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Metabolite profiling and antioxidant activity of peel extract from *Citrus amblycarpa*

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Abstract. *Kusumawati IGAW, Yogeswara IBA, Wasito H. 2025. Metabolite profiling and antioxidant activity of peel extract from* Citrus amblycarpa. *Biodiversitas 26: 2601-2610. Citrus amblycarpa* has been traditionally utilized in Balinese herbal medicine for its therapeutic properties. This study takes a unique approach to comprehensively analyzing the metabolite profile and antioxidant activity of *C. amblycarpa* peel extract. We obtained the extract through sequential extraction using solvents with various polarities, such as n-hexane, ethyl acetate, methanol, and water. The extracted metabolites were identified using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS). We also quantified the total phenolic content (TPC) and total flavonoid content (TFC) using the Folin-Ciocalteu and aluminum chloride colorimetric assays, respectively. Antioxidant activity was assessed through DPPH, FRAP, and ABTS assays. The metabolite analysis identified phenolic compounds, flavonoids, limonoids, terpenoids, coumarins, alkaloids, and carboxylic acids, with hesperidin, tangeretin, and neohesperidin emerging as the predominant flavonoids contributing to antioxidant activity. The hexane fraction exhibited the highest TPC (49.19 mg GAE/g dry weight), while the ethyl acetate fraction had the highest TFC (15.44 mg QE/g dry weight). Antioxidant assays revealed significant variation among the extracts, with the n-hexane and ethyl acetate fractions exhibiting the strongest radical scavenging activities and reducing power activities. These findings highlight the potential of *C. amblycarpa* peel as a natural source of bioactive compounds with potent antioxidant properties, suggesting its possible application in nutraceutical and pharmaceutical formulations.

Keywords: Antioxidant activity, *Citrus amblycarpa*, citrus peel, metabolite profile, sequential extraction

INTRODUCTION

The increasing prevalence of oxidative stress-related diseases has heightened the demand for natural antioxidants, positioning plant-based extracts as promising alternatives due to their bioactive compounds and minimal side effects (Wijaya et al. 2017). The *Citrus* genus, a prominent member of the Rutaceae family, thrives in tropical and subtropical regions. While the fruit of *C. amblycarpa* is commonly used as a fragrant ingredient in chili sauce preparation, its peel is often discarded and remains underutilized despite being rich in bioactive compounds (Shirisha et al. 2019; Singh et al. 2020; Dewi 2022; Vrca et al. 2024). *Citrus* peel represents a readily available, cost-effective, and valuable source of bioactive constituents with significant pharmaceutical potential (Al-Ashaal and El-Sheltawy 2011; Anticona et al. 2020; de Oliveira et al. 2022). *C. amblycarpa* has traditionally been used by the Balinese as an herbal remedy (Kusumawati et al. 2021). The lontar *Usada Taru Pramana* describes its leaves and roots as effective treatments for rheumatism (Adnyana 2021). The methanol extract of *C. amblycarpa* leaves contains bioactive compounds such as α-tocopherol, phytosterols, terpenes, butyric acid, linoleic acid, and palmitic acid, while the methanol extract of its fruit includes palmitate, sabinene, citronellal, α-limonene, and methyl oleate (Budiarto et al. 2017). Additionally, water-based extraction of *C. amblycarpa* peel yields phenolic compounds, quercetin, rutin, and gamma-aminobutyric acid (Kusumawati et al. 2021). The bioactive components present in *C. amblycarpa* exhibit various biological activities, including antihypertensive (Kusumawati et al. 2021), antibacterial (Junaedi 2022), anti-obesity (Panghiyangani et al. 2023), antioxidant, anti-aging (Stevenie et al. 2019), antidiabetic (Tambunan et al. 2020), and anti-inflammatory effects (Felim et al. 2021).

The aqueous extract of *C. amblycarpa* peel contains higher total phenolic and flavonoid content than its leaf extract (Kusumawati et al. 2021). Meanwhile, ethanol-based extraction of *C. amblycarpa* peel yields phenolics, flavonoids, steroids, and alkaloids (Tambunan et al. 2020). The elevated levels of phenolic and flavonoid compounds correlate positively with antioxidant activity. Given the presence of these compounds in *C. amblycarpa* peel extract, it has the potential to serve as a natural antioxidant by inhibiting free radicals in the body (Nawaz et al. 2020).

The extraction process and solvent selection significantly influence the resulting metabolite profile, which, in turn, affects the bioactivity of the plant extract (Xu et al. 2023; Anggraeny et al. 2024; Navarrete-Carriola et al. 2024). The maceration technique was chosen for its ease of application and its classification as a cold extraction method, which preserves compound integrity by minimizing the risk of degradation (Fadhlillah et al. 2024). To the best of our

knowledge, the metabolite profile of *C. amblycarpa* using sequential extraction has not yet been determined. In this study, we performed sequential extractions using solvents with varying polarity, a method that provides a more comprehensive metabolite profile and enhances extract yield, offering valuable insights into the bioactive potential of plant materials (Nawaz et al. 2020; Wado et al. 2022). Analysis of metabolite profiles in plant extracts can be performed using chromatographic techniques, including High-Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectrometry (GC-MS), and Liquid Chromatography-Mass Spectrometry (LC-MS). Additionally, spectroscopic methods such as UV/VIS, mass spectrometry, infrared spectroscopy, and Nuclear Magnetic Resonance (NMR) (Seger and Sturm 2022). While NMR provides excellent structural elucidation, Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) is currently regarded as the gold standard in non-targeted metabolomics due to its high sensitivity and minimal sample preparation requirements (Wasito et al. 2022). Therefore, we selected LC-HRMS for metabolite profiling in this study.

By addressing the knowledge gap regarding the underutilized peel, our findings could contribute to its valorization as a natural antioxidant source. This study aimed to identify the metabolite profile of *C. amblycarpa* peel extracts obtained through sequential extraction using solvents of different polarities and evaluate their antioxidant activity.

MATERIALS AND METHODS

Chemicals and materials

The solvents and chemicals used for analysis in this research include n-hexane, ethyl acetate, methanol, 96% ethanol, Folin-Ciocalteau reagent, sodium carbonate, aluminum chloride, sodium acetate (Merck, Germany), gallic standard, quercetin standard, ascorbic acid standard, DPPH, TPTZ, Fe^{2+}, ABTS, and potassium persulfate (Sigma Aldrich Company Ltd., England).

Plant collection

Fruits of *C. amblycarpa* were collected from Kalpataru Garden in Denpasar, Bali, Indonesia (8°40'17.8" S 115°15'09.9" E) (Figure 1). The plant was identified and certified by Herbarium Bogoriense, a division of the National Research and Innovation Agency of Indonesia. The specimen was confirmed to be *C. amblycarpa* and assigned identification number B-3020/II.6.2/IR.01.02/8/2024, classifying it within the Rutaceae family.

Citrus amblycarpa peel powder preparation

Fresh fruits were thoroughly washed under running water, manually peeled, and cut into uniform portions of 1×0.5 cm using stainless steel scissors (Gómez-Mejía et al. 2023). The peels were then dried in an oven at 50°C for 48 hours on aluminum foil. Once dried, they were ground using a grinder to obtain a fine powder. The powdered *C. amblycarpa* peels were subsequently subjected to extraction.

Plant extract preparation

The powdered *C. amblycarpa* peels were macerated in four solvents: n-hexane, ethyl acetate, methanol, and distilled water, using a 1:10 weight-to-volume ratio. This process followed the sequential cold maceration method detailed by Pagi and Patel (2017), as illustrated in the flowchart in Figure 2. The four resulting extracts (aqueous extract fraction (KLA), ethyl acetate extract fraction (KLE), hexane extract fraction (KLH), and methanol extract fraction (KLM)) were then analyzed to determine metabolite profiles and assess antioxidant activity.

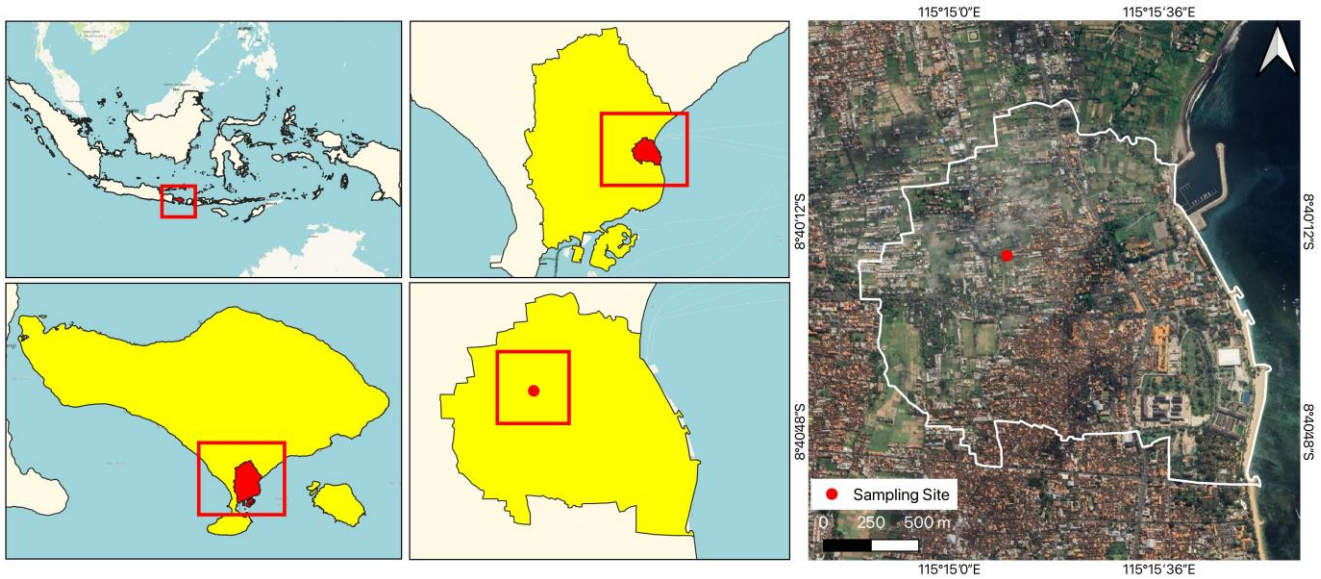

Figure 1. Sampling location in Kalpataru Garden, Denpasar, Bali, Indonesia

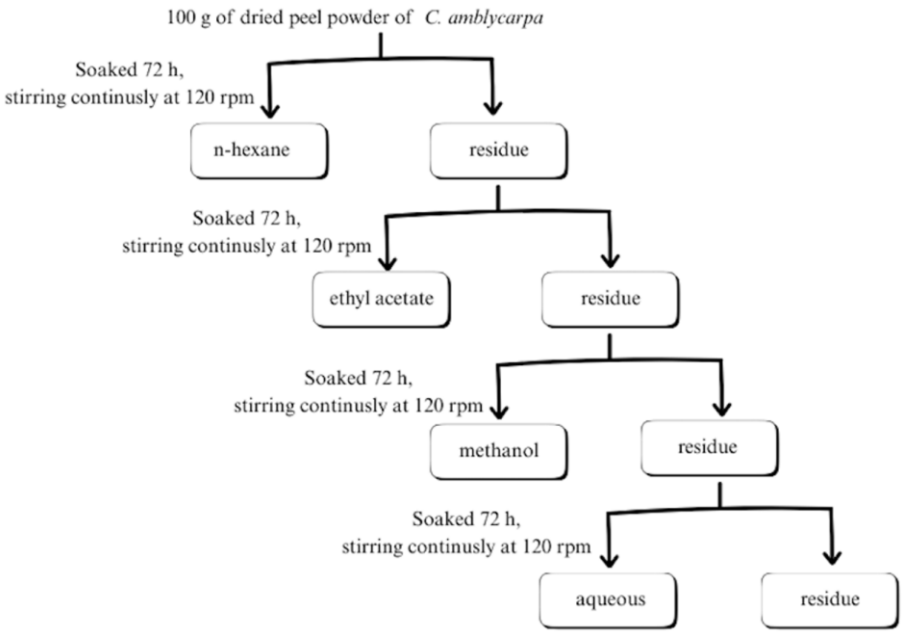

Figure 2. Sequential maceration method for the preparation of peel extracts

Metabolite analysis using LC-HRMS

Each 500 mg sample (KLM, KLE, KLH, and KLA) was diluted in 1 mL of methanol, vortexed for 2 minutes, and sonicated at 20°C for 15 minutes. Following centrifugation at 3842x g for 5 minutes, the samples were filtered through a 0.2 µm nylon filter and prepared for injection.

Non-targeted metabolite profiling was conducted using a modified version of the methodology described by Wasito et al. (2022). The analysis was performed on a Thermo Scientific Vanquish Horizon UHPLC system with a binary pump (Germering, Germany) coupled to a Thermo Scientific Orbitrap Exploris 240 HRMS (Bremen, Germany). Thermo Scientific Accucore Phenyl-Hexyl column (100x2.1 mm ID, 2.6 µm particle size, Lithuania) kept at 40°C helped to achieve chromatographic separation. The mobile phase comprised (A) 10 mmol/L ammonium formate (pH 3.0) and (B) acetonitrile containing 0.1% (v/v) formic acid. Gradient elution was performed at a flow rate of 0.3 mL/min as follows: the initial condition of 5% B was gradually increased to 90% over 16 minutes, maintained at 90% for 4 minutes, and then returned to 5% B, completing a total run time of 25 minutes. Sample injection was performed using an HTC PAL LC autosampler with a 5 µL fixed loop. The HRMS acquisition mode was full MS/dd-MS^2, with polarity switching between positive and negative. The full MS resolution was set to 60,000 FWHM, with a scan range of 70-1,000 m/z and a maximum injection time of 100 ms. The intensity threshold was 5,000, and the dd-MS^2 resolution was 22,500 FWHM. Collision energies were set at 30, 50, and 70, with nitrogen used as the collision gas. The ion source utilized was an Optamax NG Heated Electrospray Ionization (H-ESI), with spray voltages of 3,500 V (positive) and 2,500 V (negative). The temperature of the ion transfer tube was 300°C, and the vaporizer was 320°C. Compound Discoverer 3.3 (Thermo Scientific, San Jose, USA) was used to analyze the data.

Chemometric analysis for metabolomic profiling

Chemometric analysis was conducted using MetaboAnalyst 6.0, incorporating peak area data and identified metabolites from the LC-HRMS analysis. Multivariate analysis methods included Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) for sample grouping, as well as Hierarchical Cluster Analysis (HCA) to assess similarities and differences in chromatogram profiles among the samples.

Determination of total phenolic content (TPC)

The Total Phenolic Content (TPC) was determined using a spectrophotometric method with Folin-Ciocalteu reagent (Tang et al. 2020), with modifications. In this procedure, 25 µL of the extract was mixed with 25 µL of Folin-Ciocalteu reagent solution (diluted 1:3 with water), followed by the addition of 200 µL of water in a 96-well plate. The mixture was incubated at room temperature for 5 minutes. Subsequently, 25 µL of 10% (w/w) sodium carbonate was added to basify the reaction mixture, which was then incubated in the dark for 60 minutes. A spectrophotometer plate reader was used to detect absorbance at 765 nm. TPC was measured in samples using a calibration curve with gallic acid standards (0-200 µg/mL) and represented as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g dw).

Determination of total flavonoid content (TFC)

The Total Flavonoid Content (TFC) was determined using the modified aluminum chloride method (Tang et al. 2020), with modifications. In this assay, 80 µL of the extract was mixed with 80 µL of a 2% aluminum chloride

solution (diluted with ethanol) and 120 µL of a 50 g/L sodium acetate solution in a 96-well plate. Next, to experiment, a 96-well plate was filled with 80 µL of extract, 80 µL of a 2% aluminum chloride solution (diluted with ethanol), and 120 µL of a 50 g/L sodium acetate solution. The TFC was calculated as milligrams of quercetin equivalents per gram (mg QE/g dw) of sample weight using a calibration curve of quercetin standards (0-50 µg/mL).

In vitro antioxidant activity

DPPH

The DPPH scavenging activity was evaluated using the DPPH assay method described by Tang et al. (2020), with modifications. To conduct the test, a 96-well plate was filled with 40 µL of extract and 40 µL of 0.1 mM DPPH methanolic solution. The solutions were rapidly stirred and incubated at 25°C for 30 minutes before measuring absorbance at 517 nm. The DPPH radical-scavenging activity of the extracts was expressed as milligrams of ascorbic acid equivalents per gram (mg AAE/g dw), using a standard curve with concentrations ranging from 0 to 50 µg/mL.

FRAP

The FRAP assay was conducted following the method outlined by Tang et al. (2020), with modifications. This assay evaluates the ability of the test material to reduce iron in the Fe^{3+}-TPTZ complex (ferric-2,4,6-tripyridyl-s-triazine) to the Fe^{2+}-TPTZ complex. The FRAP reagent was made by combining 300 mM sodium acetate, 10 mM TPTZ, and 20 mM Fe^{3+} in a 10:1:1 ratio. A 20 µL sample of the extract or standard was added to 280 µL of the prepared FRAP reagent in a 96-well plate and incubated at 37°C for 10 minutes. The absorbance was then measured at 593 nm. The FRAP results were expressed as mg of ascorbic acid equivalents per gram (mg AAE/g dw), using a standard curve with concentrations ranging from 0 to 50 µg/mL.

ABTS

The ABTS scavenging activity was assessed using the $ABTS^+$ radical cation decolorization assay as described by Tang et al. (2020), with modifications. To generate $ABTS^+$, 5 mL of a 7 mmol/L ABTS solution was mixed with 88 mL of a 140 mM potassium persulfate solution, and the mixture was left to stand in the dark at room temperature for 16 hours. The resultant $ABTS^+$ solution was then diluted with analytical-grade ethanol to provide an initial absorbance of 0.7 at 734 nm. After that, 10 mL of the extract or standard was combined with 290 mL of the diluted ABTS solution in a 96-well plate and incubated for 6 minutes at room temperature in the dark. Absorbance was measured at 734 nm following incubation. The antioxidant capacity of the sample was measured as milligrams of ascorbic acid equivalents per gram (mg AAE/g dw) using a calibration curve with ascorbic acid concentrations ranging from 0 to 2000 µg/mL.

RESULTS AND DISCUSSION

Metabolite profile in *Citrus amblycarpa*

Metabolite profiling of *C. amblycarpa* using LC-HRMS combined with chemometric analysis revealed distinct separations among extract fractions: aqueous (KLA), ethyl acetate (KLE), hexane (KLH), and methanol (KLM). PCA, PLS-DA, and HCA were utilized to visualize sample distribution patterns. The PCA and PLS-DA score plots indicated clear differentiation among the extract fractions (Figures 3-4), while Variable Importance in Projection (VIP) score analysis identified key metabolites responsible for this separation (Figure 5). Specifically, (-)-borneol was the predominant compound observed in positive ion mode samples, whereas 5D-5-O-methyl-2,3,5/4,6-pentahydroxy-cyclohexanone was the primary metabolite in the negative ion mode. Additionally, the hierarchical dendrogram confirmed the clustering of KLA, KLE, KLH, and KLM fractions based on their chemical compositions (Figure 6).

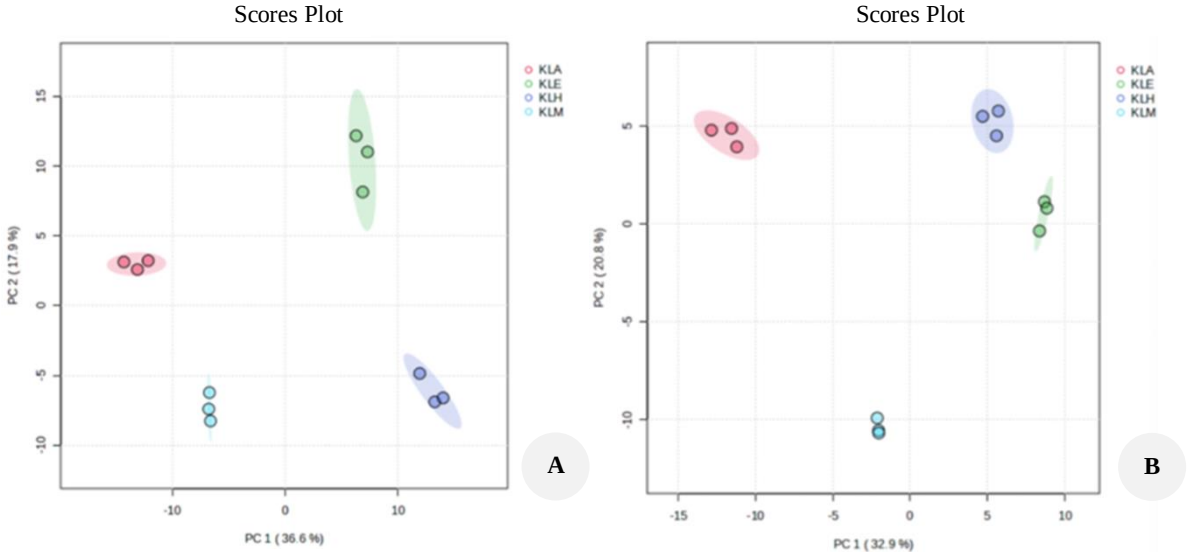

Figure 3. Score plot of PCA of Citrus amblycarpa fraction extracts. A. Positive *Citrus amblycarpa* fraction extracts, B. Negative *Citrus amblycarpa* fraction extracts

LC-HRMS analysis generated raw data in the form of peak areas and retention times for both positive and negative ion mode samples of *C. amblycarpa*. To enhance analytical accuracy, data preprocessing, including peak alignment, was performed using RStudio software (Mock et al. 2018). The dataset was then median-normalized and log10-transformed to establish a more precise distribution model using MetaboAnalyst 6.0. The PCA score plot was employed to reduce data dimensionality by transforming correlated variables into uncorrelated components while preserving essential information regarding sample variance (Saccenti 2024). As shown in Figure 3, PCA analysis of the positive ion mode samples accounted for a total variance of PC1: 36.6% and PC2: 17.9%, whereas the negative ion mode samples exhibited PC1: 32.9% and PC2: 20.8%. The sample distribution in the PCA plot reflected their compositional similarities, revealing distinct metabolite profiles among the extract fractions (Ke et al. 2018).

PLS-DA was used to enhance sample classification by maximizing group separation. Figure 4 illustrates that in the positive ion mode, the samples contributed cumulative variances of 22.5% for Component 1 and 30.3% for Component 2. In contrast, the negative ion mode samples showed cumulative variances of 23.9% for Component 1 and 28.0% for Component 2. Compared to PCA, PLS-DA provided better discrimination among extract fractions, facilitating the identification of distinct metabolomic patterns (Chong et al. 2019). The Variable Importance in Projection (VIP) score analysis derived from the PLS-DA model identified key metabolites contributing to sample discrimination, with VIP scores greater than 1 considered significant (Stoessel et al. 2018). Metabolites with high VIP scores were thus regarded as principal discriminators among the sample group and potential biomarker candidates. As presented in Figure 5, the VIP scores are displayed as a horizontal scatter plot accompanied by a side heatmap, jointly representing both the statistical significance (VIP score) and the relative abundance of each metabolite across the four extract fractions (KLH, KLE, KLM, and KLA). A color gradient indicates metabolite abundance, with red denoting high levels and dark blue indicating lower concentrations, enhancing the interpretability of variations across fractions (Song et al. 2025). In the positive ionization mode (Figure 5A), (-)-borneol and 3'-C-glucosyl were identified as the most dominant metabolites, exhibiting the highest VIP scores, and marked abundance in the KLH fraction. Conversely, in negative ionization mode (Figure 5B), 5-D-5-O-methyl and 3-O-ethylascorbate emerged as the key discriminators, showing both a high VIP score and peak relative abundance within the KLA fraction.

Hierarchical Cluster Analysis (HCA) dendograms (Figure 6) provide a visual assessment of metabolite compositional similarity among extract fractions, where shorter cluster distances indicate greater similarity (Kharyuk et al. 2018). The aqueous (KLA) and methanol (KLM) fractions exhibited the highest compositional similarity, attributable to the strong polarity of both solvents, which facilitates the extraction of polar metabolites such a flavonoid glycosides, phenolic acids, and polar alkaloids. These compounds, rich in hydroxyl and carboxyl groups, are readily soluble in polar solvents via hydrogen bonding (Gil-Martín et al. 2022), resulting in similar metabolite profiles between KLA and KLM. In contrast, the ethyl acetate (KLE) and hexane (KLH) fractions clustered closely, reflecting a shared capacity to extract nonpolar constituents such as lipids, sterols, carotenoids, and terpenoids (Dewi et al. 2022).

Despite the differences in solvent polarity-hexane being nonpolar and ethyl acetate semipolar, both are effective for isolating hydrophobic metabolites poorly soluble in polar media (Quitério et al. 2022). Consistent clustering patterns observed across both ionization modes further underscore the robustness of the metabolomic classification. Overall, the HCA results corroborate the grouping of extract fractions according to metabolite profiles and highlight the decisive role of solvent polarity in metabolite extraction Oktavianawati et al. 2023).

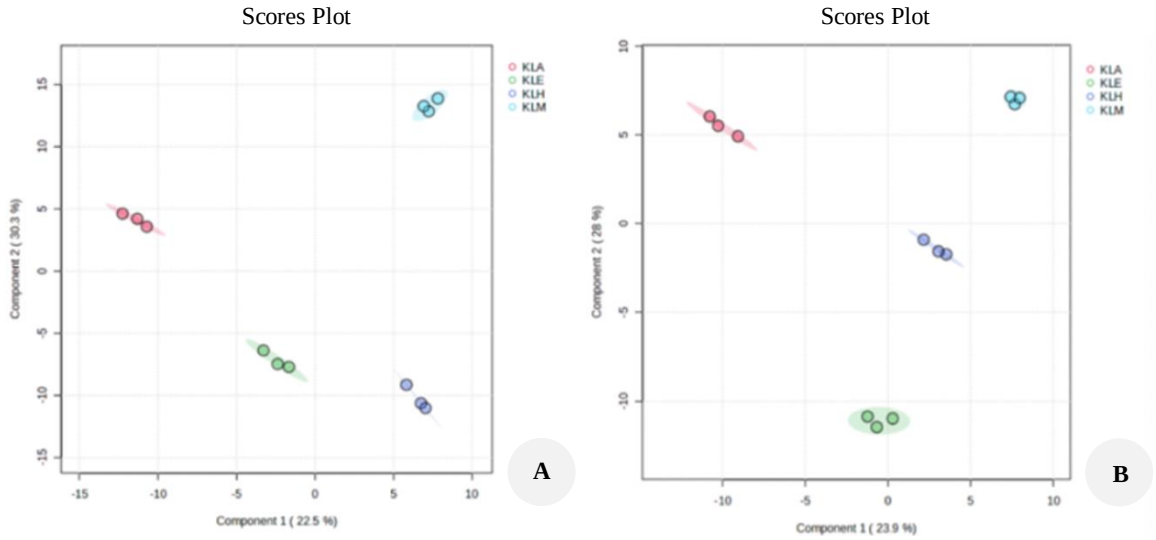

Figure 4. Score plot of PLS-DA of *Citrus amblycarpa*fraction extracts. A. Positive *Citrus amblycarpa* fraction extracts, B. Negative *Citrus amblycarpa* fraction extracts

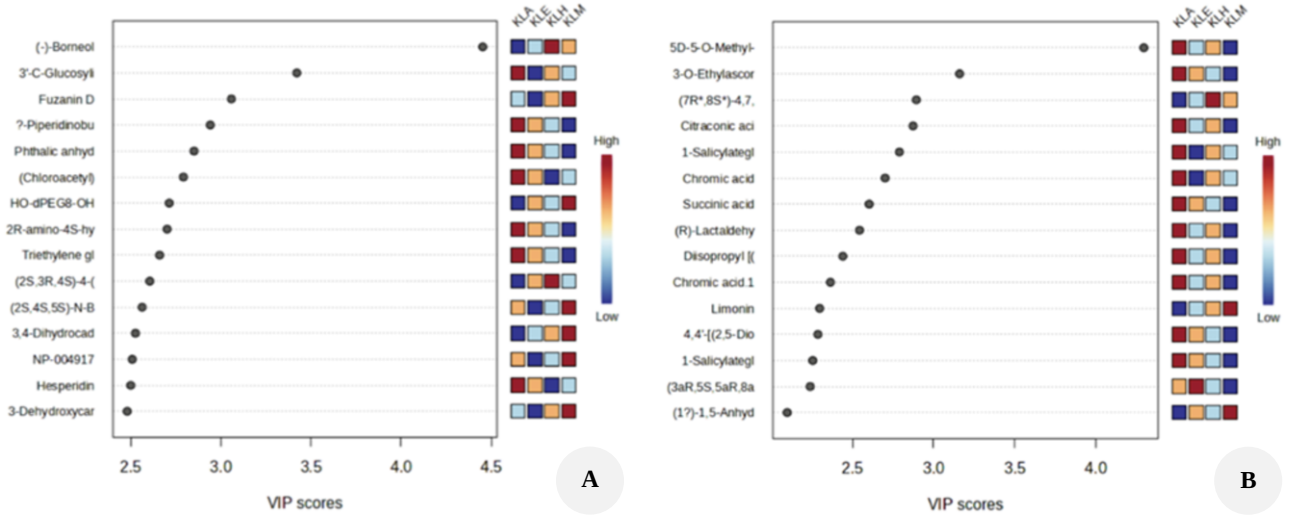

Figure 5. VIP score PLS-DA of *Citrus amblycarpa* fraction extracts. A. Positive *Citrus amblycarpa* fraction extracts, B. Negative *Citrus amblycarpa* fraction extracts

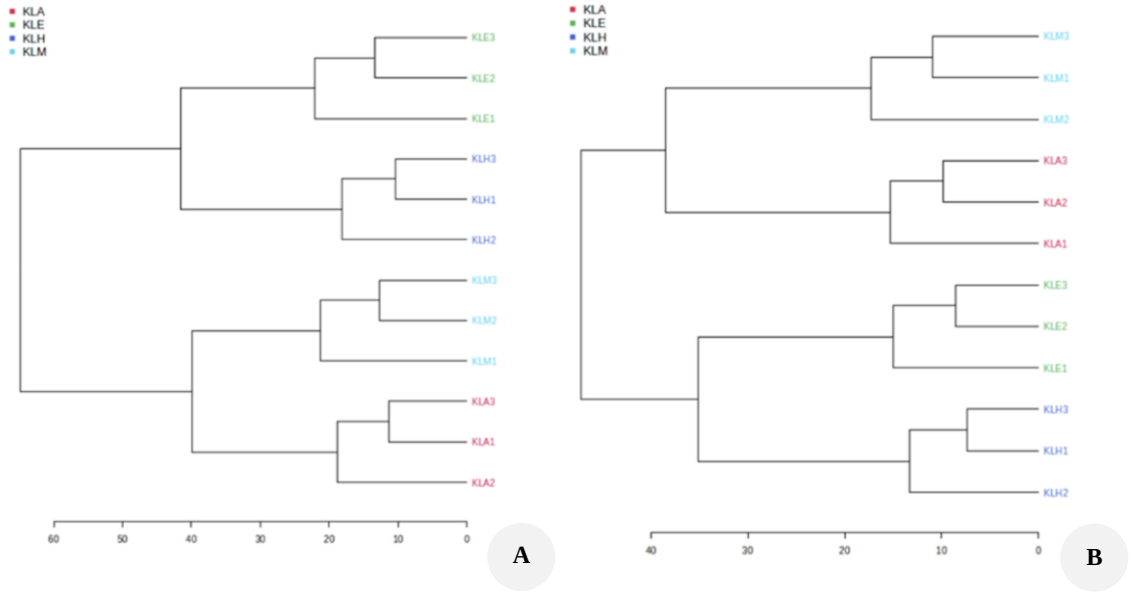

Figure 6. HCA dendrogram of *Citrus amblycarpa* fraction extracts. A. Positive *Citrus amblycarpa* fraction extracts, B. Negative *Citrus amblycarpa* fraction extracts

Determination of TPC, TFC, and antioxidant activity of *Citrus amblycarpa*

Peel extracts of *C. amblycarpa* were sequentially prepared using solvents of increasing polarity. The total phenolic content (TPC) and total flavonoid content (TFC) of each extract were determined using the Folin-Ciocalteu method and the aluminum chloride method, respectively. The TPC and TFC results for the peel extracts are summarized in Table 1. Data analysis using ANOVA and LSD tests revealed significant differences ($p<0.05$) across all measurements. The TPC and TFC in *C. amblycarpa* peel fraction extracts (n-hexane, ethyl acetate, methanol, and aqueous) ranged from 38.34±1.41 to 49.19±1.39 mg GAE/g dry weight (DW) and 5.19±1.22 to 15.44±1.29 mg QE/g DW, respectively.

Table 1. TPC and TFC values of *Citrus amblycarpa* fraction extracts

C. amblycarpa extracts	TPC (mg GAE/g dry weight)	TFC (mg QE/g dry weight)
n-hexane	49.190±1.39^{d}	10.36±1.80^{b}
Ethyl acetate	38.34±1.41^{b}	15.44±1.29^{c}
Methanol	40.50±2.02^{c}	5.79±0.35^{a}
Distilled water	-	5.19±1.22^{a}

Note: Data are expressed as mean±SD. Different superscript letters indicate significant differences within the same extract fraction at $p<0.05$. -: undetected values

The order of fractions based on their total phenolic content (TPC) was as follows: n-hexane fraction > methanol fraction > ethyl acetate fraction > aqueous. The n-hexane fraction exhibited the highest TPC, suggesting that the predominant phenolic compounds in the peel of *C. amblycarpa* are nonpolar, with fewer present in the polar extracts. The elevated TPC detected in the n-hexane extract may be attributed to interactions between the Folin-Ciocalteu reagent and non-phenolic compounds (Pérez et al. 2023), particularly (-)-borneol, which is the most dominant bioactive compound present in the n-hexane extract.

The higher TPC in the n-hexane extract compared to other solvents can be attributed to the sequential maceration process, where n-hexane was used as the initial solvent, allowing most phenolic compounds to be extracted into this phase (Akbar et al. 2021). Similarly, Wairata et al. (2022) reported a high phenolic content in the n-hexane fraction derived from the bark of *Garcinia forbesii* (187.37±0.06 mg GAE/g dry extract). The extraction of phenolic compounds from plant materials depends on their solubility in the selected solvent, which plays a crucial role in determining both yield and phenolic content (Safdar et al. 2017; Swati et al. 2024). Polyphenols in citrus peels exhibit various bioactive properties, including antioxidant, anti-inflammatory, antiproliferative, anti-allergic, antiviral, anticarcinogenic, neuroprotective, and antimicrobial activities (Higashi-Okai et al. 2002).

Flavonoids are a major group of secondary metabolites in citrus fruits, recognized for their potential health benefits. The ethyl acetate fraction exhibited the highest flavonoid content, followed by the n-hexane, methanol, and aqueous fractions. This result suggests that the primary flavonoid compounds in *C. amblycarpa* peel are semipolar, with fewer present in polar extracts. Wijaya et al. (2017) observed a similar trend, reporting a high flavonoid content (0.1147 mg RE/mg) in the ethyl acetate fraction from *Citrus hystrix* peel. Likewise, Gulo et al. (2021) reported that ethyl acetate extract of *Citrus sinensis* peel contained the highest total flavonoid content (242.48±13.83 mg QE/g DW). The flavonoid composition of plant extracts is determined by the polarity of the solvents used in the extraction process (Zazouli et al. 2016). Most flavonoids, particularly aglycone flavonoids, are highly soluble in semipolar solvents such as ethyl acetate (Andersen and Markham 2006).

The antioxidant potential of each extract was evaluated in vitro using DPPH radical scavenging, ferric reducing antioxidant power (FRAP), and ABTS cation radical scavenging assays. The antioxidant activities of *C. amblycarpa* are presented in Figure 7. The radical scavenging activity of DPPH and FRAP across all fractions ranged from 18.29±2.30% to 81.15±0.46% at 1000 µg/mL and from 7.98±0.06% to 84.13±6.79% at 20,000 µg/mL, respectively. Among all fractions, ABTS radical scavenging activity ranged from 74.70±3.03% to 87.15±2.77% at 10,000 µg/mL.

The DPPH radical scavenging assay is a widely used method for measuring antioxidant activity. This assay provides insights into the interaction between tested compounds and free radicals, with its effect attributed to the ability of compounds to donate a hydrogen atom (Swati et al. 2024). The n-hexane extract of *C. amblycarpa* demonstrated the highest DPPH radical inhibition activity, followed by the ethyl acetate, methanol, and aqueous fractions. The enhanced antioxidant activity of the n-hexane fraction correlated with its increased TPC, suggesting a synergistic contribution of phenolic compounds to DPPH radical inhibition (Ramírez-Sucre et al. 2024). Similarly, Jadid et al. (2017) reported the n-hexane extract of *Piper retrofractum* demonstrated greater potency in scavenging DPPH free radicals compared to methanol and ethyl acetate extracts. The antioxidant activity of extracts derived from peels, leaves, seeds, and flesh is influenced by multiple factors, including climate, plant variety, soil composition, maturity stage, and environmental conditions. These factors collectively affect the levels of secondary metabolites and phenolic compounds (Dikmetas et al. 2024). Additionally, both the DPPH radical scavenging activity and phenolic content are significantly influenced by the plant species and the solvent used for extraction (Shehata et al. 2021).

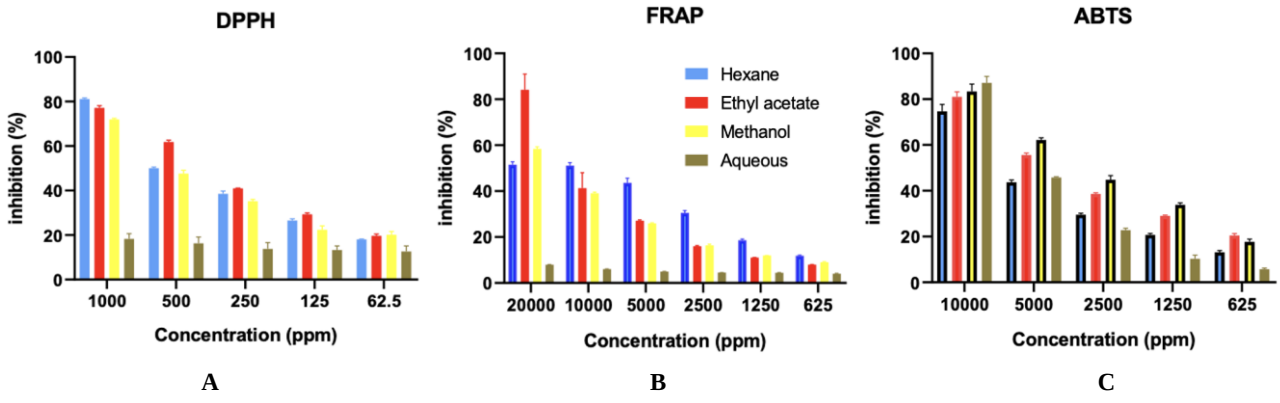

Figure 7. Antioxidant activity of *Citrus amblycarpa* fraction extracts. A. FRAP, B. DPPH, C. ABTS

The antioxidant potential of *C. amblycarpa* peel fraction extracts was further assessed by evaluating their reducing power, specifically their ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Saleem et al. 2023). The reducing power activity of *C. amblycarpa* peel extracts was observed in the ethyl acetate, methanol, n-hexane, and aqueous fractions, respectively. Similarly, Ko and Kim (2018) reported that the highest FRAP activity was found in the ethyl acetate fraction extracted from the pericarp of *Citrus grandis*. Likewise, Irawaty and Ayucitra (2018) found that the ethyl acetate fraction from *Citrus hystrix* peel exhibited notably high FRAP activity. Long et al. (2021) also reported that the ethyl acetate extract demonstrated superior FRAP activity compared to petroleum ether and water extracts. These results are consistent with the high flavonoid content observed in the ethyl acetate fraction, as flavonoids play a crucial role in the antioxidant activity of citrus peel. It is important to note that antioxidant activity is influenced not only by phenolic compounds but also by interactions between phenolic and non-phenolic compounds (Swati et al. 2024).

The ABTS radical scavenging assay was employed to evaluate the effectiveness of the samples in scavenging cation radicals (Shehata et al. 2021; Dongre et al. 2024). The scavenging activity followed the order: aqueous fraction > methanol fraction > ethyl acetate fraction > n-hexane fraction. Similarly, Higashi-Okai et al. (2002) observed that the water extract of *Citrus unshiu* peel exhibited greater antioxidant activity compared to extracts obtained using other solvents. Additionally, drying citrus peels before extraction has been shown to enhance antioxidant activity. In this study, the aqueous extract contained several compounds, including 4-(2,3-dihydroxy-3-methylbutoxy)-7H-furo[3,2-g]chromen-7-one, 3'-C-glucosylisoliquiritigenin, hesperidin, leptosin, 4-hydroxyderricin, leptosphaerin, 3,4,5-trihydroxycyclohex-1-ene-1-carboxylic acid, (+)-flavipucine, and L-(-)-3-phenyllactic acid, which are likely the main bioactive compounds responsible for its strong antioxidant potential. A combination of flavonoids, such as hesperidin and leptosin, in the aqueous extract of *C. amblycarpa* peel has been shown to contribute to ABTS radical scavenging activity. According to Chukwuma (2024), HPLC analysis identified narirutin, naringenin, prunin, nobiletin, tangeretin, hesperetin-7-O-glucoside, hesperetin, and hesperidin as the predominant flavonoids in peel extracts, which are likely to influence their bioactive properties. Hesperidin, recognized as the principal active constituent of citrus peel, has been shown to possess significant antioxidant activity (Dongre et al. 2024).

This study demonstrates that the extraction solvent profoundly influences both the chemical profile and antioxidant properties of *C. amblycarpa* peel extracts. Multivariate analyses (PCA and PLS-DA) clearly differentiated the extract fractions, with (-)-borneol predominating in the positive ion mode and 5D-5-O-methyl-2,3,5/4,6-pentahydroxycyclohexanone being the main metabolite in the negative ion mode. Notably, the n-hexane fraction exhibited the highest total phenolic content (TPC) and DPPH radical scavenging activity, while the ethyl acetate fraction contained the highest flavonoid content and demonstrated superior reducing power. Conversely, the aqueous fraction displayed the most effective ABTS radical scavenging activity. These results highlight the importance of selecting appropriate extraction solvents to optimize the recovery of specific bioactive compounds, thereby enhancing targeted antioxidant activities for potential applications in the food and pharmaceutical industries.

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