possess XO inhibitory activity. Studies to evaluate the toxicity profile of *Ficus nota* and its family should be performed to establish its safety profile. Further in-vivo studies and the isolation and identification of active compounds found in the

leaves must also be conducted to identify a potential chemical entity for clinical use in the prevention and treatment of gout.

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