Trop J Nat Prod Res, July 2025; 9(7): 3114 - 3119 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Phytochemical Screening and Antioxidant Potential of Ethanol Extract From *Piper crocatum* Leaves

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ARTICLE INFO

Article history:
Received 11 February 2025
Revised 21 May 2025
Accepted 24 May 2025
Published online 01 August 2025

ABSTRACT

Natural products play a crucial role as sources of medicinal compounds, and many modern pharmaceuticals have been developed from traditional herbal medicines. Plants in the Piperaceae family are well known for their ethnomedicinal uses. One member of this family, *Piper crocatum* Ruiz & Pav, was traditionally utilized for its diverse biological properties. However, despite its traditional uses, there is currently limited scientific literature reporting its bioactive compounds and antioxidant properties. Therefore, this study aimed to elucidate the phytochemical composition and antioxidant properties of red betel ethanol extract (RBE). RBE were processed for LC-MS analysis for compound identification. The antioxidant activity was determined using a DPPH assay. Total flavonoids and phenolics of RBE were assessed using the Aluminum Chloride and Folin-Ciocalteu techniques, respectively. The study successfully identified eighteen compounds contained in RBE, with kaempferitrin being the most abundant compound. The higher concentration of RBE exhibited high levels of antioxidant activity, with the percentage of DPPH inhibition of 52.55 ± 0.51%. The flavonoids and phenolics content of RBE was 644.44 ± 48.11 µg QE/g and 496.10 ± 1.08 µg GAE/g, respectively. These findings suggest promising bioactive potential and support further investigation into the antibacterial and anti-inflammatory properties of RBE.

Keywords: Phytochemistry, Antioxidant, Phenolic, Flavonoid, Red betel

Introduction

Over the past ten years, the use and acceptance of natural remedies have risen significantly in both developed and developing nations. This growing global interest in herbal medicine is largely driven by its perceived safety and efficacy. In many developing countries, approximately 80% of the people utilize medicinal plants as their main form of healthcare. The World Health Organization (WHO) has emphasized the vital role of traditional medicine, particularly in developing regions, to help address healthcare demands.[1] Natural products play an essential role as a source of medicinal compounds. Nowadays, several modern medicines can be derived from traditional herbal medicine. Plants can synthesize a variety of bioactives that have beneficial effects as a treatment for diseases.[1,2] Plants contain several valuable compounds, especially secondary metabolites with a broad range of phenolic compounds, which are categorized into flavonoids and non-flavonoids.[3,4]

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Citation: Maslikah SI, Lestari SR, Siri S, Yunus M, El Baroroh AR, Miasih DS, Rifa'i M, Warsito, Djati MS. Phytochemical screening and antioxidant potential of ethanol extract from *Piper crocatum* leaves. Trop J Nat Prod Res. 2025; 9(7): 3114 – 3119 https://doi.org/10.26538/tjnpr/v9i7.24

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The betel plant is one of several herbal plants that have long been utilized in traditional medicine. Betel plants belonging to the genus *Piper* (Piperaceae) are a diverse genus of tropical plants that are a rich source of various bioactive.[5] The Piperaceae family is an essential source of substances with pharmacological activity. Close to 400 compounds with varied structures and biological activities have been identified from this genus, primarily consisting of flavones, phenylpropanoids, alkaloids, chalcones, lignans, and kava-pyrones. Medicinal plants need to be analyzed to determine their phytochemical content, which will be beneficial in understanding their pharmacological importance and health risks.[6]

Piper crocatum Ruiz & Pav is a red betel plant that grows in the tropical rainforests of Indonesia. The plant belongs to the genus Piper, which has heart-shaped leaves of a silvery red color.[7] Although medicinal plants are widespread, there is a lack of literature explaining the relevance and risk of phytochemical exposure, so phytochemical screening data in plants are needed to determine how many doses are sufficient when using natural materials. Phytochemical investigations of various betel species, such as *Piper betle*, have led to the identification of several important bioactive compounds. Notable among these are kaempferol 7-methyl ether 3-[3-hydroxy-3-methylglutaryl-(1→6)]-[apiosyl-(1→2)-galactoside], isovitexin, hydrojuglone glucoside, vitexin 6"-(3-hydroxy-3-methylglutarate), and vitexin 4'-O-galactoside.[8]

Plant chemical content, characteristics, and activity might vary based on the plant's environment or the processing method. Currently, no research reveals the profile of the active compounds in the red betel (*P. crocatum*) ethanol extract (RBE). Historically, red betel has been

extensively utilized in traditional herbal therapy for managing various inflammatory conditions and microbial infections. The plant is high in phytochemical content, which presents unexplored prospects for discovering new bioactive compounds with potential as therapeutic candidates. To fully exploit this potential, phytochemical profiling was used using rapid and accurate tools such as LC-MS analysis. The antioxidant property of *P. crocatum* was also evaluated, involving measuring total phenolic and flavonoid content quantification. Therefore, this study aimed to elucidate the phytochemical composition and antioxidant properties of RBE.

Materials and Methods

Plant collection and identification

Fresh *P. crocatum* leaves were collected from the private garden of Mr. Adieb Wirahusnawan in Jatimulyo Village, Lowokwaru District, Malang, East Java, Indonesia (geographical coordinates: 7°56'06.5"S 112°36'41.6"E) in January 2024. Taxonomic identification was performed by the UPT. Balai Materia Medica Batu, Indonesia, with specimen number 000.9.3/3119/102.20/2023.

Extraction of plant material

The extraction protocol was conducted according to previous research by Djati et al.[9] with some modification on the ratio of sample and solvent. Red betel leaves were cleaned, oven-dried at 50° C, and milled into powder. The leaves that were ground were placed in an Erlenmeyer flask dan macerated with 70% ethanol in a ratio of 1:3 for 72 h. The sample was filtered using Whatman filter paper (China), and then the solvent was evaporated using a vacuum rotary evaporator (IKA® RV 10, IKA Works (Asia) Sdn Bhd, Malaysia) at 48 °C. The resulting extract was then weighed and subjected to further analysis.

Phytochemical Identification

The phytochemical compounds of RBE were identified using LC-MS analysis at the Forensic Laboratory *Center* (Puslabfor), Sentul, *Bogor*. Ten grams of dissolved RBE in 1 mL of ethanol was filtered using a 0.2 µm filter. A five µL sample was injected into a Waters® ACQUITY UPLC H-Class System (Waters Corporation, USA), utilizing a C18 reversed-phase column. The column temperature was 50 °C, with a laboratory environment at 25 °C. The mobile phase included solvent A (water + 5 mM ammonium formate) and solvent B (acetonitrile + 0.05% formic acid). A gradient elution program was applied with the mobile phases delivered at a constant flow rate of 0.2 mL/min, with a total chromatographic run time was 23 min. Data were processed using MassLynx software version 4.1 (Waters®, Water Corporation, USA) to obtain peak area values and mass-to-charge (m/z) spectra, which were then compared with reference data from the ChemSpider (https://www.chemspider.com/) and PubChem databases for compound identification.[10]

Determination of Antioxidant Activity

Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, adapted with minor modifications from the methods outlined by Okafor et al.[11] RBE was prepared at concentrations of 50, 100, 200, 400, 600, 800, and 1000 ppm, while a 50 µM dissolved DPPH in methanol was prepared. Each reaction consisted of 250 µL of the extract, standard, or control combined with 1,250 µL of DPPH solution, followed by incubation for 15 min in the dark at room temperature. Absorbance was then measured using a UV/Vis spectrophotometer (Libra S11/12, Biochrom Ltd., UK) at 517 nm. The IC_{50} value was determined from the resulting dose-response curve. To ensure accuracy and reproducibility, all experiments were performed in triplicate. The DPPH radical scavenging activity was determined using this formula:[12]

$$\%\ inhibition = \left(\frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}}\right) \times 100 \quad (1)$$

Determination of Total Phenolic Contents

Total phenolic content of RBE was assessed using the Folin-Ciocalteu assay, with minor adjustments to the method outlined by Christina et al.[13] RBE was prepared at concentrations of 50, 100, 200, 400, 600, 800, and 1000 ppm. The Folin-Ciocalteu reagent was diluted tenfold, and sodium carbonate (Na_2CO_3) was prepared at a concentration of 75 g/L. In each reaction, 500 µL of RBE was added with 500 µL of the diluted Folin–Ciocalteu reagent and allowed to incubate for 5 min. Subsequently, 500 µL of sodium carbonate solution was added to the mixture, which was then incubated in the dark at room temperature for 1 h. Absorbance was measured at 740 nm using a UV/Vis spectrophotometer (Libra S11/12, Biochrom Ltd., UK). All assays were performed in triplicate to ensure data reliability and reproducibility.

Determination of Total Flavonoid Content

Total flavonoid in RBE was assessed using aluminum chloride ($AlCl_3$) reaction based on Christina et al.[14] RBE was prepared at concentrations of 50, 100, 200, 400, 600, 800, and 1000 ppm. For each assay, 300 µL of extract was added with 420 µL of distilled water and 90 µL of 5% sodium nitrite ($NaNO_3$), and incubated for 5 min at room temperature in the dark. Subsequently, 90 µL of 10% aluminum chloride ($AlCl_3$) was added, followed by incubation for 6 min in the dark at room temperature, and addition of 600 µL of 1 M sodium hydroxide (NaOH). Absorbance was measured using a UV/Vis spectrophotometer (Libra S11/12, Biochrom Ltd., UK) at 510 nm. All assays were performed in triplicate to ensure data reliability and reproducibility.

Statistical analysis

Data were presented as the mean ± standard deviation (SD) from three independent experiments ($p<0.05$). All statistical analysis was carried out using IBM SPSS Statistics for Windows version 22.0 (IBM Corp., Armonk, NY).

Results and Discussion

LCMS Results

Figure 1 demonstrates the metabolite profile of red betel (*P. crocatum*) ethanolic extract with 18 peaks (Figure 1). Based on LC-MS results, 17 compounds were detected in red betel extract, with 1 unknown compound (Table 1). Compounds are identified qualitatively by analyzing their molecular ion peaks, ion fragments, and mass-to-charge ratios (m/z).

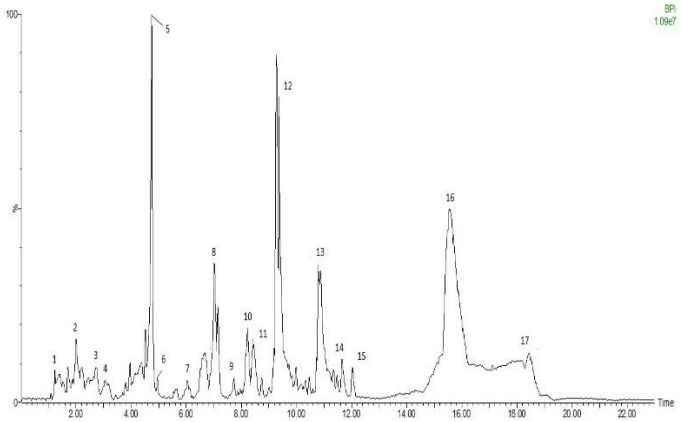

Figure 1: The chromatographic profiles of red betel (*P. crocatum*) extract

LCMS discovered several types of compounds that have been identified, as well as compounds that are not yet known. Unknown compounds refer to those that are detected by instruments but cannot be identified, either because they are impure or because they have not yet been included in existing databases, particularly when these compounds are present in high concentrations.[15] The study found that the highest chromatogram peak of red betel extract was observed at peak number

5, which was identified as kaempferitrin. Kaempferitrin is a class of flavonoids widely reported as antioxidants.[16] Mitragynine is found in red betel extract. Mitragynine is reportedly one of the derivatives of alkaloid compounds that have anti-inflammatory, antihypertensive, local anesthetic, and hypoglycemic functions.[17] In addition, piperbetol compounds were also found at a retention time of 8.22. Piperbetol is one of the active compounds isolated from the plant and has been found to play a role in anti-inflammatory/immunomodulatory, anti-ulcer, and antibacterial.[18] According to Maslikah et al.[19], red betel extract contains main compounds, including kampferitin, sesamin, piperine, and piperbetol. Sesamin, one of these compounds, has potential as an anti-inflammatory agent by blocking NF-κB activity.[20]

Table 1: The identified compounds of red betel (*P. crocatum*) extract based on LC-MS results

Peak No	Retention Time	% Area	(m/z)	Molecular Weight (g/mol)	Formula	Compound
1	1.238	1.48	266.12	265.26	$C_{10}H_{19}NO_7$	2-N-(Carboxypropylamino)-2-deoxyglucopyranose
2	2.01	4.01	178.09	177.58	$C_5H_{11}N_3O_4$	O-(Carbamoylamino)homoserine
3	2.73	2	174.15	173.25	$C_9H_{19}NO_2$	2-Aminononanoic acid
4	3.06	0.84	310.13	209.31	$C_{15}H_{19}NO_6$	2,6-dimethyl-3,5-di(propanoyl)-4H-pyridine-1,4-dicarboxylic acid
5	4.75	10.26	579.17	578.50	$C_{27}H_{30}O_{14}$	Kaempferitrin
6	4.95	0.23	236.17	235.29	$C_{10}H_{17}N_7$	N-[2-(6-Amino-9H-purine-9-yl)ethyl]-1,3-propane diamine
7	6.03	0.49	355.17	354.35	$C_{13}H_{26}N_2O_9$	Methyl Beta-Chitobioside
8	6.66	2.4	589.23	Unknown	Unknown	Unknown
9	7.01	5.33	785.23	784.70	$C_{34}H_{36}N_6O_{16}$	1-[3-[[[[2'-O-Acetyl-3'-[[(1,3-dioxo-1,3-dihydro-2H-isoindole-2-yl)oxy]methyl]-5'-oxo-3'-deoxy-5'-uridyl]amino]oxy]methyl]-5-O-tert-butyl-3-deoxy-beta-D-ribofuranuronosyl]uracil
10	7.73	0.38	399.23	398.50	$C_{23}H_{30}N_2O_4$	Mitraciliatine/Mitragynine
11	8.22	2	387.18	386.41	$C_{22}H_{26}O_6$	Piperbetol
12	8.44	1.87	717.23	716.60	$C_{30}H_{40}N_2O_{18}$	N,N'-Bis(2-O,3-O,4-O,6-O-tetraacetyl-beta-D-glucopyranosyl)-1,2-ethanediimine
13	9.37	13.24	441.19	440.50	$C_{21}H_{24}N_6O_5$	Caffeine N-Acetyl-L-Tryptophan
14	10.79	7.14	483.20	482.48	$C_{22}H_{30}N_2O_{10}$	adipic acid; 1,3-diisocyanate-2-methyl-benzene; ethanol; 2-hydroxyethyl prop-2-enoate
15	11.65	0.9	493.17	438.4	$C_{20}H_{26}N_2O_9$	TC 2559 difumarate
16	12.02	0.58	277.22	276.4	$C_{18}H_{28}O_2$	Stearidonic acid
17	15.56	31.58	758.56	774.05	$C_{42}H_{67}N_{11}O_3$	L-Arginyl-2,5,7-tris(2-methyl-2-propanyl)-L-tryptophyl-N-benzyl-L-argininamide
18	18.44	13.34	577.52	576.90	$C_{37}H_{68}O_4$	phenolic phthiocerol

Antioxidant Activity of RBE

DPPH assay revealed that RBE demonstrated stronger antioxidant activity at higher concentrations, with an IC_{50} value of 284.880 µg/mL (Figure 2). RBE exhibited the highest antioxidant activity at 200 ppm, with the percentage of DPPH inhibition of 52.55 ± 0.51%.

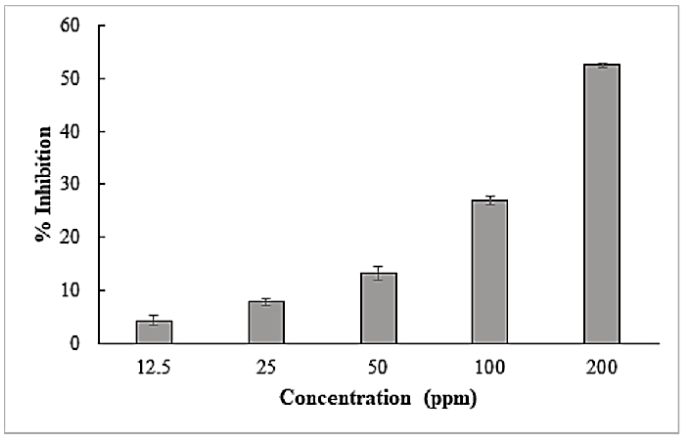

Figure 2: The antioxidant activity level of red betel extract (*P. crocatum*) extract

Another study revealed that *P. cubeba* essential oil has antioxidant activity against DPPH radicals. This study determined an IC_{50} value of 110.00 ± 0.08 µg/mL, indicating a moderate level of antioxidant activity.[21] *P. ecuadorense* has an IC_{50} value of more than 2.5 mg/mL.[22] Nguyen et al.[2] showed that the ethanol extract of *P. betel* leaves has an IC_{50} value of 20.57±0.48 µg/mL. The strong antioxidant capacity of RBE is largely due to its high levels of phenolic compounds and flavonoids, which play an essential role in neutralizing free radicals and reactive oxygen species. This helps reduce oxidative stress, directly supporting the prevention or slowing of chronic disease processes closely related to oxidative stress.[23]

The DPPH radical, which contains unpaired electrons, absorbs at 517 nm, resulting in a deep purple color in solution. When antioxidants neutralize the radicals by donating electrons or hydrogen atoms, the color changes to pale yellow. The extent of absorbance reduction is directly related to the antioxidant strength of the sample.[24] Compounds known as antioxidants help shield cells from the harm that free radicals can do by stopping molecules from oxidizing. Free radicals are unstable, which means highly reactive molecules with unpaired electrons, consequently, they have a short lifespan. As a result, they seek stability by bonding with other molecules.[25] The radical-eradication activity is often correlated with the presence of phenolic compounds containing several hydroxyl groups in their molecular structure. Eugenol, hydroxycavicol, and gallic acid, especially hydroxykavikol, contribute strongly to antioxygen activity with two hydroxyl groups.[3] The findings underscore the importance of red betel nut in developing new therapeutic strategies to address chronic diseases, promoting a more natural and plant-based approach in healthcare.

Total Phenolic and Flavonoid Content

The study showed that the standard gallic acid curve follows a linear regression equation of y = 0.0429x + 0.0273, with a regression coefficient of 0.9999. From this equation, the total phenol content of RBE was calculated, and the sample absorbance can be plotted in a standard curve. Based on Table 2, the average total phenolic content of red betel was 496.10 ± 1.08 µg GAE/g.

Table 2: Total phenolic and flavonoid levels of red betel (*P. crocatum*) extract

Sample	Extract weight (g)	Average of total phenolic content (µg GAE/g)	Average of total flavonoid content (µg QE/g)
Red Betel Extract	0.01	496.10 ± 1.08	644.44 ± 48.11

Note: GAE: Galic Acid Equivalent; QE: Quercetin Equivalent

The total phenol content was determined using the Folin-Ciocalteu method, which relies on the ability of Folin-Ciocalteu reagents to oxidize the hydroxyl groups (OH-) of phenolic compounds. Phenolic compounds reduce phosphotungstic and phosphomolybdic acids in Folin-Ciocalteu to form a molybdenum-tungsten complex. Gallic acid serves as the standard for measurement due to its classification as a simple phenolic acid and its stability.[26] Folin-Ciocalteu reagent is employed because phenolic compounds interact with it, producing a colored solution or blue molybdenum-tungsten complex compound, which is measured at 765 nm.[27] This reaction involves oxidizing phenolic groups (ROH) with a mixture of phosphotungstic acid in reagents to a quinoid form (R=O). Phenolic compounds react with Folin-Ciocalteu reagents only in an alkaline atmosphere to dissociate protons in phenolic compounds into phenolic ions. To create alkaline conditions, 7% Na_2CO_3 is used.[28,29] Phenolic compounds with a large hydroxyl functional group (OH) or in their free form (aglycone) are capable of producing a high total phenol content.[28,30] Phenolics are natural compounds that accumulate in plant tissues and are essential in bioactivity.[31] Based on previous research *P. betle* had phenolic levels of 137.6 ± 2.54 mg GAE/g. While *P. sarmentosum* has phenolic levels of 53.19 ± 2.16 54 mg GAE/g, extracted with 70% ethanol.[32] The phenolic content value of *P. nigrum* and *P. trichostachyon* reaches 6.26 ± 0.31 mg and 7.28 ± 0.36 mg TAE/g, respectively.[33]

The quercetin curve has a linear regression equation y = 0.00003x + 0.0536, with its linear regression coefficient of 0.9906. Based on Table 2, the average total phenolic content was 496.10 ± 1.08 µg GAE/g. The total phenol obtained from RBE, which was extracted with 70% ethanol, affects the results. As the concentration of ethanol increases, the total phenol or flavonoid content also increases, since ethanol, containing a hydroxyl group (OH), can form hydrogen bonds with the hydroxyl groups of phenolic compounds. The presence of intramolecular hydrogen bonding can enhance the solubility of phenolic compounds in ethanol.[34] Other studies also revealed that *P. betle* ethanol extract has total flavonoid levels of 108.94 ± 4.47 mgCE/g.[10] While *P. nigrum* and *P. trichostachyon* leaves have total flavonoid levels of 5.61 ± 0.28 and 5.14 ± 0.26 mg TAE/g FW, respectively.[33]

The $AlCl_3$ technique determines total flavonoid concentration by forming a complex between aluminum chloride and quercetin.[35] The flavonoid quantification on RBE is important to evaluate the content and activity of its bioactive compounds. Flavonoids, known for their powerful antioxidant activities, serve an important role in maintaining health and avoiding disease.[36] This is confirmed by Alfarabi et al.[37] research, which reveals that *P. crocatum* reduces the oxidation process of fatty acids and captures free radicals. Lister et al.[38] studies consistently show that flavonoids, with their antioxidant potential, play an essential role in disease prevention and health maintenance. The current investigation indicated a significant flavonoid concentration in the extract, indicating its potential as an alternative source of natural antioxidants. These findings confirmed the value of RBE in pharmacognosy research and its potential in the development of plant-based health products, focusing on exploiting its antioxidant and therapeutic properties.

Conclusion

This study successfully identified 18 phytochemical constituents in RBE, with kaempferitrin being the most abundant compound. The higher concentration of RBE exhibited high antioxidant activity, with the percentage of DPPH inhibition of 52.55 ± 0.51%. The flavonoids and phenolics content of RBE was 644.44 ± 48.11 µg QE/g and 496.10

± 1.08 µg GAE/g, respectively. Further research should focus on investigating the potential antibacterial and anti-inflammatory properties of RBE, as well as elucidating the underlying mechanisms of action and evaluating its efficacy *in vivo*.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors thank Universitas Negeri Malang for funding this research through the Internal grants of Malang State University research fund (contract number 5.4.623/UN32.20.1/LT/2023).

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