Hue University Journal of Science: Agriculture and Rural Development
pISSN: 2588-1191; eISSN: 2615-9708
Vol. 134, No. 3D, 2025, P. 17–28, DOI: 10.26459/hueunijard.v134i3D.7869

MICROWAVE-ASSISTED EXTRACTION OF POLYPHENOL-RICH EXTRACT AND EVALUATION OF DPPH RADICAL SCAVENGING ACTIVITY FROM LEAVES OF *VITEX NEGUNDO* L. (VERBENACEAE)

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(*Received: May 18, 2025; Accepted: June 10, 2025*)

Abstract. This research investigates the ultrasound-assisted extraction (UAE) of polyphenol-rich extracts from leaves of *Vitex negundo* L. (Verbenaceae) and evaluates their antioxidant activity. Dried leaf powder, cultivated in Ho Chi Minh City, Vietnam, was extracted using 60% ethanol under varying conditions of sample-to-solvent ratio, temperature, and time. Optimal extraction was achieved at a ratio of 1:10 (g/ml), 50°C, and 40 minutes, resulting in the highest total polyphenol (72.90 mg GAE/g) and flavonoid contents (179.94 mg QE/g). The final concentrated extract showed a moisture content of 4.90%, extraction yield of 27.94%, and retained 42.84 mg GAE/g of polyphenols and 165.67 mg QE/g of flavonoids. Antioxidant capacity was assessed via DPPH radical scavenging assay. The extract exhibited moderate activity with an IC_{50} of 66.08 μg/ml, compared to 12.61 μg/ml for ascorbic acid and 4.48 μg/ml for BHA. The results demonstrate that *V. negundo* leaves are a promising source of natural antioxidants. UAE was shown to be an effective and green technique for enhancing extraction efficiency. These findings support the potential application of *V. negundo* leaf extract in functional foods and phytopharmaceuticals.

Keywords: *Vitex negundo*, ultrasound-assisted extraction, polyphenols, flavonoids, DPPH, antioxidant activity

1 Introduction

In recent years, interest in plant-derived bioactive compounds has increased significantly due to their potential health benefits and applications in functional foods, pharmaceuticals, and cosmetics. Among these compounds, polyphenols and flavonoids are known for their strong antioxidant, anti-inflammatory, and disease-preventing properties [1, 2]. *Vitex negundo* L. (Verbenaceae), commonly known as Chinese chaste tree or "Ngũ Trảo" in Vietnam, is a widely distributed medicinal plant in Asia. Traditionally, its leaves have been used to treat fever, inflammation, asthma, and skin disorders. Phytochemical analyses have confirmed the presence

of phenolics, flavonoids, essential oils, and terpenoids in the leaves, many of which exhibit significant antioxidant activity [3, 4].

The efficiency of bioactive compound extraction depends on several factors, including solvent type, extraction method, temperature, and duration. Ultrasound-assisted extraction (UAE) has emerged as a green, efficient, and time-saving technique that enhances mass transfer and disrupts plant cell walls, allowing for improved recovery of target compounds [5–7]. Moreover, using ethanol–water mixtures is considered safe and effective for extracting both polar and moderately polar compounds [8]. Despite increasing scientific interest, studies optimizing UAE conditions for *V. negundo* leaves, especially in the Vietnamese context, remain limited. Furthermore, there is a need to correlate total polyphenol and flavonoid contents with antioxidant activity, particularly using standard radical scavenging assays like 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Therefore, this research aims to (i) determine the appropriate ultrasound-assisted extraction parameters (solvent ratio, temperature, and time) for recovering polyphenol-rich extracts from *V. negundo* leaves, (ii) evaluate the extract's antioxidant capacity through DPPH radical scavenging assay, and (iii) identify the potential of *V. negundo* as a natural source of antioxidants for future food applications.

2 Materials and methods

2.1 Materials

Plant material: Fresh semi-ripe leaves of *V. negundo* were harvested in District 12, Ho Chi Minh City, Vietnam. The leaves were throughly washed and subjected to convective drying at 50–52 °C for approximately 10 hours until the moisture content reached approximately 9.8%, in compliance with the Vietnamese Pharmacopoeia V standard (<12%) [9, 10]. The dried leaf samples were ground into fine powder and passed through a 0.5 mm mesh sieve. The resulting powder was stored in zip-lock bags together with silica gel desiccants to prevent moisture absorption.

2.2 Extraction procedure

The dried leaf powder (0.5000 ± 0.0002 g) was extracted using ultrasound-assisted extraction (UAE) with 60% ethanol as solvent. The extraction conditions were optimized across different factors: sample-to-solvent ratios (1:5, 1:10, 1:15, 1:20, 1:25, 1:30 and 1:35 g/ml), extraction temperature (30, 40, 50, 60, 70 and 80 °C), extraction times (10, 20, 30, 40, 50 and 60 minutes). Ultrasound was applied using a probe-type system operating at 37 kHz. After filtration, the

extracts were concentrated under reduced pressure using a rotary evaporator to obtain the final crude extract.

2.3 Determination of total polyphenol and flavonoid contents

Total Polyphenol Content (TPC) of the extracts was determined using the Folin–Ciocalteu colorimetric method, as described by Rao et al. [11], with slight modifications. Briefly, 0.5 mL of appropriately diluted extract was mixed with 2.5 mL of 10% (v/v) Folin–Ciocalteu reagent. After 5 minutes of incubation at room temperature, 2.0 mL of 7.5% (w/v) sodium carbonate solution was added. The mixture was incubated in the dark at room temperature for 30 minutes, and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer. Gallic acid was used as the standard, and the results were expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g).

Total Flavonoid Content (TFC) was quantified using the aluminum chloride colorimetric method, following the procedure by Chang et al. [12]. In brief, 0.5 mL of the extract was mixed with 0.1 mL of 10% (w/v) aluminum chloride, 0.1 mL of 1 M potassium acetate, and 4.3 mL of distilled water. The mixture was incubated at room temperature for 30 minutes, and absorbance was recorded at 415 nm. Quercetin was used as the standard, and TFC was expressed as mg quercetin equivalents per gram of dry extract (mg QE/g).

2.4 Antioxidant activity

The antioxidant capacity was evaluated using the DPPH radical scavenging assay following the method of Gulcin and Alwasel [13], with slight modifications. In brief, 1.0 mL of extract at various concentrations was mixed with 1.5 mL of 0.1 mM DPPH solution in methanol and incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The percentage of DPPH radical scavenging activity was calculated, and IC_{50} values were obtained from dose–response curves. Ascorbic acid and BHA served as reference antioxidants.

2.5 Statistical Analysis

All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation (SD). Significant differences among treatments were determined using one-way ANOVA followed by Tukey's HSD test at $p < 0.05$.

3 Results and Discussion

The freshly harvested leaves of *V. negundo* had an initial moisture content of 61.7%. After preliminary processing, the leaves were dried using a convective drying method at 50–52 °C for approximately 10 hours to reduce the moisture content to the target range of 8–10%. The final moisture content after drying was 9.8%, which complies with the standard specified in the Vietnamese Pharmacopoeia V [10], requiring moisture levels not to exceed 12.0%. Furthermore, this value aligns with the predetermined moisture target of the study. Therefore, the dried *V. negundo* leaf samples were considered satisfactory for use in subsequent experiments.

3.1 Effect of Solvent on Polyphenol and Flavonoid Content

The experiment investigating the effect of solvent type on the extraction efficiency of polyphenols and flavonoids from leaves of *V. negundo* was conducted using 0.5000 ± 0.0002 g of dried powdered sample. Ultrasound-assisted extraction conditions were kept constant throughout the study, including a sample-to-solvent ratio of 1:10 (g/ml), extraction temperature of 50 °C, and duration of 20 minutes. The variable factor in this experiment was the solvent type, comprising distilled water and ethanol at concentrations of 40, 50, 60, 70, 80, and 90%. The quantitative results of polyphenol and flavonoid contents, along with statistical comparisons among treatments, are presented in the following Table 1.

The results showed that the choice of solvent significantly influenced the extraction efficiency of polyphenols and flavonoids from *V. negundo* leaves. Among the tested solvents, 60% ethanol yielded the highest content of both polyphenols (63.60 mg GAE/g) and flavonoids (181.70 mg QE/g), significantly higher than other ethanol concentrations and water ($p < 0.05$). This trend indicates the suitability of moderately polar ethanol–water mixtures for the extraction of medium-polarity bioactive compounds such as polyphenols and flavonoids.

This finding aligns with the study by Saklani et al. [4], where 60% ethanol extract of *V. negundo* leaves showed significantly higher polyphenol content and antioxidant activity compared to 80% ethanol and aqueous extracts, confirming that 60% ethanol is optimal for solubilizing and releasing active phytochemicals. Similarly, Lakshmanashetty et al. reported that *V. negundo* leaf extracts obtained using ethanol concentrations of 50–60% exhibited stronger antioxidant activity, while 90% ethanol was less effective due to its lower polarity [3].

Table 1. Total Polyphenol and Flavonoid Contents in different solvents

Solvents	Total Polyphenol Content (mg GAE/g dry weight)	Total Flavonoid Content (mg QE/g dry weight)
Water	$42.76^{a} \pm 0.49$	$74.94^{a} \pm 0.78$
40% Ethanol	$56.71^{c} \pm 0.24$	$145.18^{c} \pm 0.62$
50% Ethanol	$59.06^{c} \pm 0.45$	$161.02^{d} \pm 0.55$
60% Ethanol	$63.60^{d} \pm 0.50$	$181.70^{e} \pm 0.68$
70% Ethanol	$58.77^{c} \pm 0.48$	$152.93^{d} \pm 0.61$
80% Ethanol	$56.31^{c} \pm 0.46$	$143.28^{c} \pm 0.79$
90% Ethanol	$48.07^{b} \pm 0.51$	$106.47^{b} \pm 0.66$

Values in the same column followed by the same letter are not significantly different from each other at $p < 0.05$.

These results are consistent with the chemical nature of polyphenol and flavonoid compounds in *V. negundo*, which mostly exist in glycosylated forms or bound to organic acids, requiring a solvent of intermediate polarity for effective extraction [14]. Furthermore, 60% ethanol is considered a safe, volatile, and environmentally friendly solvent, making it suitable for applications in food, pharmaceuticals, and cosmetics. Therefore, 60% ethanol was determined to be the most effective extraction solvent, meeting the criteria of high extraction yield, safety, and practical applicability for subsequent experiments involving *V. negundo* leaf extracts.

3.2 Effect of Sample-to-Solvent Ratio on Total Polyphenol and Flavonoid Contents

The experiment was conducted under fixed ultrasonic-assisted extraction conditions (50 °C, 20 min, 60% ethanol). The sample-to-solvent ratio ranged from 1:5 to 1:35 (g/ml). The effect of this ratio on extraction efficiency was evaluated. Total polyphenol and flavonoid contents were quantified for each condition. Results and comparisons are presented in the following Table 2.

The highest polyphenol content was obtained at a ratio of 1:10 (67.49 mg GAE/g dry weight), followed by 1:20 and 1:25. Similarly, the maximum flavonoid content was also observed at the 1:10 ratio (178.40 mg QE/g dry weight), which was significantly higher than other treatments ($p < 0.05$). These findings suggest that a moderate dilution of the sample facilitates better diffusion and mass transfer of polyphenol compounds from plant matrix into the solvent. When the solvent volume was too low (e.g., 1:5), the extraction efficiency decreased, likely due to saturation effects and limited solvent capacity to dissolve all target compounds. Conversely, overly high dilution (e.g., 1:35) also reduced extraction efficiency, possibly due to lowered interaction between solvent and matrix and excessive dilution of target compounds [15, 16].

Table 2. Effect of Sample-to-Solvent Ratio on Total Polyphenol and Flavonoid Contents

Sample-to-Solvent Ratio (g/ml)	Total Polyphenol Content (mg GAE/g dry weight)	Total Flavonoid Content (mg QE/g dry weight)
1:5	$54.21^{b} \pm 0.49$	$138.36^{a} \pm 0.78$
1:10	$67.49^{e} \pm 0.24$	$178.40^{d} \pm 0.68$
1:15	$55.61^{b} \pm 0.45$	$158.82^{bc} \pm 0.61$
1:20	$65.13^{de} \pm 0.51$	$163.88^{c} \pm 0.76$
1:25	$64.36^{cd} \pm 0.65$	$164.98^{c} \pm 0.79$
1:30	$62.31^{c} \pm 0.48$	$153.54^{b} \pm 0.61$
1:35	$45.79^{a} \pm 0.55$	$144.96^{a} \pm 0.63$

Values in the same column followed by the same letter are not significantly different from each other at $p < 0.05$.

These results are consistent with previous findings that show moderate sample-to-solvent ratios (1:10 to 1:25) are ideal for polyphenol compound extraction, especially under ultrasound-assisted conditions [4, 6, 7]. Therefore, a ratio of 1:10 g/ml is considered the optimal condition for extracting polyphenols and flavonoids from *V. negundo* leaves in subsequent studies.

3.3 Effect of Extraction Temperature on Total Polyphenol and Flavonoid Contents

The experiment was carried out using the weight of dried powdered sample under fixed ultrasound-assisted extraction conditions: 60% ethanol, a sample-to-solvent ratio of 1:10 (g/ml), and 20 minutes of extraction. The variable factor was temperature, tested at 30, 40, 50, 60, 70, and 80 °C. Total Polyphenol and Flavonoid contents were quantified under each condition. The influence of extraction temperature on the yield of polyphenols and flavonoids from *V. negundo* leaf extracts is presented in Table 3.

The results indicate that temperature has a significant effect ($p < 0.05$) on the extraction efficiency. The highest TPC was observed at 50 °C (71.53 mg GAE/g), followed closely by 60 °C (70.53 mg GAE/g). Similarly, the highest flavonoid content was also obtained at 50 °C (178.40 mg QE/g), followed by 60 °C (152.66 mg QE/g). These findings suggest that moderate temperatures enhance solvent penetration, improve solubility of polyphenol compounds, and activate the release of cell wall-bound polyphenols under ultrasonic waves [6, 7]. At lower temperatures such as 30 °C, the extraction yields were significantly lower (polyphenols: 54.11 mg GAE/g; flavonoids: 127.39 mg QE/g), likely due to reduced molecular diffusion and lower solvent activity. On the other hand, temperatures above 60 °C, particularly 70–80 °C, resulted in a marked decline in both polyphenol and flavonoid contents. This can be attributed to thermal degradation and oxidation of heat-sensitive polyphenol compounds [16, 17]. These results are consistent with earlier studies

Table 3. Effect of Temperature on Total Polyphenol and Flavonoid Contents

Temperature (°C)	TPC (mg GAE/g dry weight)	TFC (mg QE/g dry weight)
30	$54.11^{a} \pm 0.54$	$127.39^{a} \pm 0.79$
40	$65.72^{c} \pm 0.58$	$140.78^{b} \pm 0.68$
50	$71.53^{d} \pm 0.48$	$178.40^{d} \pm 0.55$
60	$70.53^{d} \pm 0.50$	$152.66^{c} \pm 0.64$
70	$57.81^{b} \pm 0.40$	$137.54^{b} \pm 0.70$
80	$57.09^{ab} \pm 0.54$	$138.07^{b} \pm 0.57$

Values in the same column followed by the same letter are not significantly different from each other at $p < 0.05$.

that suggest extraction temperatures around 50–60 °C are appreciate for ultrasonic-assisted recovery of polyphenols without compromising structural integrity. Therefore, 50 °C is recommended as the appreciate temperature for further extractions to balance efficiency and compound stability.

3.4 Effect of Extraction Time on Total Polyphenol and Flavonoid Contents

The effect of extraction time on the recovery of total polyphenols and flavonoids from *V. negundo* leaves using ultrasound-assisted extraction (UAE) with 60% ethanol is presented in Table 4. The extraction time varied from 10 to 60 minutes, while all other parameters (solvent concentration, temperature, and sample-to-solvent ratio) were kept constant.

The results demonstrate that extraction time significantly influenced the yield of both polyphenols and flavonoids ($p < 0.05$). The highest polyphenol (72.90 mg GAE/g) and flavonoid (179.94 mg QE/g) contents were obtained at 40 minutes, indicating that this duration was the most favorable for releasing polyphenol compounds under ultrasonic conditions. This can be attributed to sufficient time for solvent penetration, cavitation-induced disruption of plant cell walls, and effective diffusion of intracellular compounds [5–7].

Shorter extraction times, such as 10 or 20 minutes, resulted in lower yields, likely due to incomplete diffusion or insufficient breakdown of plant tissues. For example, polyphenol content at 10 minutes was only 64.88 mg GAE/g, while flavonoids reached 162.34 mg QE/g, suggesting that extraction was still ongoing. Interestingly, extraction durations beyond 40 minutes (i.e., 50–60 minutes) showed a decline in yield, particularly for flavonoids (137.48 and 136.1 mg QE/g, respectively). This could be attributed to degradation or oxidation of heat- and ultrasound-sensitive compounds, especially flavonoids, during prolonged exposure [17, 18]. Overall, these

Table 4. Effect of time on Total Polyphenol and Flavonoid Contents

Time (min)	Total Polyphenol Content (mg GAE/g dry weight)	Total Flavonoid Content (mg QE/g dry weight)
10	$64.88^{a} \pm 0.18$	$162.34^{c} \pm 0.79$
20	$69.82^{c} \pm 0.59$	$167.40^{c} \pm 0.68$
30	$66.07^{ab} \pm 0.62$	$149.14^{b} \pm 0.73$
40	$72.90^{d} \pm 0.69$	$179.94^{d} \pm 0.68$
50	$67.43^{b} \pm 0.61$	$137.48^{a} \pm 0.67$
60	$67.21^{b} \pm 0.56$	$136.16^{a} \pm 0,57$

Values in the same column followed by the same letter are not significantly different from each other at $p < 0.05$.

findings support previous reports that ultrasound-assisted extraction is time-dependent, and that there exists an optimal time beyond which compound degradation may outweigh release benefits. Therefore, 40 minutes is identified as the optimal extraction time for maximizing both polyphenol and flavonoid yields in *V. negundo* leaf extraction using UAE.

3.5 Comparison of Extract Before and After Concentration

After identifying the appreciate conditions, 10.00 g of dried *V. negundo* leaf powder was extracted using 100 ml of 60% ethanol at a sample-to-solvent ratio of 1:10 (g/ml), at 50 °C for 40 minutes with ultrasound assistance at 37 kHz. Following extraction, the TPC and total flavonoid content (TFC) of the extract were measured. The extract was then concentrated using rotary evaporation at 50 °C to remove the solvent, yielding 2.65 g of crude dry extract. The extract had a thick consistency and a dark brown color.

The concentration step plays a crucial role in converting the liquid extract into a solid or semi-solid form (referred to as the final extract or crude extract), suitable for long-term storage and biological activity testing. In this study, post-concentration results showed a moisture content of 4.90%, which meets the acceptable criteria for botanical extracts, typically required to be under 5–10% for stability and microbial safety [19].

The extract yield was calculated to be 27.94%, indicating an efficient recovery of solid materials from the liquid phase. This yield is within the expected range for polyphenol-rich plant extracts processed by ethanol-based ultrasound-assisted extraction [6, 7]. While TPC and TFC decreased after the concentration step (from 71.39 to 42.84 mg GAE/g and 206.11 to 165.67 mg QE/g, respectively), the final extract still retained a high level of bioactive compounds. The reduction in content is likely due to thermal degradation, oxidation, or loss of volatile components during solvent evaporation [17, 20].

Table 5. Comparison of Extract Before and After Concentration

Parameter	Extract Before Concentration	Final Extract (Post-concentration)
Moisture content (%)	–	4.9
Extract yield (%)	–	27.94
TPC	71.39 ± 1.24 mg GAE/g dry weight	42.84 ± 0.72 mg GAE/g dry weight
TFC	206.11 ± 2.22 mg QE/g dry weight	165.67 ± 1.99 mg QE/g dry weight

Nonetheless, the final concentrated extract remained rich in polyphenols and flavonoids, suggesting good stability and suitability for downstream applications such as antioxidant assays, formulation into functional foods or phytopharmaceuticals.

3.6 Evaluation of DPPH Radical Scavenging Activity

The IC_{50} values represent the concentration required to inhibit 50% of DPPH free radicals, with lower IC_{50} values indicating stronger antioxidant activity.

The results indicate that BHA exhibited the strongest antioxidant activity (IC_{50} = 4.48 μg/ml), followed by ascorbic acid (12.61 μg/ml). The ethanolic extract of *V. negundo* leaves showed moderate antioxidant potential, with an IC_{50} value of 66.08 μg/ml, which is significantly higher than the standards. Although the extract was less potent than the synthetic antioxidant (BHA) and ascorbic acid, it still demonstrated notable free radical scavenging capacity. This activity is likely attributed to its high content of polyphenols and flavonoids, as previously quantified. The results align with findings by Lakshmanashetty et al. [3] and Saklani et al. [4], which reported similar moderate antioxidant capacity of *V. negundo* extracts in DPPH assays. Therefore, while not as potent as standard antioxidants, the extract retains natural antioxidant potential, making it a promising candidate for functional food or phytopharmaceutical applications, especially where safety and natural origin are prioritized.

4 Conclusion

Ultrasound-assisted extraction using 60% ethanol proved effective in recovering high levels of polyphenols and flavonoids from leaves of *V. negundo*. Optimal extraction conditions were identified as a sample-to-solvent ratio of 1:10 (g/ml), an extraction temperature of 50 °C, and an extraction time of 40 minutes. The final extract exhibited low moisture content (4.90%), a satisfactory yield (27.94%), and retained significant amounts of total polyphenols (42.84 mg GAE/g) and flavonoids (165.67 mg QE/g), indicating good stability and extraction efficiency. In terms of antioxidant capacity, the extract demonstrated a moderate ability to scavenge DPPH free radicals, with an IC_{50} value of 66.08 μg/ml. Although this value is higher

Table 6. IC_{50} evaluates the DPPH free radical scavenging capacity

Parameter	IC_{50} (μg/ml)
Ascorbic acid	12.61
BHA	4.48
Plant extract	66.08

than that of standard antioxidants such as ascorbic acid (12.61 μg/ml) and BHA (4.48 μg/ml), the extract still shows promising natural antioxidant potential. This activity is attributed to its high polyphenol and flavonoid content and supports its potential application in the development of functional foods, nutraceuticals, and herbal formulations. Overall, the findings suggest that *V. negundo* leaf extract, when obtained under optimized ultrasonic-assisted conditions, can serve as a valuable source of natural antioxidants.

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