**RESEARCH ARTICLE**

# Evaluation of Xanthine Oxidase Inhibitory Activity, Antioxidant Activity, and Quantification of Total Phenolic and Flavonoid Contents in *Phyllanthus reticulatus* Poir

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## ABSTRACT
*Phyllanthus reticulatus* is an important medicinal plant in traditional medicine, used for treating bone and joint disorders, pain relief, anti-inflammatory purposes, liver protection, diabetes management, and antioxidant activities. This investigation focuses on analyzing the antioxidant and enzyme inhibitory activities of total and fractionated extracts of *Phyllanthus reticulatus*, through DPPH radical scavenging and xanthine oxidase inhibition assays, in addition to measuring their phenolic and flavonoid contents. The results indicate that the ethyl acetate (EA) fraction exhibited superior biological activities with the lowest $IC_{50}$ values, reaching 46.48±8.85 µg/ml for xanthine oxidase and 9.35±0.66 µg/ml for DPPH. The polyphenol and flavonoid content in this fraction were the highest, measuring 365.58±15.57 mg GAE/g and 21.67±1.92 mg QE/g, respectively, highlighting a strong correlation between chemical composition and biological efficacy. The total extract and BuOH and water fractions also demonstrated antioxidant and enzyme inhibitory activities at varying levels. These findings confirm the potential of the *Phyllanthus reticulatus* in developing pharmaceutical and functional food products, particularly for treating oxidative stress-related diseases.
**Keywords:** *Phyllanthus reticulatus,* TPC, TFC, antioxidant activity, xanthine oxidase.
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## INTRODUCTION
Gout is a prevalent type of arthritis resulting from the accumulation of sodium urate crystals, commonly linked to hyperuricemia arising from purine metabolism abnormalities or compromised kidney function. This results in increased serum uric acid concentrations, leading to joint inflammation[1-3].

Although various treatments such as colchicine, corticosteroids, and nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to manage acute gout attacks, these medications may cause adverse effects including skin rashes, gastrointestinal disturbances, and renal impairment with long-term use[4,5]. In contrast, traditional medicine approaches such as acupuncture, cupping therapy, and herbal remedies have been used for thousands of years and have shown therapeutic effectiveness and a favorable safety profile in managing gout[6,7].

*Phyllanthus reticulatus* is a widely occurring shrub native to Vietnam and various regions of East Asia[8,9]. Traditionally, this plant has been utilized in ethnomedicine for the treatment of musculoskeletal ailments such as spondylosis, rheumatoid arthritis, and joint pain. In addition, *Phyllanthus reticulatus* has been reported to exhibit analgesic, antioxidant, anti-inflammatory, hepatoprotective, antidiabetic, antidiarrheal, antimalarial, and wound-healing activities[10,11]. Its major chemical constituents include triterpenoids, phytosterols, coumarins, flavonoids, and phenolic compounds, which are believed to contribute significantly to its pharmacological effects[12].

This research aimed to investigate the xanthine oxidase inhibitory effects and antioxidant capacity of *Phyllanthus*

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*reticulatus*, along with the determination of its total phenolic and flavonoid contents. The findings are expected to offer a scientific basis for the potential therapeutic application of this medicinal plant in managing diseases associated with oxidative stress and inflammation, including gout and arthritis.

## MATERIALS AND METHODS

*Materials*

The aerial parts of *Phyllanthus reticulatus* (commonly known as "Phèn đen") were collected from Ben Tre province, Vietnam. Botanical identification was confirmed by Dr. Vu Huynh Kim Long, Faculty of Pharmacy, Ton Duc Thang University. Upon collection, the plant material was manually chopped into small fragments and initially air-dried in the shade for 24 hours to reduce surface moisture. Subsequently, At Can Tho University of Medicine and Pharmacy, the samples underwent additional drying using a hot-air oven maintained at 50 °C for a duration of 4 hours. The final moisture content of the dried material was assessed using an infrared moisture analyzer and recorded at 10.5%. The processed plant material was then stored under dry conditions at Faculty of Pharmacy - Ton Duc Thang University, for subsequent phytochemical and biological activity evaluations.

*Methods*

*Plant extraction*

*Phyllanthus reticulatus* was extracted using 96% ethanol by maceration. Twenty grams of dried plant material were soaked in ethanol at a ratio of 1:10 (materials:ethanol) for three consecutive 7-day periods. After each maceration, the extract was filtered and pooled. The combined extract was concentrated under reduced pressure at 40 °C to eliminate ethanol, yielding 3.19 g of crude extract. This extract was subsequently subjected to liquid–liquid partitioning using ethyl acetate and n-butanol, resulting in three distinct fractions: ethyl acetate (0.7 g), n-butanol (0.9 g), and aqueous (1.39 g). These fractions were employed in biological activity evaluations as well as measuring of total phenolic (TPC) and total flavonoid contents (TFC).

*Assay for evaluating xanthine oxidase inhibitory activity*

XO inhibition was recorded using a spectrophotometer at 295 nm by tracking uric acid formation in a 96-well plate assay. Samples diluted in phosphate buffer were combined with XO enzyme and buffer, pre-incubated at 37 °C, then treated with xanthine to initiate the reaction. Absorbance was measured every 30 seconds for 10 minutes. Allopurinol was used as a reference inhibitor. Percent inhibition was calculated by comparing absorbance with control wells, and $IC_{50}$ values were obtained using GraphPad Prism[13].

XO inhibition (%) was calculated using the formula:

$$XO\ inhibition\ (\%) = (AO - AT)/AO \times 100$$

Where $A_O$ and $A_T$ are the absorbance values of the blank and test sample, respectively, at 295 nm.

*Assay for evaluating DPPH radical scavenging activity*

Antioxidant capacity was assessed via a modified DPPH assay in 96-well microplates.[14] A 0.6 mM DPPH solution was used, and extract concentrations (60–200 µg/mL) along with ascorbic acid (20–200 µg/mL) as a positive control were prepared in methanol. Each well contained 25 µL of sample, 150 µL methanol, and 25 µL DPPH. After 30 minutes of dark incubation at room temperature, absorbance at 517 nm was recorded using a Varioskan™ LUX reader. Antioxidant activity was determined by comparing sample and control absorbance, and $IC_{50}$ values were calculated with GraphPad Prism 9.5.1[14].

Antioxidant activity-DPPH (%) was calculated as:

$$Antioxidant\ activity - DPPH\ (\%) = (AC - AT)\ Ac\ x\ 100$$

$A_C$ and $A_T$ denote the absorbance at 517 nm for the control and sample, respectively. Lower $IC_{50}$ values reflect higher antioxidant effectiveness.

*Determination of TPC*

TPC of the crude extract and its fractions was assessed using a modified Folin–Ciocalteu method. Briefly, 10 µL of sample or gallic acid standard was mixed with 10 µL methanol and 80 µL of diluted Folin–Ciocalteu reagent (1:10). After 10 minutes of dark incubation, 100 µL of 7.5% sodium carbonate was added. The mixture was kept in the dark at room temperature for 90 minutes, and absorbance was measured at 760 nm using a Varioskan™ LUX reader. A blank with methanol was used as control. Results were expressed in mg GAE/g[15,16].

*Determination of TFC*

TFC was measured using a modified aluminum chloride colorimetric method based on Lim et al. Briefly, 10 µL of sample or quercetin standard was mixed with 170 µL methanol and 20 µL of 10% aluminum chloride, then incubated in the dark for 30 minutes at room temperature. Absorbance at 415 nm was recorded using a Varioskan™ LUX microplate reader. A blank lacking aluminum chloride was used for comparison. Results were expressed as mg quercetin equivalents per gram (mg QE/g)[17].

## RESULTS AND DISCUSSION

*Evaluation of Crude Extract and Fractional XO Inhibition*

Involved in purine metabolism and uric acid generation, XO is known to be a major factor in disorders including gout, hyperuricemia, and oxidative damage. Table 1 shows the XO inhibition results for the crude extract and its ethyl acetate, butanol, and water fractions.

The $IC_{50}$ values revealed significant differences in the inhibitory efficiency among the samples. The EA fraction showed the highest inhibitory effect, with an $IC_{50}$ of 46.48 ± 8.85 µg/ml, while the crude extract ranked next ($IC_{50}$ = 80.36 ± 6.64 µg/ml). The BuOH and aqueous fractions did not reach 50% inhibition at the tested concentrations, indicating considerably weaker activity. Among all samples at 150 µg/ml, the EA fraction displayed the most significant inhibitory activity (83.56 ± 2.81%), whereas the crude extract reached 70.01 ± 1.94%. In contrast, the BuOH and aqueous fractions achieved only 34.83 ± 4.84% and 17.55 ± 3.39% inhibition, respectively, at 200 µg/ml. Polyphenols and flavonoids present in the EA fraction may contribute to its enhanced XO inhibitory effect[18], which are known for their XO inhibition, resulting in reduced uric acid and associated reactive oxygen species[19]. The lower activity of the BuOH and aqueous fractions may be due to a lack of active components or the presence of inactive or non-relevant compounds.

Table 1: Evaluation of xanthine oxidase inhibitory effects in the crude extract and its fractions

| Samples | Concentration (μg/ml) | Inhibition (%) | $IC_{50}$ (μg/ml) |
|---|---|---|---|
| Crude extract | 150 | 70.01 ± 1.94 | 80.36 ± 6.64 |
| | 100 | 63.48 ± 1.76 | |
| | 75 | 41.60 ± 6.22 | |
| | 50 | 31.49 ± 3.58 | |
| | 20 | 10.32 ± 1.36 | |
| EA fraction | 150 | 83.56 ± 2.81 | 46.48 ± 8.85 |
| | 100 | 63.60 ± 0.29 | |
| | 75 | 59.74 ± 0.69 | |
| | 50 | 48.99 ± 7.75 | |
| | 20 | 30.77 ± 0.11 | |
| BuOH fraction | 200 | 34.83 ± 4.84 | / |
| | 100 | 32.36 ± 5.41 | |
| | 75 | 28.80 ± 0.64 | |
| | 50 | 17.76 ± 3.01 | |
| | 20 | 11.60 ± 2.39 | |
| Aqueous fraction | 200 | 17.55 ± 3.39 | / |
| | 100 | 0.77 ± 0.17 | |

The enhanced activity of the EA fraction compared to the crude extract suggests the enrichment of bioactive compounds through solvent partitioning. These results highlight the potential of the EA fraction for further phytochemical investigation to identify specific compounds responsible for XO inhibition. The crude extract of *Phyllanthus reticulatus*, with moderate activity, also warrants further in vivo studies to assess its efficacy and safety for possible therapeutic use in diseases related to xanthine oxidase, such as gout.

*Antioxidant Activity – DPPH Free Radical Scavenging*

The potential to act as antioxidants, notably through DPPH radical scavenging, plays an essential role in protecting the body against oxidative stress-induced damage, which is a major contributor to chronic and degenerative diseases[20]. Table 2 summarizes the results of the DPPH assay used to evaluate antioxidant activities in the crude extract and its fractions.

$IC_{50}$ values indicated significant differences in antioxidant potency among the samples. The EA fraction exhibited the strongest activity, as indicated by its $IC_{50}$ value of 9.35 ± 0.66 μg/ml, indicating high radical scavenging capacity at low concentrations. The crude extract also exhibited strong activity ($IC_{50}$ = 14.47 ± 0.73 μg/ml), next were the BuOH and water fractions. At 25 μg/ml, all samples showed high radical scavenging activity (>88%), with the EA fraction achieving the highest inhibition (93.02 ± 1.18%). However, a dose-dependent decrease in activity was observed as concentration decreased, particularly in the BuOH and aqueous fractions. These differences in antioxidant activity among fractions may be explained by variations in both the levels and kinds of bioactive constituents. The EA fraction's lowest $IC_{50}$ value suggests it contains high levels of polyphenols, flavonoids, or other antioxidant compounds[18], which are widely recognized for their strong ability to scavenge DPPH radicals. Although slightly less active, the BuOH and aqueous fractions still demonstrated noteworthy antioxidant capacity.

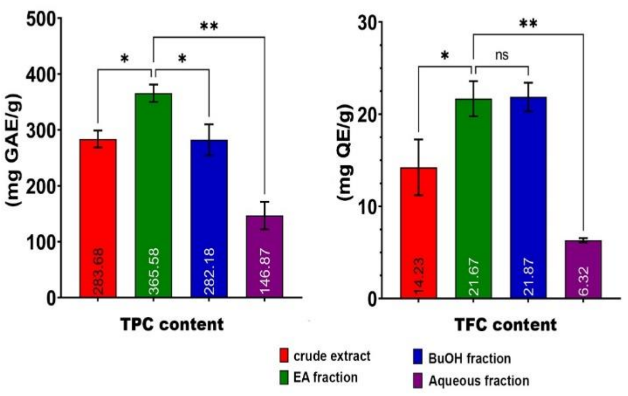


Figure 1: Analysis of phenolic and flavonoid content in the crude extract and its various fractions. Statistical significance was observed compared to the EA fraction (*p < 0.05, **p < 0.01); error bars indicate mean ± standard deviation (SD)

Table 2: Scavenging effect on DPPH radicals by the crude extract and its fractions

| Samples | Concentration (μg/ml) | Inhibition (%) | $IC_{50}$ (μg/ml) |
|---|---|---|---|
| Crude extract | 25 | 94.80 ± 0.62 | 14.47 ± 0.73 |
| | 12.5 | 44.11 ± 4.27 | |
| | 10 | 35.71 ± 5.34 | |
| | 7.5 | 29.43 ± 4.35 | |
| | 5 | 23.45 ± 9.32 | |
| EA fraction | 25 | 93.02 ± 1.18 | 9.35 ± 0.66 |
| | 12.5 | 64.31 ± 2.26 | |
| | 10 | 62.83 ± 8.49 | |
| | 7.5 | 35.17 ± 3.59 | |
| | 5 | 20.13 ± 1.87 | |
| BuOH fraction | 25 | 92.43 ± 2.58 | 15.59 ± 0.70 |
| | 12.5 | 38.72 ± 5.04 | |
| | 10 | 38.19 ± 3.93 | |
| | 7.5 | 34.94 ± 4.34 | |
| | 5 | 21.91 ± 7.22 | |
| Aqueous fraction | 25 | 88.73 ± 2.90 | 15.59 ± 6.93 |
| | 18.75 | 66.05 ± 0.75 | |
| | 12.5 | 30.61 ± 0.88 | |
| | 10 | 28.97 ± 2.06 | |
| | 7.5 | 10.39 ± 2.62 | |

These findings highlight *Phyllanthus reticulatus* as a promising natural source of antioxidant agents. Further studies should focus on chemical profiling of the EA fraction to isolate and identify the key constituents, as well as on evaluating their safety and biological efficacy in appropriate biological models.

*Total Phenolic and Flavonoid Contents*

Phenolic, flavonoid compounds are two major classes of natural substances well-known for their diverse biological activities, particularly in protecting the body against oxidative stress and related diseases[21-23]. The crude extract and its fractions' total polyphenol and flavonoid contents are shown in Figure 1.

The results revealed significant differences in compound content among the samples, reflecting the chemical diversity of each fraction. The most abundant total phenolic content was recorded in ethyl acetate (EA) fraction (365.58

± 15.57 mg GAE/g), followed by the butanol (BuOH) fraction (282.18 ± 27.8 mg GAE/g), and minimal in the aqueous fraction (146.87 ± 24.5 mg GAE/g). The EA fraction differed significantly from other samples ($p < 0.05$), indicating that the EA fraction was the richest in polyphenolic compounds.

Similarly, the richest flavonoid content was observed in EA fraction (21.67 ± 1.92 mg QE/g), which was comparable to the BuOH fraction (21.87 ± 1.54 mg QE/g), while aqueous fraction displayed the lowest TFC (6.32 ± 0.23 mg QE/g). The differences between the EA and BuOH fractions, and the crude extract or aqueous fraction, were statistically significant ($p < 0.05$).

These findings suggest that polyphenol and flavonoid compounds are predominantly concentrated in the EA fraction, while the aqueous fraction contains much lower levels. These results can be explained by the inherent polarity of polyphenols and flavonoids. These compounds typically exhibit moderate polarity and are therefore more efficiently extracted with ethyl acetate and butanol, rather than water[24-26]. The high polyphenol and flavonoid content in the EA fraction is consistent with the strong antioxidant activities observed in this sample during the xanthine oxidase inhibition and DPPH radical scavenging assays.

## CONCLUSION

This study provided evidence supporting the efficacy of the crude extract and solvent fractions of *Phyllanthus reticulatus* in inhibiting xanthine oxidase and scavenging DPPH free radicals. Among the samples, ethyl acetate fraction showed the highest level of biological activity, as reflected by the lowest $IC_{50}$ values in both assays. The elevated levels of polyphenols and flavonoids in this fraction are likely the major contributors to its bioactivity, highlighting a strong correlation between chemical composition and biological efficacy.

These results highlight the value of *Phyllanthus reticulatus* as a promising natural source for the development of antioxidant agents and therapeutic products targeting xanthine oxidase-related disorders. Further research is warranted to isolate and characterize the active compounds and evaluate their pharmacological effects in *in vivo* and clinical models.

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