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# *Piper crocatum* Leaves Extracted with Different Ethanol Concentrations: Yield, DPPH Antioxidant Activity, and ATR-FTIR Metabolomic Profiling

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***ABSTRACT***

***Introduction:*** *Although Piper crocatum shows antioxidant activity, studies integrating ATR-FTIR metabolomic fingerprinting and chemometric modeling to link spectra–activity across solvent polarities remain limited.* ***Aims:*** *This study evaluated extraction yield, DPPH-based antioxidant activity, and ATR-FTIR metabolic fingerprints of P. crocatum extracts obtained using solvents with different polarities and established a PLS-R spectrum–activity relationship using chemometrics.* ***Method:*** *Leaves were extracted using ultrasound-assisted extraction with ethanol at three concentrations (100%, 75%, and 50%). Extracts were concentrated to constant weight and evaluated for antioxidant capacity using DPPH method (mg AEAC/g extract) and ATR-FTIR metabolic profiles. Statistical analysis was performed using one-way ANOVA ($p < 0.05$) followed by Tukey's post hoc test, along with PCA and PLS-R.* ***Result:*** *The antioxidant activities of the asignificant difference was observed among the extracts ($p < 0.05$). The 75% ethanol extract produced the highest yield (23.51 %), whereas the 100% ethanol extract showed the strongest antioxidant activities capacity, followed by 75% and 50% ethanol, with values of 21.30, 15.22, and 13.96 mg AEAC/g extract, respectively. FTIR spectra showed similar band positions with different intensities in the O–H, C–H, C=O, C=C, and C–O regions. PCA clearly discriminated the extract groups, while PLS-R identified O–H, C=C, and C–O bands as major positive contributors to antioxidant activity.* ***Conclusion:*** *Solvent polarity strongly affects the yield and antioxidant activity of P. crocatum extracts. ATR-FTIR fingerprinting combined with chemometric analysis provides a rapid approach to discriminate extracts and identify functional groups that contribute to antioxidant capacity.*

***KEYWORDS***: Antioxidant, *piper crocatum*, metabolomic, FTIR, DPPH

## INTRODUCTION

An imbalance between pro-oxidants and antioxidants in the body can lead to oxidative stress, a condition that damages essential biological macromolecules such as proteins, lipids, and DNA, and contributes to the development of degenerative diseases, including cancer, diabetes, and

atherosclerosis (Al-Abd et al., 2015; Raunsai et al., 2022). This imbalance may result from multiple factors, including psychological stress, radiation exposure, and alcohol consumption (Raunsai, Fathoni, & Agusta, 2022). Therefore, the exploration and development of plant-derived natural antioxidants have become increasingly important as safer alternatives to synthetic antioxidants (Baharfar, Azimi, & Mohseni, 2015).

Red betel (*Piper crocatum*) has been reported as a medicinal plant rich in phenolic and flavonoid compounds with potential antioxidant properties (Damanik et al., 2024; Irawan et al., 2024). Several studies have demonstrated that ethanol extracts of *P. crocatum* leaves exhibit significant free radical scavenging activity, which correlates with their total phenolic and flavonoid contents (Astyka et al., 2024; Nursamsiar et al., 2023). Moreover, variations in ethanol concentration during the extraction process have been reported to influence the resulting antioxidant capacity (Yasa et al., 2019), indicating that solvent polarity plays a crucial role in obtaining optimal bioactive compounds.

Although the antioxidant activity of *P. crocatum* has been widely reported, studies integrating ATR-FTIR spectral fingerprinting with chemometric analysis to establish a spectrum–activity relationship across extracts obtained using different solvent polarities remain limited. Ethanol was selected as the extraction solvent owing to its classification as generally recognized as safe (GRAS) status, particularly in food and pharmaceutical applications (Rahman et al. 2018). Compared to other organic solvents such as methanol and acetone, ethanol is considered safer for human consumption and handling, making it ideal for potential food or medicinal uses of *P. crocatum* extracts determine antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, and establish a spectrum-activity relationship to identify the FTIR bands/functional groups most strongly associated with antioxidant potency. This study aimed to investigate the effect of ethanol concentration (50%, 75%, and 100%) on extraction yield, DPPH-based antioxidant activity, and ATR-FTIR metabolomic fingerprints of *P. crocatum* leaves, and subsequently to establish a spectrum–activity relationship using a chemometric approach.

## METHODS

### Materials

Pro analysis (PA)-grade ethanol was purchased from SmartLab. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Tokyo Chemical Industry (TCI, Tokyo,

Japan), while ascorbic acid was purchased from Sigma-Aldrich (St. Louis, USA). A 0.45 µm syringe filter was purchased from Microlab Scientific (Zhejiang, China). The instruments used included standard laboratory glassware and equipment, a Powersonic 610 ultrasonicator (Hwashin Technology, Korea), an FDH-10A dehydrator (Wirastar, Surabaya, Indonesia), and an ATR-FTIR Alpha II spectrometer (Bruker, Massachusetts, USA). UV-Vis spectrophotometric measurements were carried out using a Shimadzu UV-Vis spectrophotometer (Shimadzu Corporation, Japan) equipped with disposable polystyrene (PS) cuvettes with a 1 cm (10 mm) optical path length.

**Sample Preparation and Extraction**

Leaves of red betel (*Piper crocatum*) were collected from the Kelayan area, Banjarmasin, South Kalimantan, Indonesia (−3.343245, 114.589951). Botanical authentication was performed at the Biology Education Laboratory, Faculty of Teacher Training and Education, Universitas Lambung Mangkurat, under certificate No. 088/UN8.1.2.3.2/PG/Lab.PMIPA/Bio/2025, to confirm the plant identity as *P. crocatum*. The leaves were rinsed with water, cut into small pieces, and dried in a drying cabinet at approximately 50°C for 24 h. The dried material was then ground into powder and sieved through an 80-mesh screen. The powdered simplicia was extracted at a 1:10 (w/v) material-to-solvent ratio using ultrasound-assisted extraction (UAE) for 90 min, employing ethanol with three polarity levels (i.e., 100%, 75%, and 50% ethanol). The extraction procedure was conducted with technical replication (n = 5). The resulting extracts were filtered through a 0.45 µm syringe filter, and the filtrates were concentrated using a dehydrator at 50 °C until a viscous extract with constant weight was obtained. The extracts were subsequently evaluated for antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and chemically characterized by ATR-FTIR (attenuated total reflectance–fourier transform infrared) spectroscopy.

**Antioxidant Activities**

Antioxidant activity was assessed using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay measured with a UV–Vis spectrophotometer, following a previously published method with modifications (Choirunnisa et al., 2025). To prepare the DPPH solution, first ensure all procedures were carried out protected from light to minimize reagent (DPPH) degradation. Turn off all lights and use indirect lighting during preparation. DPPH solution was prepared with a concentration of 100 µg/mL using PA-grade ethanol. Ascorbic acid was prepared as a positive control in a concentration series of 0.5–5 µg/mL. For

Table 1. The DPPH assay conditions

| Parameter | Condition |
|---|---|
| Concentration | 75 µg/mL |
| Sample concentration range | 100 µg/mL |
| Incubation time | 30 minutes |
| Incubation temperature | 20°C-25°C (Room Temperature) |
| Wavelength | 517 nm |
| Ascorbic acid concentration range | 0.5-5 µg/mL |

the assay, 400 µL of each standard or sample solution was mixed with 1200 µL of DPPH solution and incubated for 30 minutes at room temperature (20°C–25°C). The DPPH concentration was 75 µg/mL at final concentration mixture. The DPPH reagent is temperature-sensitive and should be stored under low-temperature conditions to maintain its stability. The absorbance was then recorded at 517 nm using a UV–Vis spectrophotometer. The same protocol was applied to *P. crocatum* extracts, where ethanol extracts obtained using different levels of polarity (100%, 75%, and 50% ethanol) were prepared at a concentration of 100 µg/mL in PA-grade ethanol. Measurements were performed with technical replicates (n = 5). The detailed conditions of the DPPH assay used in this study are summarized in Table 1. The percentage of DPPH radical scavenging activity was determined and subsequently converted using the regression equation derived from the ascorbic acid calibration curve. The results were expressed as milligrams of ascorbic acid equivalent antioxidant capacity per gram of extract (mg AEAC/g extract).

**ATR-FTIR Spectra Analysis**

The sample extract was positioned on the ATR crystal surface and pressed using the integrated pressure device to achieve optimal contact with the crystal. Prior to each measurement, the ATR crystal surface was cleaned with an alcohol swab and lint-free tissue. The infrared spectra were recorded over a wavenumber range of 4000–400 $cm^{-1}$. The FTIR spectra were pre-processed using OPUS software (version 6; Bruker, Massachusetts, USA) prior to multivariate analysis. Pre-processing steps included conversion to absorbance, baseline correction, normalization using the offset correction option, and smoothing with a 25-point setting. The 25-point smoothing was applied to reduce high-frequency instrumental noise while preserving the integrity and shape of characteristic absorption bands, thereby preventing distortion of chemically relevant spectral information. Offset correction was performed to shift the spectral baseline so that the minimum absorbance value was set to zero, minimizing baseline offsets and systematic intensity variations among spectra. This procedure ensured that

subsequent multivariate analyses (PCA and PLS-R) reflected genuine chemical differences among samples rather than instrumental artifacts. Absorbance values were then exported as a data point table (DPT) file and used as variables for downstream chemometric analysis.

**Data Analysis**

The extraction yield and antioxidant activity data were subjected to descriptive analysis and expressed as mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance (ANOVA) with a significance threshold of $p < 0.05$, implemented in GraphPad Prism (version 9). Multivariate analysis was performed using principal component analysis (PCA) to examine clustering patterns among extracts, while partial least squares regression (PLS-R) was employed to identify the dominant spectral bands/functional groups associated with antioxidant activity. All chemometric models were built using The Unscrambler X (version 10.1; Camo, Oslo, Norway). The FTIR spectra subjected to chemometric analysis were those that had been pre-processed using OPUS software (version 6; Bruker, Massachusetts, USA).

**RESULTS AND DISCUSSION**

**Results**

*Piper crocatum* leaves extracts

In this study, red betel (*Piper crocatum*) leaf extraction was performed using ultrasound-assisted extraction (UAE) with ethanol at 50%, 75%, and 100% (Table 2). Differences in ethanol concentration modulate the solvent polarity, and therefore ethanol at 50%, 75%, and 100% is expected to preferentially extract different classes of compounds according to their polarity-related characteristics (Do et al., 2014).

Table 2. Extraction yield of *P. crocatum*

| *P. crocatum* extract | Yield (%) |
|---|---|
| EtOH 100% | 15.70 ± 0,66[a] |
| EtOH 75% | 23.51 ± 2,67[b] |
| EtOH 50% | 16.30 ± 1,54[c] |

Note: Mean ± SD (n = 5, technical replicates). Different letters within the same column denote statistically significant differences among the means (Tukey's test, $p < 0.05$).

In this work, ethanol-based extraction was conducted for 90 min using ultrasound-assisted extraction (UAE). UAE employs ultrasonic waves that generate cavitation, which can disrupt plant cell walls and enhance mass transfer, thereby promoting the diffusion of target constituents into the solvent and accelerating the extraction process. Compared with conventional maceration, UAE typically requires less solvent and enables extract production within a shorter processing time (Zhang, Lin, & Ye, 2018).

**Antioxidant Activity**

The antioxidant activity of *P. crocatum* leaf extracts was determined using the DPPH assay. DPPH is a stable free radical at

Table 3. Antioxidant activities of *P. crocatum*

| *P. crocatum* extract | mg AEAC/g extract |
|---|---|
| EtOH 100% | 21.30 ± 0.52[a] |
| EtOH 75% | 15.22 ± 0.39[b] |
| EtOH 50% | 13.96 ± 0.39[c] |

**Note:** Mean ± SD (n = 5, technical replicates). Different letters within the same column denote statistically significant differences among the means (Tukey's test, $p < 0.05$). Ascorbic acid $IC_{50}$ value of 3.20 µg/mL.

room temperature and can be measured by UV–Vis spectrophotometry at 517 nm. Upon reaction with antioxidant molecules, DPPH is reduced, which is typically indicated by a color change from deep purple to pale yellow accompanied by a decrease in absorbance, a greater absorbance reduction reflects stronger antioxidant capacity (Choirunnisa et al., 2025). The DPPH method is widely applied due to its simplicity, rapid execution, high sensitivity, low sample requirement, and the practical stability of the reagent. Nevertheless, the assay also has limitations because DPPH is light-sensitive, and light exposure may compromise the accuracy of the reduction measurement; it is also temperature-sensitive, requiring storage under low-temperature conditions (Artika & Siregar, 2024). The antioxidant assay results are summarized in Table 3.

The antioxidant capacity of *P. crocatum* leaf extracts differed markedly across ethanol polarity levels (Table 3). The extract prepared with 100% ethanol exhibited the highest antioxidant activity compared with 75% and 50% ethanol extracts, with values of 21.30 ± 0.52, 15.22 ± 0.39, and 13.96 ± 0.39 mg AEAC/g extract, respectively. The ascorbic acid standard curve showed excellent linearity ($y = 26.539x - 34.939$; $R^2 = 0.9943$), and the $IC_{50}$ value was 3.20 µg/mL.

**Metabolomic based on FTIR**

FTIR spectroscopy is an analytical technique that employs Fourier transformation to detect and characterize compounds from both natural sources and synthetic materials by generating an infrared spectrum as the primary output. The resulting spectrum provides distinctive features, including absorption band positions (wavenumbers), intensities, and overall peak patterns that reflect specific functional groups (Hikma, Burhan, Ulfah, & Awaluddin, 2023).

FTIR is also recognized as a high-resolution method that effectively assists in identifying chemical components and elucidating molecular structural features (Rahmawati, Maryam, & Hidayanti, 2023). In the context of non-targeted spectroscopy, FTIR serves as a valuable tool for producing rapid, cost-efficient, and high-throughput metabolic fingerprints, thereby supporting metabolomic characterization of sample constituents (Cebi, Bekiroglu, & Erarslan, 2023).

The FTIR results showed broadly similar spectral patterns across the extracts, but with differences in band intensities, most notably between the 100% and 75%

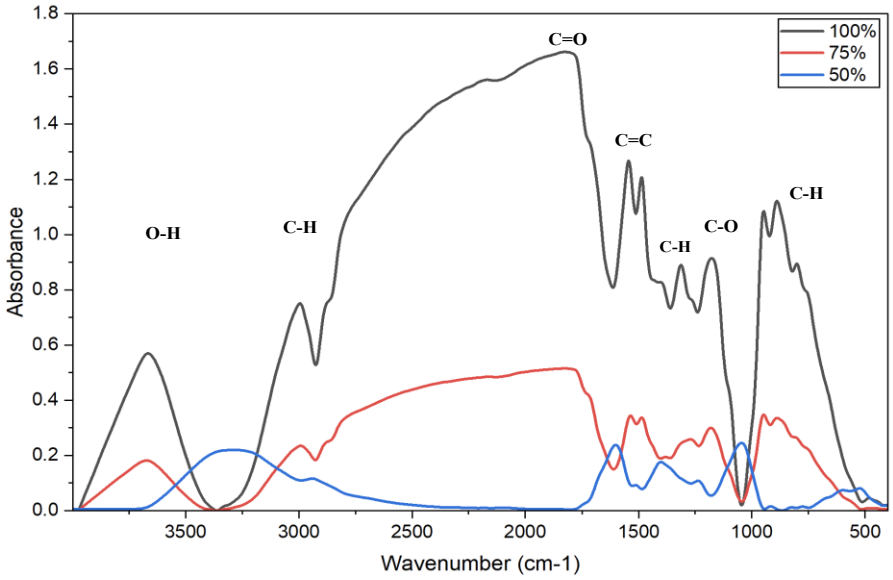



Figure 1. ATR-FTIR spectra of *P.crocatum* leaf extracts obtained using different ethanol concentrations. Black line=100% ethanol; Red line=75% ethanol; Blue line=50% ethanol.

Table 4. Functional group identification of *P. crocatum* (Shriner, Hermann, Morrill, Curtin, & Fuson, 2003)

| Wavenumber ($cm^{-1}$) | Functional group |
|---|---|
| 3200–3650 | O–H (Stretch) |
| 2990–3100 | C–H (Stretch) |
| 1680–1750 | C=O (Stretch) |
| 1400–1500 | C=C (Stretch) |
| 1620–1680 | C=C (Stretch) |
| 1365–1370 | C–H (Bend) |
| 1000–1260 | C–O (Stretch) |
| 650–1000 | C–H (Out Of Plane Bend) |

ethanol extracts, while the 50% ethanol extract exhibited a more pronounced deviation (Figure 1). The overall spectral similarity suggests a consistent absorption fingerprint among extracts, indicating comparable functional-group profiles of the detected secondary metabolites. Nevertheless, differences in peak heights (intensities) imply variations in the relative abundance of bioactive constituents, which may contribute to the observed differences in antioxidant activity among samples (Rohaeti, Karunina, & Rafi, 2021). The functional group assignments are summarized in Table 4.

The $IC_{50}$ of ascorbic acid (3.20 µg/mL) indicates very strong antioxidant activity and should be directly compared with the extracts to provide context. Since the extracts have higher $IC_{50}$ values, their antioxidant potency is lower than the pure standard. Extracts with $IC_{50}$ values closer to 3.20 µg/mL will have higher AEAC values and stronger relative activity. Therefore, comparing extract $IC_{50}$ values to ascorbic acid helps clarify their relative antioxidant strength.

Principal component analysis (PCA) revealed a clear separation among sample groups extracted with 50%, 75%, and 100% ethanol, with the total explained variance reaching 100%. The first principal component (PC-1) accounted for most of the total variation (99%), indicating that the

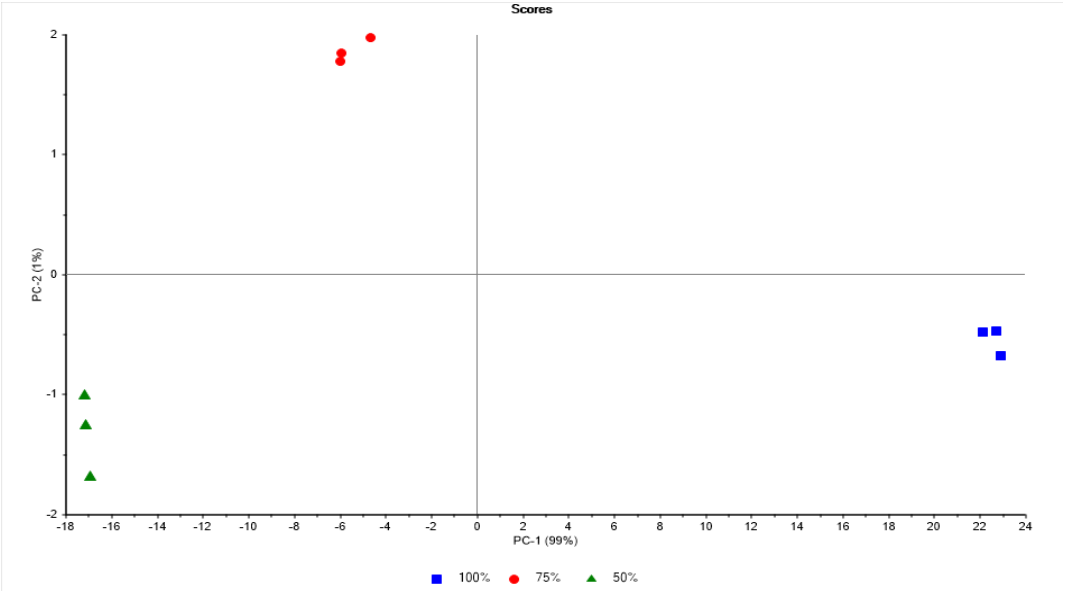



Figure 2. PCA score plot based on FTIR spectra of *P.crocatum* leaves extracts with various solvents polarity

model captured nearly all discriminative information associated with the treatment conditions. Samples were arranged in an approximately linear trajectory along the PC-1 axis as ethanol concentration increased, demonstrating the sensitivity of the approach to the treatment gradient (Greenacre et al., 2022). The corresponding loading plot revealed that wavenumbers 3200-3650 $cm^{-1}$ (O-H stretching) and 1400-1680 $cm^{-1}$ (C=C stretching) exhibited the highest positive loadings on PC1 (>0.85), indicating these phenolic and neolignan-associated spectral regions primarily drive the discrimination of 100% ethanol extracts from more aqueous counterparts along the solvent polarity gradient (Rohaeti et al., 2021). Overall, the PCA score plot confirms pronounced differences among the three ethanol concentrations (Matwijczuk et al., 2019; Rohman et al., 2020), and the distinct clustering pattern indicates that each group possesses a different metabolite fingerprint (Figure 2).

Partial least squares regression (PLS-R) was employed to determine the key FTIR spectral variables associated with DPPH-based antioxidant activity (Figure 3). In this model, bands with positive (upward) regression coefficients indicate wavenumbers positively related to higher antioxidant capacity, whereas negative (downward) coefficients suggest an inverse relationship, given that antioxidant activity was expressed as AEAC. The optimal number of latent variables was set to LV = 5 based on the lowest RMSEP, and the X-regression coefficients from Factor-4 were subsequently used to pinpoint discriminant FTIR wavenumbers. According to the weighted regression coefficient profile, the spectral regions predicted to contribute positively to antioxidant activity were

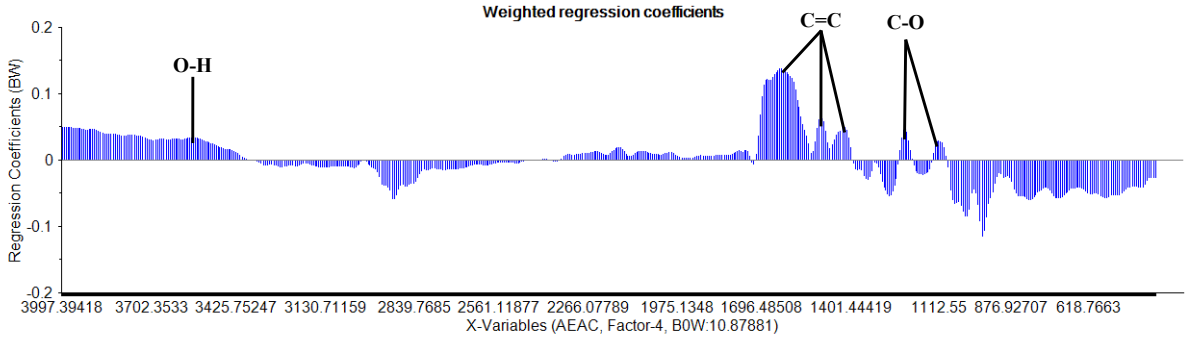



Figure 3. PLS-R identification of key variables linking FT-IR spectra to DPPH antioxidant activity

mainly located at 3200–3650 $cm^{-1}$ (O–H), 1400–1680 $cm^{-1}$ (C=C), and 1000–1260 $cm^{-1}$ (C–O). The presence of O–H functional groups is typically related to phenolic and flavonoid compounds, which are known for their antioxidant properties due to their capacity to donate hydrogen atoms and scavenge free radicals (Maslikah et al., 2025).

## Discussion

Differences in ethanol polarity significantly influenced the extraction yield of *P. crocatum* leaves (Table 2). In the present study, 75% ethanol produced the highest yield compared to 100% and 50% ethanol. This finding is in agreement with previous studies showing that intermediate ethanol concentrations generally produce higher extract yields than very dilute or nearly absolute ethanol (Do et al., 2014). Other studies have also shown that 70% ethanol is effective in extracting most components from pepino melon fruit, leading to high extraction yields. With its optimal water content of around 30%, 70% ethanol successfully extracts secondary metabolites (Firsty, Sugihartini, & Mulyaningsih, 2023). Mechanistically, 75% ethanol is effective because its intermediate polarity allows it to dissolve a wide range of secondary metabolites, while the water content enhances plant cell wall swelling, improves solvent penetration, and facilitates mass transfer. These combined effects explain why 75% ethanol produced the highest extract yield from *P. crocatum* leaves. Our findings reinforce the idea that 75% ethanol is an effective solvent for maximizing extract yield from *P. crocatum* leaves.

The extraction yields obtained using different ethanol concentrations decreased in the following order: 75% ethanol (23.51%) > 50% ethanol (16.30%) > 100% ethanol (15.70%). It can be observed that the 75% ethanol extract produced a markedly higher yield than both 50% and 100% ethanol extracts. This observation indicates that solvent polarity is an important factor influencing extraction efficiency, and that an intermediate ethanol concentration provides optimal conditions

for maximizing extract recovery. The significantly higher yield obtained with 75% ethanol suggests that the presence of water enhances extraction performance. Compared to absolute ethanol, aqueous ethanol improves plant matrix swelling and solvent penetration, thereby facilitating the release of intracellular constituents. Furthermore, higher water content in the solvent system may enhance the co-extraction of non-phenolic compounds, other than phenolics, such as carbohydrates, proteins, and other polar metabolites, which contribute to the overall mass of the extract (Zielinski & Kozłowska, 2000).

In contrast, absolute ethanol (100%) lacks sufficient polarity to efficiently solubilize highly polar compounds, resulting in a lower yield. Similarly, 50% ethanol, although more polar, may not effectively dissolve certain semi-polar secondary metabolites compared to 75% ethanol. These findings suggest that the combination of water and ethanol improves extraction yield by facilitating the solubilization of a wider range of compounds that are soluble in either water or organic solvents. This observation is consistent with previous reports showing that aqueous organic solvents often provide higher extraction yields than pure solvents in various plant matrices (Chatha et al., 2006).

The antioxidant capacity values were calculated using the linear regression equation. Statistical analysis indicated that these three values of antioxidant activity were significantly different, suggesting that solvent polarity plays a critical role in extracting antioxidant constituents from *P. crocatum* leaves. This is likely because 100% ethanol, being less polar, preferentially extracts non-polar flavonoid aglycones and lipophilic phenolics key antioxidants compared to more aqueous 75% and 50% ethanol solvents that co-extract diluting polar glycosides and carbohydrates. Higher ethanol concentrations may enhance the antioxidant activity of medicinal plant extracts determined by more effectively solubilizing relatively less polar phenolic compounds, thereby enriching antioxidant-active fractions (Januarti, Wijayanti, Wahyuningsih, & Nisa, 2019). In the case of *P. crocatum*, its phenolic profile has been reported to include flavonoids, tannins, and other polyphenolic constituents with varying polarity. Several studies have shown that these compounds particularly flavonoid aglycones and certain condensed tannins exhibit stronger antioxidant activity and are more efficiently extracted using hydroethanolic solvents with higher ethanol proportions. Therefore, the increased antioxidant activity observed at higher ethanol concentrations may be

attributed to the improved extraction of these less-polar phenolic constituents present in *P. crocatum* leaves.

Our findings are consistent with earlier studies reporting the DPPH radical scavenging activity of *P. crocatum*. Previous study reported 59.34% DPPH inhibition at 100 µg/mL using a 70% ethanol extract (Alfarabi, Bintang, Suryani, & Safithri, 2010), and 65.65% inhibition at 80 µg/mL with a 96% ethanol extract (Artika & Siregar, 2024). Another study indicated that the most favorable $IC_{50}$ was obtained from a 90% ethanol extract of red betel leaves (81.61 µg/mL), and that antioxidant potency weakened as solvent polarity increased, with the least potent extract produced using 50% ethanol ($IC_{50}$ = 143.33 µg/mL) (Yasa, Putra, & Wiadnyani, 2019). Collectively, these reports support our results, which demonstrate that the 100% ethanol extract exhibits stronger antioxidant activity than the 75% and 50% ethanol extracts. Furthermore, the $IC_{50}$ value of ascorbic acid (3.20 µg/mL) was substantially lower than those of all extracts, indicating markedly stronger antioxidant activity. This comparison provided important context for the AEAC values, as it showed that although the extracts possessed considerable radical scavenging capacity, their potency remained significantly lower than that of the standard antioxidant.

Functional-group interpretation of the FTIR spectra (Figure 1) revealed a broad O–H stretching band in the 3200–3650 $cm^{-1}$ region across all samples. This O–H signal is a key indicator of phenolic and flavonoid constituents, which are commonly associated with antioxidant activity (de Oliveira, Santos-Buelga, Aquino, Barros, & Heleno, 2025). Bands observed at 2840–3000 $cm^{-1}$ correspond to C–H stretching, suggesting the presence of aliphatic carbon chains. The presence of carbonyl (C=O) groups was supported by a sharp absorption in the 1680–1700 $cm^{-1}$ range, a feature frequently attributed to flavonoids and/or organic acids. Signals at 1365–1370 $cm^{-1}$ are consistent with C–H bending, followed by bands in the 1000–1260 $cm^{-1}$ region assigned to C–O stretching, which collectively suggests the occurrence of alcohol and/or ether linkages in the extract constituents. In the lower wavenumber region, absorptions at 650–1000 $cm^{-1}$ are attributable to out-of-plane C–H bending vibrations. Overall, FTIR absorption features are governed by molecular vibrational patterns of constituent functional groups; for phenolic and flavonoid compounds, characteristic spectral fingerprints typically arise from O–H, C–O, and C=O functionalities (Kusumadewi et al., 2022; Nurani et al., 2024; Umar, Syahruni, Ranteta'dung, & Rafi, 2023). Differences in peak intensities among the black (100% ethanol), red (75% ethanol), and blue (50% ethanol) spectra indicate variations in the relative abundance of detectable constituents,

with more concentrated ethanol extracts generally exhibiting higher absorbance at key bands.

In the context of *P. crocatum*, the O-H stretching band (3200-3650 $cm^{-1}$) corresponds to phenolic compounds such as piperol A and B, the C=C stretching region (1400-1680 $cm^{-1}$) is consistent with aromatic neolignan derivatives like pipcroside A-C, and the C-O stretching (1000-1260 $cm^{-1}$) indicates flavonoid glycosides, strengthening the metabolomic fingerprint of these antioxidant-rich extracts (Saputra, Andayani, & Nursyam, 2016).

Partial least squares regression (PLS-R) was employed to determine the key FTIR spectral variables associated with DPPH-based antioxidant activity (Figure 3). In this model, bands with positive (upward) regression coefficients indicate wavenumbers positively related to higher antioxidant capacity, whereas negative (downward) coefficients suggest an inverse relationship, given that antioxidant activity was expressed as AEAC. The optimal number of latent variables was set to LV = 5 based on the lowest RMSEP. The PLS-R model was validated using leave-one-out cross-validation implemented in The Unscrambler X. The calibration model showed excellent goodness of fit with $R^2$(cal) = 0.9925 and RMSEC = 0.2658, while cross-validation results showed strong predictive performance with $R^2$(CV) = 0.9199 and RMSECV = 0.9751. The regression slope was close to 1 with a minimal intercept, indicating good agreement between predicted and measured antioxidant activity values and confirming the robustness and predictive capability of the model. The X-regression coefficients from Factor-4 were subsequently used to pinpoint discriminant FTIR wavenumbers. According to the weighted regression coefficient profile, the spectral regions predicted to contribute positively to antioxidant activity were mainly located at 3200–3650 $cm^{-1}$ (O–H), 1400–1680 $cm^{-1}$ (C=C), and 1000–1260 $cm^{-1}$ (C–O). The O–H functionality is commonly linked to phenolic and flavonoid compounds, which are well- recognized antioxidants due to their capacity to donate hydrogen atoms and scavenge free radicals (Maslikah et al., 2025).

Overall, the combined evidence from extraction yield data, antioxidant assays, comparative literature, and FTIR spectral analysis reinforces the conclusion that 75% ethanol is optimal for maximizing extract yield, while higher ethanol concentrations may enhance antioxidant potency by selectively extracting less polar, highly active phenolic constituents.

## CONCLUSION

Ultrasound-assisted extraction of *P. crocatum* leaves using different ethanol

concentrations significantly affects extraction yield and antioxidant capacity. The 75% ethanol extract produces the highest yield (23.51 ± 2.67%), while 100% ethanol shows the strongest antioxidant activity (21.30 ± 0.52 mg AEAC/g extract). ATR-FTIR fingerprints show similar band positions across extracts, with intensity differences indicating variations in bioactive compound abundance. PCA clearly separates the extracts according to ethanol concentration, while PLS-R identifies O–H (3200–3650 $cm^{-1}$), C=C (1400–1680 $cm^{-1}$), and C–O (1000–1260 $cm^{-1}$) regions as key contributors to antioxidant activity. These findings highlight that solvent polarity optimization is important for maximizing either extraction yield or antioxidant potency. Further research should aim to identify the specific compounds responsible for the observed activity activity using LC-MS–based metabolomics and validating antioxidant potential through additional assays such as FRAP and ABTS.

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