© 2021 Journal of Pharmacy & Pharmacognosy Research, 9 (6), 846-858, 2021
ISSN 0719-4250
http://jppres.com/jppres

Original Article

Antioxidant activity, xanthine oxidase inhibition and acute oral toxicity of *Dillenia philippinensis* Rolfe (*Dilleniaceae*) leaf extract

[Actividad antioxidante, inhibición de la xantina oxidasa y toxicidad oral aguda del extracto de hoja de *Dillenia philippinensis* Rolfe (*Dilleniaceae*)]

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Abstract

Context: Hyperuricemia is a metabolic syndrome characterized by a high serum uric acid level with an increased risk of gout, diabetes, cardiovascular and renal disease. Allopurinol is a widely known xanthine oxidase inhibitor that lowers serum uric acid production. Documented adverse effects of the drug raise the need for a natural alternative for xanthine oxidase inhibitor that is safe and effective. This research is driven to address the need by capitalizing on previous studies made on the *Dillenia* species showing nephroprotective and antihyperuricemic activity.

Aims: To evaluate the antioxidant capacity, xanthine oxidase inhibitory activity, and acute toxicity of *Dillenia philippinensis*; a plant locally known as *Katmon* and endemic to the Philippines.

Methods: The *D. philippinensis* ethanolic leaf extract (DPELE) was partitioned using solvents of different polarities, hexane, ethyl acetate, and butanol. The antioxidant activity of the DPELE and the sub-extracts was assessed using nitric oxide, hydrogen peroxide, and hydroxyl radical scavenging assays against ascorbic acid, while the xanthine oxidase inhibitory activity was examined against allopurinol as a standard. The acute toxicity was performed using Sprague-Dawley rats following OECD 425 guidelines.

Results: The *D. philippinensis* ethyl acetate fraction (DPEAF) had the highest scavenging activity of nitric oxide, hydrogen peroxide, and hydroxyl (IC_{50} = 210.00, 70.92 and 59.88 µg/mL) free radicals, respectively. Also, the xanthine oxidase dose-dependent inhibitory activity was observed to be highest in the DPEAF with an IC_{50} = 23.09 µg/mL. Lastly, neither toxicity nor mortalities were observed in rats treated with the DPELE and the DPEAF for 14 days. The approximate lethal dose for DPELE and DPEAF was higher than 2000 mg/kg BW.

Conclusions: This study shows that the DPELE and DPEAF are potential sources of natural antioxidants and xanthine oxidase inhibitors, which may be used in the treatment of hyperuricemia and are presumed safe to be used orally.

Keywords: allopurinol; ascorbic acid; free radical; hyperuricemia; inhibitor.

Resumen

Contexto: La hiperuricemia es un síndrome metabólico caracterizado por un nivel alto de ácido úrico en suero con un mayor riesgo de gota, diabetes, enfermedades cardiovasculares y renales. El alopurinol es un inhibidor de la xantina oxidasa ampliamente conocido que reduce la producción de ácido úrico en suero. Los efectos adversos documentados del fármaco plantean la necesidad de una alternativa natural al inhibidor de la xantina oxidasa que sea segura y eficaz. Esta investigación tiene como objetivo abordar la necesidad aprovechando los estudios previos realizados sobre la especie *Dillenia* que muestran actividad nefroprotectora y antihiperuricemiante.

Objetivos: Evaluar la capacidad antioxidante, la actividad inhibidora de la xantina oxidasa y la toxicidad aguda de *Dillenia philippinensis,* una planta conocida localmente como Katmon y endémica de Filipinas.

Métodos: El extracto de hoja etanólica de *D. philippinensis* (DPELE) se particionó utilizando disolventes de diferente polaridad, hexano, acetato de etilo y butanol. La actividad antioxidante del DPELE y los sub-extractos se evaluó usando ensayos de eliminación de radicales hidroxilo, peróxido de hidrógeno y óxido nítrico contra el ácido ascórbico, mientras que la actividad inhibidora de la xantina oxidasa se examinó contra el alopurinol como estándar. La toxicidad aguda se realizó utilizando ratas Sprague-Dawley siguiendo las directrices de la OCDE 425.

Resultados: La fracción de acetato de etilo de *D. philippinensis* (DPEAF) tuvo la mayor actividad de eliminación de radicales libres de óxido nítrico, peróxido de hidrógeno e hidroxilo (IC_{50} = 210,00, 70,92 y 59,88 µg/mL, respectivamente). Además, se observó que la actividad inhibidora dependiente de la dosis de xantina oxidasa era más alta en el DPEAF con una CI_{50} = 23,09 µg/mL. Por último, no se observó toxicidad ni mortalidad en ratas tratadas con DPELE y DPEAF durante 14 días. La dosis letal aproximada para DPELE y DPEAF fue superior a 2000 mg/kg de peso corporal.

Conclusiones: Este estudio muestra que el DPELE y el DPEAF son fuentes potenciales de antioxidantes naturales e inhibidores de la xantina oxidasa que pueden usarse en el tratamiento de la hiperuricemia y se presume que son seguros para usarse por vía oral.

Palabras Clave: alopurinol; ácido ascórbico; hiperuricemia; inhibidor; radicales libres.

ARTICLE INFO
Received: May 16, 2021.
Received in revised form: June 13, 2021.
Accepted: June 15, 2021.
Available Online: June 30, 2021.

INTRODUCTION

Hyperuricemia has been identified as one of the leading causes of work disability among USA citizens (Zhang et al., 2020). The prevalence rate of hyperuricemia in the Philippines is 25% (Prasad and Krishnan, 2014), which is a risk factor most importantly to hypertension, diabetes, congestive heart failure, coronary heart disease, hyperlipidemia, gouty arthritis, non-alcoholic fatty liver disease, kidney stones, and renal impairment (Foster et al., 2020). It is a metabolic disorder characterized by increased levels of uric acid >6.8 mg/dL in the blood. There is homeostasis in the production and the excretion of uric acid; however, an imbalance in uric acid production and excretion leads to hyperuricemia (Rahim et al., 2020). Recent studies suggest that the occurrence of gout and hyperuricemia is increasing worldwide drastically, and the possible reason may be lifestyle changes such as a diet rich in foods and drinks containing fructose, which increase purine synthesis in the body (Caliceti et al., 2017). The high level of these sugar compounds and uric acid competes for the excretion from the kidneys (Yan et al., 2020). Food rich in purine, such as red meat, seafood, spinach, and cauliflower, contributes to hyperuricemia. Purine nucleotides are obtained from both endogenous (nucleic acid synthesis and breakdown) and exogenous (dietary intake) sources. Deoxyribonucleotides and purines nucleotides catabolism lead to uric acid production in which hypoxanthine and xanthine are the intermediate products (Abdullhafiz et al., 2020). Increased uric acid levels in the blood cause the accumulation of monosodium urate (MSU) crystals in the joints and the soft tissues surrounding them, which leads to disorders such as gout and nephrolithiasis. The increase of oxidative stress has been observed in gouty patients (Yao et al., 2020).

Uric acid-lowering agents, including xanthine oxidase inhibitors and uricosuric agents, are used most commonly to manage gouty arthritis. The most frequently used drug for the management of hyperuricemia and gout is allopurinol. However, it has serious side effects such as gastrointestinal toxicity, renal toxicity, gastrointestinal bleeding, liver function abnormalities, Stevens-Johnson syndrome, and Toxic Epidermal Necrolysis Syndrome (Brucato et al., 2020). Around 80% of the global population depends on traditional and herbal remedies for their main medical care. Owing to these utmost medical needs, the success of alternative herbal medicines to prevent hyperuricemia is an important field of study (Yang et al., 2020). The combined effect of antioxidants and xanthine oxidase inhibitors would become an encouraging act for the management of hyperuricemia (Chi et al., 2020). Thus, there is a need for alternative natural products with lesser side effects and higher therapeutic activity.

The xanthine oxidase is the rate-limiting enzyme in purine metabolism and is present in the gastrointestinal tract and liver in its reduced form. It consists of two independent subunits, each with one molybdenum, one adenine nucleotide, and two iron-sulfur centers. It catalyzes the purine catabolism in two steps; the oxidation of hypoxanthine to xanthine and xanthine to uric acid with the release of two reactive oxygen species (ROS), superoxide radical (O_2^-) and hydrogen peroxide. Therefore, xanthine oxidase is an important enzyme for forming uric acid and ROS, which leads to the progression of cancer, inflammation, atherosclerosis, and aging (Furuhashi, 2020). These reactions are as showed in Fig. 1 (Schmidt et al., 2019).

$$\text{Hypoxanthine} + H_2O + O_2 \rightarrow \text{Xanthine} + H_2O_2$$
$$\text{Xanthine} + H_2O + 2O_2 \rightarrow \text{Uric acid} + 2O_2^{\bullet -} + 2H^+$$
$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{Uric acid} + H_2O_2$$
$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Figure 1. Reaction of xanthine oxidase.

Oxidative stress arises from a disturbance in the balance between prooxidant-antioxidant, in favor of the former resulting in ROS formation. Under cellular physiological conditions, redox reactions are highly regulated as ROS are formed continuously within the cells. These ROS are captured by the endogenous antioxidants acting as the natural defense of the body. The ROS becomes toxic and can damage cells when the rate of their production

increases and the capacity of cells to capture them is saturated. Therefore, scavenging ROS will increase xanthine oxidase inhibitory activity, leading to a decrease in uric acid production (Liu et al., 2021).

Ethnobotanical studies in the Philippines showed that *D. philippinensis* leaves have been traditionally used to treat inflammation, hyperglycemia, and cough (Dante et al., 2019). *D. philippinensis*, has been reported to contain 11 compounds, including one new sulfated glycoside and oleanane-type terpenoid, and flavonoid, tiliroside, and dillenetin, which exhibit several pharmacological activities. The most abundant compounds isolated from this family are flavonoids and terpenoids (Macahig et al., 2011). In a study conducted in 2009, silica gel chromatography indicated the presence of betulinic acid and 3-oxoolean-12-en-30-oic acid in *D. philippinensis*. These compounds exhibited antibacterial and antifungal activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Bacillus subtilis*, and *Candida albicans* (Ragasa et al., 2009). The most extensively studied species among the genus *Dillenia* is *D. indica*. In 2017, a study was conducted on the ethanolic leaf extract of *D. indica* (100, 200, and 400 mg/kg BW) showed remarkable enhancement in the control of glucose level, kidney parameter, lipid profile, and level of antioxidant enzymes in diabetic neuropathy (Kaur et al., 2017).

The levels of uric acid, serum urea, creatinine, and BUN are significantly high during diabetic neuropathy (Li et al., 2018). Treatment of diabetic rats with *D. indica* leaf extract effectively lowered the levels of uric acid, creatinine, and urea, suggesting their increased clearance from the kidney (Kaur et al., 2017). The fruit of *D. indica* is a good source of xanthine oxidase inhibitor to manage gout and other metabolic disorders (Abu Bakar et al., 2018). Three isolated flavonols (keamferide, dillenetin, and quercetin) from *D. indica* showed a xanthine oxidase inhibitory activity almost identical to the standard drug allopurinol. There is a strong correlation between antioxidant activity and total flavonoid content, indicating that flavonoids are crucial secondary metabolites that possess a number of bioactive compounds that largely contribute to xanthine oxidase inhibitory activity (Khamee et al., 2019). There is currently no published scientific evidence to prove that the leaves of *D. philippinensis* have an antioxidant and xanthine oxidase activity. Therefore, this study evaluates the principal metabolites present, their free radical scavenging activity, xanthine oxidase inhibition, followed by an acute toxicity study. This result may lead to *in vivo* animal models and preformulation studies of the plant once proven scientifically as a natural source remedy.

MATERIAL AND METHODS

Chemicals

Solvents such as hexane, ethanol, ethyl acetate, and butanol of analytical grade were purchased from Belman Laboratories (Manila, Philippines). Chemicals such as Griess reagent, sodium nitroprusside (SNP), xanthine, xanthine oxidase from bovine milk, allopurinol, ascorbic acid, gallic acid, and quercetin were acquired from Sigma Aldrich (Singapore). The following reagents were purchased from Laboratory Supplies and Equipment Office (UST-LESO) (Manila, Philippines): phenanthroline, ferrous sulfate, aluminum chloride, hydrogen peroxide, and phosphate buffer saline (PBS).

Collection of plant sample

The *D. philippinensis* fresh leaves were collected in the morning on December 8, 2019, from Barangay Anoling of General Nakar, Quezon Province (14.7648°N, 121.6356°E). The leaves were authenticated by the herbarium of the Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, and a voucher specimen with control no. ISO: S037-00-F047 was deposited at the University of Santo Tomas. The collected leaves were air-dried for 14 days, pulverized using a Willey mill (Thomas Scientific, New Jersey, USA), and further sieved using mesh No. 10 to obtain uniform particle size.

Extract preparation of plant materials

Around 2.5 kg of powdered leaves were subjected to extraction using 95% ethanol, three times at 25°C for 3 days. The collected crude extract was filtered and evaporated to dryness using an Eyela N-1200BR (EYELA, Tokyo, Japan) rotary evaporator at 40-45°C. Liquid-liquid partitioning was performed by mixing 5 g of *D. philippinensis* ethanolic leaf extract (DPELE) in 100 mL of water, and the latter partitioned successively with 100 mL hexane, ethyl acetate, and butanol to yield *D. philippinensis* hexane fraction (DPHF), ethyl acetate fraction (DPEAF), and butanol fraction (DPBF) respectively. The sub-extracts were filtered prior to concentration under reduced pressure using a rotary evaporator (EYELA, Tokyo, Japan) (Nguyen et al., 2017).

Phytochemical studies

Total flavonoid content determination

The method utilizes aluminum chloride as the agent to quantify flavonoids (Aryal et al., 2019). A wavelength of 420 nm was utilized after incubating the mixture of the sample or standard (100 µL) and aluminum chloride (100 µL) for 60 min at 25°C using Multiskan Go Spectrophotometer manufactured by Thermo Fisher Scientific (Japan). For the determination of total flavonoid content, a line regression equation from the plotted standard curves was used, and the results were expressed as milligram quercetin equivalents per gram extract (mg QE/g).

Total phenolic content determination

The procedure utilizes the Fast-Blue BB [4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi(zinc chloride) salt] method (Pico et al., 2020). In a 96-well microplate, 100 µL of the sample or standard was mixed with 10 µL of 0.1% Fast-Blue BB reagent and 10 µL of 5% sodium hydroxide. A wavelength of 420 nm was utilized after incubating the mixture for 90 min at 25°C in Multiskan Go Spectrophotometer manufactured by Thermo Fisher Scientific, Japan. A standard calibration curve was observed to determine the total phenolic content, and the results were expressed as milligram gallic acid equivalents per gram extract (mg GAE/g).

Antioxidant activity analysis

Nitric oxide scavenging assay

The *D. philippinensis* crude and sub-extracts were prepared from a 10 mg/mL stock solution (Nguyen et al., 2017). These were serially diluted with PBS to make concentrations from 63-1000 µg/mL. The SNP was prepared in 100 mL of PBS. A volume of 75 µL of SNP was mixed with 25 µL of the different concentrations of the sample and incubated at 25°C for 150 min. Griess reagent was prepared by adding 250 mL of ultrapure water and stored at room temperature. A volume of 100 µL of freshly prepared Griess reagent was mixed with the extract and SNP. Blank was prepared by adding buffer instead of the extract with SNP and Griess reagent. The results were compared to the standard L-ascorbic acid using concentrations from 63-1000 µg/mL and the absorbance utilized in this method was 546 nm using Multiskan Go Spectrophotometer manufactured by Thermo Fisher Scientific, Japan. The formula used in the calculation of percentage nitric oxide scavenging activity of the extracts and ascorbic acid is as follows [1]:

$$\text{Inhibition (\%)} = \frac{(A\ control\ -\ A\ test)}{A\ control} \times 100 \qquad [1]$$

Where $A_{control}$ is the absorbance of the control sample and A_{test} is the absorbance in the presence of the extracts or standards.

Hydrogen peroxide assay

In a 96-well microplate, different concentrations (39-1250 µg/mL) of the *D. philippinensis* crude and sub-extracts were mixed with 120 µL of 40 mmol/L hydrogen peroxide in each well. The plate was incubated for 10 min at 25°C, and absorbance was recorded at 230 nm using Multiskan Go Spectrophotometer manufactured by Thermo Fisher Scientific, Japan. The inhibitory activity of the test samples was compared to that of the standard L-ascorbic acid using concentration from 39-1250 µg/mL (Borquaye et al., 2020) using the formula [1].

Hydroxyl radical scavenging assay

The reaction mixture contained 25 µL of different concentration of the *D. philippinensis* crude and sub-extracts (39-1250 µg/mL), 50 µL of 1,10-phenanthroline (0.75 mmol/L), 50 µL of PBS (pH 7.4) and 50 µL of ferrous sulfate (0.75 mmol/L). A volume of 25 µL of 0.01% hydrogen peroxide was added to the reaction mixture and incubated for 60 min at 37°C. The standard utilized in the assay was L-ascorbic acid using a concentration range from 39-1250 µg/mL, and the absorbance was measured at 536 nm using Multiskan Go Spectrophotometer manufactured by Thermo Fisher Scientific, Japan (Barreto et al., 2020). The hydroxyl radical scavenging activity was measured using the following equation [1].

In vitro *xanthine oxidase inhibitory assay*

The inhibitory activity of xanthine oxidase was measured at 290 nm using Multiskan Go Spectrophotometer (Thermo Fisher Scientific, Japan). Ethanol was used to dilute the *D. philippinensis* crude and sub-extracts and accordingly diluted with PBS (pH 7.4) to achieve a concentration containing less than 1% ethanol. The reaction mixture contained 50 µL of different concentration of samples (5, 10, 20, 50, 100, 200, and 300 µg/mL), 35 µL PBS, and 30 µL of xanthine oxidase solution freshly prepared (0.1 U/mL) was incubated for 15 min at 25°C prior to the start of the experiment. The mixture was incubated for another 30 min at 25°C after the addition of 60 µL xanthine solution (150 µM). The reaction was terminated by mixing 25 µL of hydrochloric acid (1 N) solution, and percentage inhibition was calculated utilizing different concentrations of allopurinol (5, 10, 20, 50, 100, 200, and 300 µg/mL) as the standard. The control solution was prepared similarly, but the addition of hydrochloric acid solution was prior to the addition of xanthine solution (Quy and Xuan, 2019).

All the samples were run in triplicates, and their respective percentage inhibitions were determined using the formula below [1].

Where $A_{control}$ is the activity of xanthine oxidase without the presence of sample and A_{test} is the activity of xanthine oxidase in the presence of the extracts or standards.

Source and housing conditions of tested animals

Twelve male Sprague-Dawley (SD) rats (5-8 weeks) were purchased from Mots animal house with Bureau of Animal Industry (BAI) registration number LAF-0036. The rats were kept at the animal handling facility of Thomas Aquinas Research Center one week before the start of the experimentation for animal acclimatization with free access to rat pellets and water. Metallic cages with separate containers for rat pellet and water were used. The room temperature was maintained at 23 ± 2°C with a relative humidity of 40%, and an artificial light source for providing 12 hours of light and darkness was utilized. Experiments were performed according to the protocol approved by the UST Institutional Animal Care and Use Committee (UST-IACUC) with BAI certification number RC2018-64109.

Acute toxicity testing

Initially, one animal was tested on the test dose (2000 mg/kg) administered using oral gavage. It was observed for signs of clinical toxicity for 24 to 48 hours (Acute Oral Toxicity: 425, Organisation for Economic Cooperation and Development, 2008). Experimental animals were observed for fur characterization, ophthalmologic findings, central nervous system-autonomic, and cardiovascular manifestations, and weekly changes in the body weight were also determined. After the survival of the first animal, additional five animals were administered with the same test dose and were observed for 14 days. The approximate lethal dose (ALD) was higher than 2000 mg/kg since more than three animals survived. Gross necropsy was done in all test animals after the 14-day observation period. The animals were sacrificed through cervical dislocation. The liver and kidney samples were collected and immediately fixed for hematoxylin and eosin (H & E) staining. An electron microscope Eclipse E100, Nikon, USA) with high-intensity illumination, providing uniform brightness in the entire field of view, with 40X objective (CP-Achromat, Zeiss, Germany) connected to

Ziess application through a camera (Iphone 7, Apple, USA) was utilized. A blinded histopathological evaluation was performed independently by a pathologist.

Statistical analysis

The mean and its standard error were used to summarize the data. Analysis of variance was used to compare groups with Tukey's HSD for *post hoc* analysis. P-values less than 0.05 indicate significant differences. Further, IC_{50} estimates were generated using a four-parameter logistic regression model. The Statistical Package for Social Sciences Program (SPSS, Inc, version 20) and GraphPad Prism (version 8) were used as statistical software.

RESULTS

Plant extraction

A total of 2635 g of dried *D. philippinensis* leaf powder and 12.5 L of ethanol were used for extraction. The crude ethanolic leaf extract of *D. philippinensis* manifested a yield of 10% (265 g). The sub-extract DPHF extracted 5%, whereas sub-extract DPEAF and DPBF extracted approximately 1% yield, respectively.

Phytochemical studies

Total flavonoid content determination

With the use of the calibration curve, y = 0.0093x - 0.0512, R^2 = 0.9996 of quercetin (4-500 µg/mL), the results of the total flavonoid content in the *D. philippinensis* crude and sub-extracts were calculated. Among the samples, the highest amount of flavonoid content was found in DPEAF (44.44 mg QE/g), followed by DPELE (23.07 mg QE/g), DPHF (20.03 mg QE/g), and DPBF (10.06 mg QE/g). The total flavonoid content of the samples significantly differed ($p<0.001$), indicating that DPEAF was significantly the highest ($p<0.001$), while DPBF was significantly the lowest ($p<0.001$), as presented in Table 1.

Total phenolic content determination

Phenolic compounds are important for antioxidant activities in plants through their redox properties. In plant extracts, the hydroxyl groups are responsible for facilitating ROS (Borquaye et al., 2020). The results of total phenolic content determination of the samples were calculated using the calibration curve equation, (y = 0.002x - 0.0526, R^2 = 0.995) of gallic acid (4-500 µg/mL). Among the samples, the highest amount of phenolic content was found in DPEAF (426.08 mg GAE/g), followed by DPELE (316.72 mg GAE/g), DPHF (304.88 mg GAE/g), and DPBF (97.62 mg GAE/g). Similar to the total flavonoid content, the total phenolic content of the samples significantly differed ($p<0.001$), which indicates that the total phenolic content of DPEAF was significantly the highest ($p<0.001$), while the total phenolic content of DPBF was significantly the least ($p<0.001$). The total phenolic content of DPHF was significantly higher ($p<0.001$) than DPBF, but significantly less than DPELE ($p=0.044$). The total phenolic content of DPELE was significantly less than DPEAF ($p<0.001$), as presented in Table 1.

Table 1. Total flavonoid and total phenolic content of *D. philippinensis* leaf extracts.

Extracts	mg QE/g of the sample	mg GAE/g of the sample
DPELE*	23.07 ± 0.35	316.72 ± 1.30
DPHF*	20.13 ± 0.78	304.88 ± 1.51
DPEAF*	44.44 ± 0.80	426.08 ± 0.71
DPBF*	10.06 ± 0.43	97.62 ± 0.33

Values are mean ± SEM , n = 3, *p<0.001. DPELE: *D. philippinensis* ethanolic leaf extract; DPHF: *D. philippinensis* hexane fraction; DPEAF: *D. philippinensis* ethyl acetate fraction; DPBF: *D. philippinensis* butanol fraction.

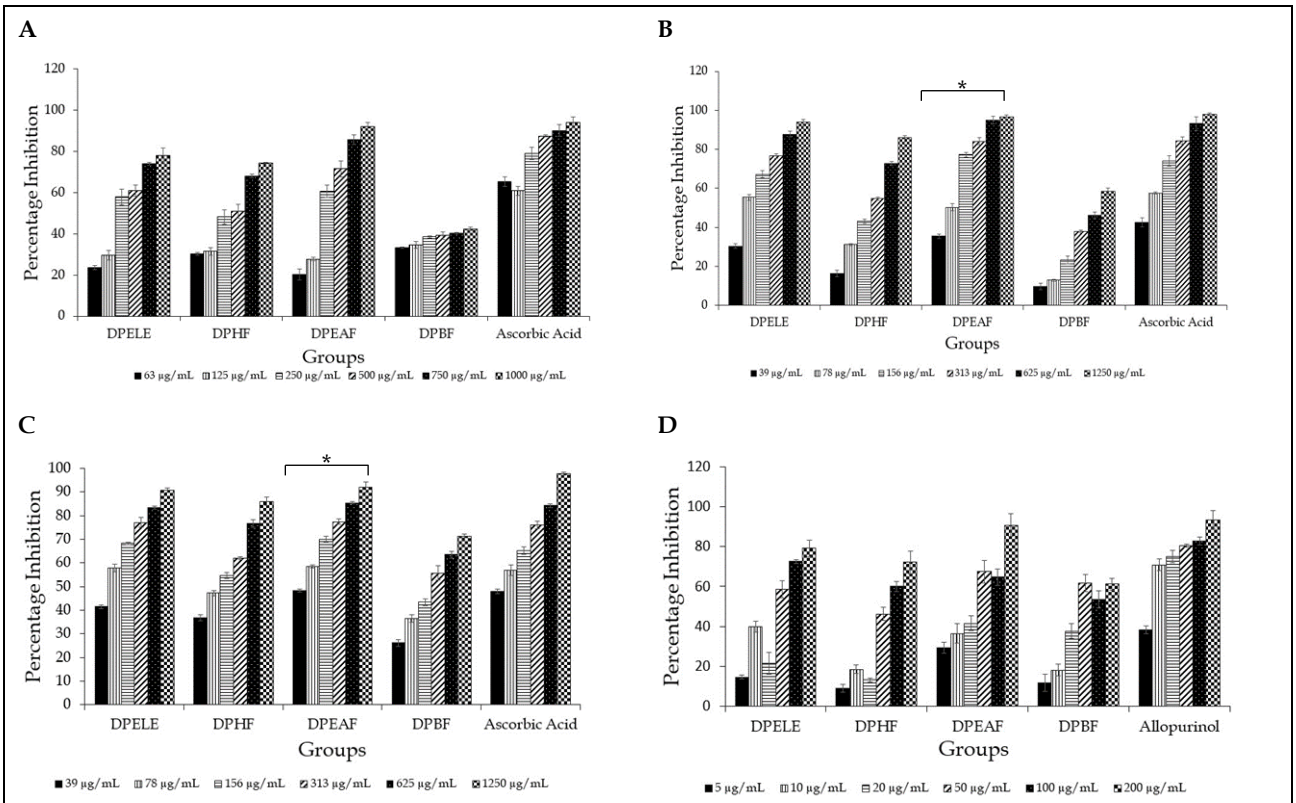


Figure 2. Antioxidant and xanthine oxidase inhibitory activity of the *D. philippinensis* leaf fractions.

(A) A comparison of nitric oxide radical scavenging activity plotted against different solvent samples and ascorbic acid as standard; **(B)** A comparison of hydrogen peroxide scavenging activity plotted against different solvent samples and ascorbic acid as standard; **(C)** A comparison of hydroxyl radical scavenging activity plotted against different solvent samples and ascorbic acid as standard; **(D)** A comparison of xanthine oxidase inhibitory activity plotted against different solvent samples and allopurinol as standard.

The data are expressed as the mean ± SEM, n = 3, *p<0.05 compared to ascorbic acid, **p<0.05 compared to allopurinol. DPELE: *D. philippinensis* ethanolic leaf extract; DPHF: *D. philippinensis* hexane fraction; DPEAF: *D. philippinensis* ethyl acetate fraction; DPBF: *D. philippinensis* butanol fraction.

Antioxidant activity analysis

Nitric oxide scavenging assay

The nitric oxide radical scavenging assay was carried out on the crude, and the sub-extracts of *D. philippinensis* leaves and L-ascorbic acid as a standard compound from a concentration range of 63-1000 µg/mL. Percentage inhibition was plotted against the concentration of the extracts as shown in Fig. 2 to give results as shown in Table 2. DPEAF was the most potent with an IC_{50} of 210.00 µg/mL as it removed the nitrite free radical at a lower concentration as compared to the other plant extracts followed by DPELE (247.50 µg/mL), DPHF (298.70 µg/mL), and DPBF (11879.00 µg/mL). The percentage inhibition of nitric oxide radical scavenging activity of the samples significantly differed ($p<0.001$), which shows that DPBF was significantly the least ($p<0.001$). The percentage inhibition of DPHF and DPELE did not differ ($p=0.073$) but were both significantly higher than DPBF ($p<0.001$). The percentage inhibition of ascorbic acid was significantly the highest ($p<0.001$), while DPEAF was significantly less than ascorbic acid ($p<0.001$) but significantly higher than DPELE ($p<0.001$), DPHF ($p<0.001$), and DBPF ($p<0.001$).

Hydrogen peroxide assay

The hydrogen peroxide scavenging potency of the *D. philippinensis* crude and sub-extracts was determined and presented in Fig. 2. All samples showed a direct relationship with the increase in the concentration and radical inhibiting activity.

Table 2. Antioxidant and xanthine oxidase inhibitory activity, expressed as percentage inhibition and IC_{50}, estimated by nitric oxide radical, hydrogen peroxide, hydroxyl radical, and xanthine oxidase of different solvent samples and standards.

Sample	Antioxidant and xanthine oxidase inhibitory activity							
	Nitric oxide radical		Hydrogen peroxide		Hydroxyl radical		Xanthine oxidase	
	Inhib. (%)	IC_{50} (µg/mL)	Inhib. (%)	IC_{50} (µg/mL)	Inhib. (%)	IC_{50} (µg/mL)	Inhib. (%)	IC_{50} (µg/mL)
DPELE	53.98 ± 5.09	247.50 ± 10.79	60.65 ± 1.39	72.77 ± 2.18	59.82 ± 1.21	73.74 ± 0.31	47.76 ± 6.02	37.10 ± 4.56
DPHF	50.55 ± 4.10	298.70 ± 16.93	44.19 ± 1.10	223.35 ± 5.24	51.31 ± 1.07	119.06 ± 2.67	55.07 ± 5.38	69.25 ± 2.66
DPEAF	59.62 ± 6.65	210.00 ± 4.91	65.80 ± 1.47	70.92 ± 2.78*	63.91 ± 0.97	59.88 ± 1.76*	55.07 ± 5.38	23.09 ± 5.83
DPBF	38.04 ± 0.85	11879.00 ± 17.21	27.62 ± 1.26	701.20 ± 15.48	43.95 ± 1.26	221.36 ± 6.08	40.73 ± 5.01	59.51 ± 8.92
Ascorbic acid	81.73 ± 2.52	24.70 ± 2.08	67.92 ± 1.84	54.32 ± 2.30	65.10 ± 1.28	55.80 ± 1.23		
Allopurinol							73.49 ± 4.29	5.77 ± 3.54

Values are mean ± SEM, n = 3, *p<0.05 compared to ascorbic acid, **p<0.05 compared to allopurinol.

DPELE: *D. philippinensis* ethanolic leaf extract; DPHF: *D. philippinensis* hexane fraction; DPEAF: *D. philippinensis* ethyl acetate fraction; DPBF: *D. philippinensis* butanol fraction. Inhib.: Inhibition.

The highest radical scavenging activity with a minimum IC_{50} value was recorded for DPEAF (70.92 µg/mL), followed by DPELE (72.77 µg/mL), DPHF (223.35 µg/mL), and DPBF (701.20 µg/mL). The results from the crude and sub-extracts were compared with the IC_{50} value of ascorbic acid (54.32 µg/mL), as shown in Table 2. The percentage inhibition of the extracts significantly differed (p<0.001), which shows that DPBF was significantly the least (p<0.001). The percentage inhibition of DPHF was significantly higher than DBPF (p<0.001) but less than DPELE (p<0.001), DPEAF (p<0.001), and ascorbic acid (p<0.001). The percentage inhibition of DPEAF and ascorbic acid did not differ (p=0.119), while the ascorbic acid was significantly higher (p<0.001) as compared with the other groups.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was carried out on the crude, and the sub-extracts of *D. philippinensis* leaves from a concentration of 39-1250 µg/mL and ascorbic acid as a standard compound. Percentage inhibition was plotted against the concentration of the extracts, as shown in Fig. 2, to give results as shown in Table 2. The highest percentage inhibition with a minimum IC_{50} value was recorded for DPEAF (59.88 µg/mL), followed by DPELE (73.74 µg/mL), DPHF (119.06 µg/mL), and DPBF (221.36 µg/mL). The results from the crude and sub-extracts were compared with the IC_{50} value of ascorbic acid (55.80 µg/mL). The percentage inhibition of hydroxyl radical scavenging activity of the samples significantly differs (p<0.001), which showed that DPBF was significantly the least (p<0.001). The percentage inhibition of DPHF was significantly higher than DPBF (p<0.001) but less than DPELE (p<0.001), DPEAF (p<0.001), and ascorbic acid (p<0.001). The percentage inhibition of DPEAF and ascorbic acid did not differ (p=0.423), while the ascorbic acid was significantly higher (p<0.001).

In vitro *xanthine oxidase inhibitory assay*

To determine the ability of *D. philippinensis* crude and sub-extracts to inhibit xanthine oxidase with allopurinol, as a positive control, the *in vitro* xanthine oxidase inhibitory activity was investigated. Table 2 indicates the IC_{50} value of DPEAF (23.09 µg/mL), which exhibited a high xanthine oxidase inhibitory activity followed by DPELE (37.10 µg/mL), DPHF (69.25 µg/mL), and DPBF (59.51 µg/mL), resulting in decreased production of uric acid crystals. The xanthine oxidase inhibitory activity of DPELE and its fractions was dose-dependent, as shown in Fig. 2. The percentage inhibition of xanthine oxidase inhibitory activity of the samples significantly differed (p<0.001), which showed that the percentage inhibition of DPHF and DPBH did not differ (p=0.291) and were significantly the least (p<0.05) compared to DPELE and DPEAF. The percentage activity of DPELE was significantly higher compared to DPHF (p<0.001) and DPBH (p=0.013), but significantly less than DPEAF (p=0.009), while the percentage inhibition of allopurinol was significantly higher

($p<0.001$) among the samples. The inhibition of the xanthine oxidase enzyme by DPEAF showed a modest correlation between the phenolic content and xanthine oxidase inhibitory activity.

Acute toxicity testing

The SD rats induced with a dose of 2000 mg/kg of DPELE and DPEAF were found to be safe after a period of 14 days. No signs or symptoms of clinical toxicity and no death were documented in the SD rats during the observation period. The gross necropsy was documented by Dr. Judith Cabigan, DVM. All test animals induced with DPELE were healthy with no macroscopic parasites. There was no observation of any lesions on the liver and kidneys. The liver and kidneys exhibited a firm and smooth surface with well-defined borders. Histopathologic examination of the DPELE induced rat liver and kidneys are shown in Fig. 3, indicating an intact capsule with numerous red blood cells in the hepatic veins and in the equally spaced sinusoids. Hepatocytes were not swollen, and no inflammation nor steatosis was noted. The microscopic examination of the left and the right kidney shows an intact renal capsule, glomerulus, and tubules, and no diagnostic abnormality was seen. The observations showed that DPELE was safe at 2000 mg/kg; therefore, the ALD was beyond 2000 mg/kg.

All test animals induced with DPEAF were healthy with no macroscopic parasites. There was no observation of any lesions on the liver and kidneys. The liver and kidneys exhibited a firm and smooth surface with well-defined borders. Histopathologic examination of the DPEAF induced rat liver and kidneys are shown in Fig. 4, indicating an intact capsule with numerous red blood cells in the hepatic veins and in the equally spaced sinusoids. Hepatocytes were not swollen, and no inflammation nor steatosis was noted. The microscopic examination of the left and the right kidney shows renal tissues with intact renal capsule, glomerulus, and tubules, and 10% congestion was seen.

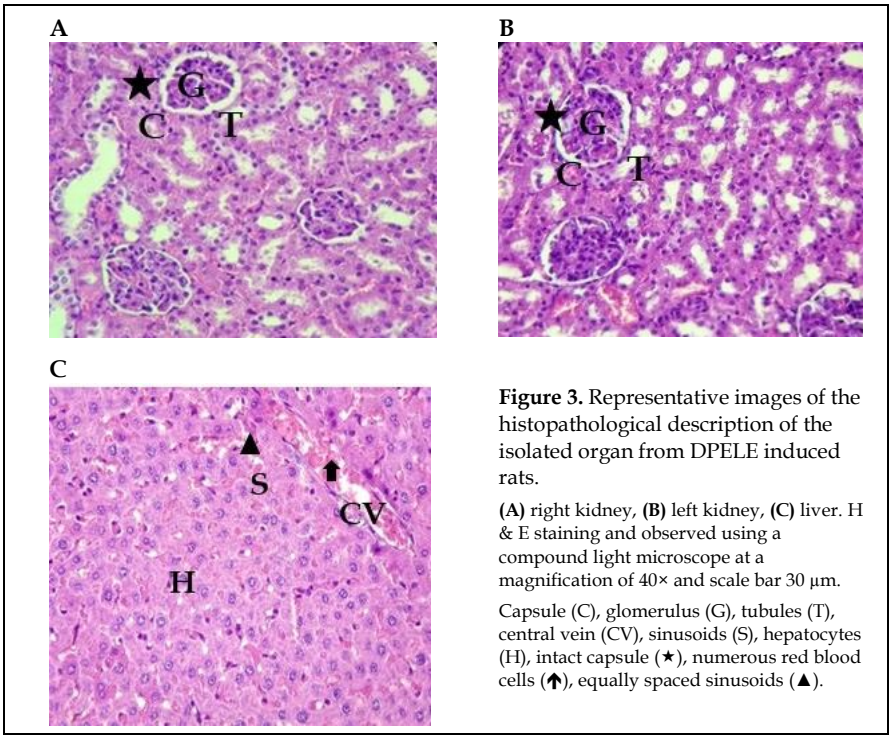
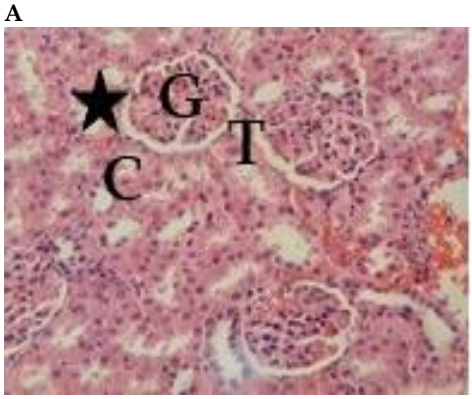
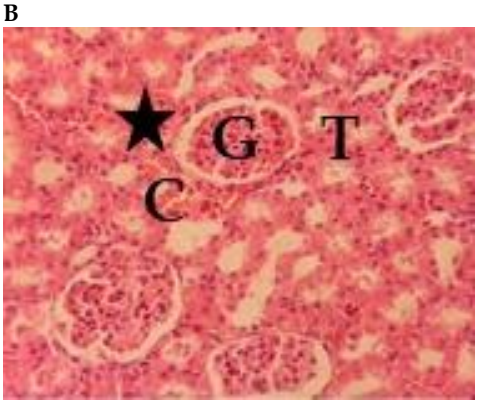


Figure 3. Representative images of the histopathological description of the isolated organ from DPELE induced rats.

(A) right kidney, **(B)** left kidney, **(C)** liver. H & E staining and observed using a compound light microscope at a magnification of 40× and scale bar 30 µm.

Capsule (C), glomerulus (G), tubules (T), central vein (CV), sinusoids (S), hepatocytes (H), intact capsule (★), numerous red blood cells (🡅), equally spaced sinusoids (▲).





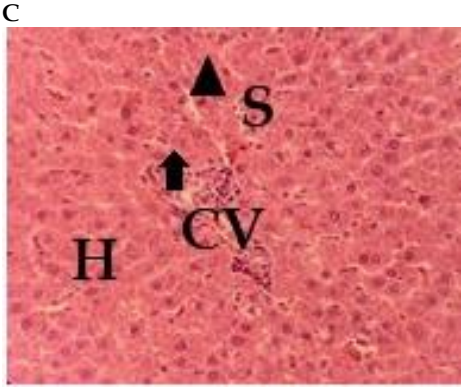


Figure 4. Representative images of the histopathological description of the isolated organ from DPEAF induced rats.

(A) right kidney, **(B)** left kidney, **(C)** liver. H & E staining and observed using a compound light microscope at a magnification of 40× and scale bar 30 µm.

Capsule (C), glomerulus (G), tubules (T), central vein (CV), sinusoids (S), hepatocytes (H), intact capsule (★), numerous red blood cells (🡅), equally spaced sinusoids (▲).

DISCUSSION

Dilleniaceae plants contain various flavonoids and phenolic compounds (Lima et al., 2014). Some of them reduce ROS production and have antioxidant activity. Flavonoids have been documented to possess the ability to act as potent inhibitors of xanthine oxidase. They are phytochemical constituents with antioxidant activity. The position and number of free hydroxyl radical groups determine their potency. The flavonoid can bind to the xanthine oxidase inhibitor through the process of hydroxylation, hydrogenation, glycosylation, and hydroxylation methoxylation (Mehmood et al., 2019). In the leaves of *D. suffruticosa*, flavonoids are mainly noticed in the semi-polar fraction, such as butanol and methanol (Shah et al., 2020). For *D. pentagyna* the ethanolic fruit extract is enriched with phenolics, flavonoids, tannin, saponin, alkaloid, and terpenoids (Patle et al., 2020). The phenolic content of *D. indica* is highest in the methanolic leaf extract (Das et al., 2012). Based on these results, it is recommended that the polarity of the extracting solvent has an effect on the extractability of polyphenols. Here, we report for the first time the total flavonoid and total phenolic content of *D. philippinensis*, which is found to be the highest in the DPEAF.

The crude and the sub-extracts from *D. philippinensis* leaves were evaluated for their *in vitro* antioxidant activity against nitric oxide, hydrogen peroxide, and hydroxyl radical. There is a direct relationship between oxidative stress and hyperuricemia (Abdulhafiz et al., 2020). The present study showed that DPEAF reduced the formation of nitrite ions by donating a proton to it and scavenged the nitric oxide free radical. The antioxidant activity was exhibited at physiological pH through scavenging oxygen that was generated from SNP releasing free nitrite radical (Ali et al., 2019). Nitric oxide acts as a necessary component for regulating various biological processes such as vasoconstriction, immune regulation, and neural response. The increased nitric oxide generation can cause tissue damage and is associated with other meta-

bolic diseases such as hyperuricemia (Adebayo et al., 2019). Therefore, identifying a compound to suppress nitric oxide production can be a potential source of a xanthine oxidase inhibitor.

The hydrogen peroxide is a non-radical ROS. It is a powerful natural antioxidant that can give rise to hydroxyl radical, leading to lipids' oxidation and damage to the DNA, causing extreme conditions like cancer, diabetes, and cardiovascular diseases. The antioxidant activity of hydrogen peroxide is due to phenolic compounds in DPEAF, which donate electrons to the hydrogen peroxide to reduce it to water (Zhang et al., 2020). Therefore, scavenging hydrogen peroxide can also reduce oxidative stress caused by oxidative enzymes.

In this experiment, hydroxyl radicals were generated by Fenton reaction in the presence of H_2O_2-Fe^{2+} confirmed with the formation of an orange-colored complex. This radical is the most reactive oxygen-centered species that cause lipid peroxidation and mutagenicity, resulting in cancer, atherosclerosis, neurological disorders, and other diseases (Muthoni et al., 2020). The DPEAF exhibited the highest quenching activity due to the presence of the highest number of flavonoids as the reactive hydroxyl radicals were stabilized by interacting with the reactive hydroxyl groups of flavonoids (Mehmood et al., 2019).

The highest *in vitro* xanthine oxidase activity was exhibited by DPEAF. Phenols and flavonoids play a vital role in inhibiting free radical species that are released during the oxidation of hypoxanthine to xanthine with a production of O_2^- and hydrogen peroxide (Zhang et al., 2020). The strong correlation between antioxidant activity and total flavonoid content indicates that flavonoids are essential secondary metabolites that possess a number of bioactive compounds that largely contribute to the DPEAF high xanthine oxidase inhibitory activity. The results from this study recommend that *D. philippinensis* can also lower uric acid due to the abundance of flavonoids. However, further *in vivo* studies are needed to prove the antihyperuricemic activity of the leaf extracts of *D. philippinensis*.

CONCLUSIONS

Among the sub-extracts, *D. philippinensis* ethyl acetate fraction is proven to be the highest among these areas: total phenolic content, total flavonoid content, free radical scavenging activity, and xanthine oxidase inhibitory activity. There was no recorded toxicity nor fatality at a dose of 2000 mg/kg for both *D. philippinensis* ethanolic leaf extract and *D. philippinensis* ethyl acetate fraction. Thus, this study concludes that DPEAF has the potential to lower the levels of uric acid that can be beneficial for the management of hyperuricemia and gout. However, further animal model studies should be performed to validate the reported inhibitory activities under *in vivo* conditions and to recognize and determine the potentially active phytochemicals involved in the xanthine oxidase inhibitory activity reported in the present study.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

ACKNOWLEDGMENTS

The authors are grateful to the Philippine Institute of Traditional and Alternative Health Care for funding the external research grant.

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AUTHOR CONTRIBUTION:

Contribution	Ansari SS	Diño PH	Castillo AL	Santiago LA
Concepts or ideas	x		x	x
Design	x		x	x
Definition of intellectual content	x	x	x	x
Literature search	x		x	x
Experimental studies	x	x	x	x
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x	x	x	x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

Citation Format: Ansari SS, Diño PH, Castillo AL, Santiago LA (2021) Antioxidant activity, xanthine oxidase inhibition and acute oral toxicity of *Dillenia philippinensis* Rolfe (*Dilleniaceae*) leaf extract. J Pharm Pharmacogn Res 9(6): 846–858.