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LC-HRMS Profiling and Antibacterial Activity of Extract and Fraction of *Phaleria macrocarpa* (Scheff.) Boerl. Fruit

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ABSTRACT

Antibiotics are widely used in treating infectious diseases, yet rising drug resistance requires alternative therapies. *Phaleria macrocarpa* (Scheff.) Boerl. has emerged as a promising source of antibacterial agents, and this study assessed the activity of water extracts and solvent-partitioned fractions from its fruit, peel, and seeds. Extracts produced by infusion and subsequent ethyl acetate and n-butanol partitioning were tested via broth microdilution against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis*, and *Propionibacterium acnes* ATCC 6919 to determine MIC and MBC values. The ethyl acetate fraction of the fruit flesh (EAFFF) showed the strongest activity, with MIC values of 12.500, 6.250, 3.125, and 12.500 ppm and MBC values of 12.500, 6.250, 6.250, and 25.000 ppm against the respective bacteria. UHPLC–Orbitrap LC-HRMS profiling identified 77 putative metabolites in EAFFF and 110 in the butanol fraction of the fruit flesh (BFFF), including flavonoids and phenolic compounds such as eriodictyol, (±)-naringenin, apigenin, gallic acid, glycitein, and L-(−)-carvone, many of which are supported by literature as antibacterial agents. Overall, these findings point to *P. macrocarpa* water extract as a sustainable, halal-compliant source of natural antibacterial chemicals and encourage more research into it for the creation of safe and efficient plant-based therapeutics.
Keywords: LC-HRMS; untargeted profiling; antimicrobial activity; *Phaleria macrocarpa;* broth microdilution

INTRODUCTION

Antibiotics have been widely used in modern medicine to both prevent and treat bacterial infections by either killing certain bacteria (bactericidal) or preventing the development (bacteriostatic) of various diseases and infections (Punchihewage-Don et al., 2022; Retnaningrum & Rahmawati, 2023). Unfortunately, multidrug resistance from inappropriate dosages, types, and timing of antibiotic administration may occur (Mittal et al., 2020). The use of plant-based alternatives for disease treatment has been extensively explored to measure the potentials of herbs in treating microbial infections (Mundy et al., 2016). *P. macrocarpa* (Scheff.) Boerl (local Indonesian name: *Mahkota Dewa*) is a medicinal plant that belongs to *Thymelaceae* family that is found in Indonesia (Retnaningrum & Rahmawati, 2023; Agustin et al., 2023). Almost every part of the plant contains bioactive compounds that have anticancer, antihyperglycemic, antihypertensive, anti-inflammatory, antidiarrheal, antioxidant, antiviral, antibacterial, antifungal, and vasorelaxant properties. Ethanol extracts from *P. macrocarpa*'s fruits and leaves have modest antibacterial action against both gram-positive and gram-negative bacteria (Othman et al., 2014). These studies revealed moderate inhibition against key bacterial strains, including *Pseudomonas putida, Bacillus subtilis, S. aureus,* and *E. coli*, with inhibition zones ranging from 8.0 to 9.01 mm. Meanwhile, streptomycin as the positive control displayed a larger inhibition zone of 16.5–19.5 mm. Another study found that methanol extracts from the seeds, fruits, and peels of *P. macrocarpa* exhibited variable inhibitory activity at a concentration of 0.3 mg/disc, with inhibition zones between 0.93 to 2.33 cm, compared to 1 ug/disc of kanamycin, which had zone diameters ranging from 1.0 to 1.73 cm (Hendra et al., 2011).

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The use of methanol has raised concerns about the halal status of these treatments, particularly among Muslim communities worldwide (Aslan, 2023). The use of water as an alternative extraction solvent represents a promising approach to maintain halal compliance while preserving the therapeutic potential of herbal formulations. Both ethanol and water extracts of *P. macrocarpa* fruit exhibit antibacterial activity. Ethanol extracts at concentrations of 10–40% inhibited bacterial growth with inhibition zones of 3.10–17.46 mm, whereas water extracts produced inhibition zones of 2.81–11.14 mm. A 10% iodide control yielded an inhibition zone of 2.72 mm (Astriyani et al., 2017). Water extracts at concentrations of 25%, 50%, and 75% also achieved inhibition zones between 14.00 and 17.33 mm, whereas chloramphenicol produced an inhibition zone of 36.42 mm, and the negative control (distilled water) exhibited no inhibition (0 mm) (Buulolo, 2018). Despite these promising results, there is a lack of comprehensive profiling of the chemical constituents in the water extracts of *P. macrocarpa* and their solvent-partitioned fractions. Most researchers mainly focused on ethanol or methanol extracts, while the knowledge regarding the specific bioactive constituents present in water extracts remains limited. In addition, systematic analyses that use sophisticated analytical tools like liquid chromatography–high-resolution mass spectrometry (LC-HRMS) to correlate antibacterial activity with expected chemical profiles were also limited. To address this gap, this study was performed to evaluate the antibacterial properties of water extracts and derived fractions from *P. macrocarpa*, alongside untargeted LC-HRMS profiling in order to identify the putative bioactive compounds. The use of this integrated approach is expected to provide novel insights into the antibacterial potential of *P. macrocarpa* as a halal herbal therapeutic.

MATERIALS AND METHODS

The solvent used in this study was purchased from Rofa Laboratory, while ethyl acetate, *n*-butanol, NaCl, NaH_2PO_4, and Na_2HPO_4 were the reagents purchased from Merck. Furthermore, Nutrient Broth (NB), Nutrient Agar (NA), McFarland standard, Mueller Hinton Broth (MHB), tetracycline, and Mueller Hinton Agar (MHA) were purchased from Himedia, and ciprofloxacin antibiotic was purchased from OGB Dexa, and sterile cotton swabs from OneMed.

Plant material

P. macrocarpa was collected from Java Island, Indonesia at latitude 6.92˚ 53’ 47.770000” S and longitude 107.69˚ 41’ 12.610000” E. This plant was identified at Herbarium Bandungense Laboratory, Bandung Institute of Technology, College of Life Sciences with specimen voucher number FIPIA-DEP113.

Sampling preparation

Different components of *P. macrocarpa* (including fruits, peels, and seeds) were systematically sorted, thoroughly cleaned, and subjected to sun drying for 72 hours. The sun drying process offers greater cost-effectiveness, energy efficiency, and accessibility, and it minimizes the thermal degradation of heat-sensitive phytochemicals. Unlike oven drying, which may induce thermal breakdown of certain bioactive components such as flavonoids and saponins, sun drying at ambient temperatures effectively preserves the integrity of the polar compounds targeted for extraction.

Extraction and fractionation

Each component of the *P. macrocarpa* fruit was subjected to infusion extraction using distilled water (1:10, w/v) at 90 °C for 15 minutes. After cooling, the extracts were filtered through Whatman filter paper, concentrated by evaporating the water solvent, and subsequently dried using a freeze-drying method (Krakowska-Sieprawska et al., 2022).

Fractionation of the extracts was performed by dissolving each sample in distilled water at the predetermined ratio, followed by sonication to fully dissolved and homogeneous before being transferred into a separating funnel and extracted with an equal volume of ethyl acetate. The funnel was shaken five times, vented after each shake, and allowed to stand overnight to facilitate optimal phase separation. The ethyl acetate fraction was collected in three replications to ensure complete extraction (Hasim et al., 2022). The remaining aqueous phase was then subsequently extracted with an equal volume of n-butanol using the same procedure as for the ethyl acetate fraction, which resulting fractions were each concentrated in a water bath until the solvent removal completed (Hasim et al., 2022).

Antibacterial activity screening

The antibacterial activity of the ethyl acetate fraction, water fraction, and *n*-butanol

fraction in the extract were screened using broth microdilution method (Hossain, 2024). The bacterial strains were obtained from IPB culture collection, UNPAD culture collection, Biotechnology Laboratory of Muhammadiyah University of Bandung, and Agavi Laboratory Bandung.

Bacterial strains

E. coli ATCC 8739 as the gram-negative bacteria and three gram-positive bacteria: *S. aureus* ATCC 29213*, S. epidermidis,* and *P. acnes* ATCC 6919 were tested as bacterial panel. Prior to the assay, bacterial strains were cultured in Nutrient Agar (NA; Himedia) at 37˚C for 18 – 24 hours. Each bacterial culture was injected into sterile NaCl to gain a concentration of 1 x 10^8 CFU/mL, or the 0.5 McFarland standard. The bacterial suspension was then put into a 96-well microtiter plate.

Sample preparation for antibacterial assay

Preparation of the extracts and fractions was performed based on the Clinical and Laboratory Standards Institute (CLSI) protocol (Weinstein, 2019). A total of 12 samples were tested; water extract of peel (WEP), water extract of fruit flesh (WEFF), water extract of seeds (WES), ethyl acetate fraction of peel (EAFP), ethyl acetate fraction of fruit flesh (EAFFF), ethyl acetate fraction of seeds (EAFS), water fraction of peel (WFP), water fraction of fruit flesh (WFFF), water fraction of seeds (WFS), n-butanol fraction of peel (BFP), n-butanol fraction of fruit flesh (BFFF), and n-butanol fraction of seeds (BFS). Each sample was dissolved in DMSO to obtain a final concentration of 100 mg/mL.

Ciprofloxacin was used as the reference antibiotic against *E. coli, S. aureus,* and *S. epidermidis* in the antibacterial activity assays, while tetracycline was used as the reference for *P. acnes.* Stock solutions of each antibiotic were prepared at 1,000 µg/mL in sterile phosphate-buffered saline (PBS) (Mulqie et al., 2022).

Broth microdilution assay
MIC value determination

A total 100µL of MHB was added to all columns, while the 10µL of bacterial culture that had been diluted 1 x 10^8 CFU/mL was pipetted and added to column 2 to 12, rows A to H. First sample was added to column 12, rows A, B, C, while the second sample was added to column 12, rows F, G, H (each treatment was replicated three times). The antibiotics solution was added in duplicate in column 12, rows D and E. Subsequently, 100µL of the solution from the mixture was taken to fill column 11. The dilution was then carried out sequentially up to column 3. Column 1 of the microplate was used as a negative control, while column 2 as a positive control.

The culture was incubated for 18 to 24 hours at 37˚C, and the clarity of the culture was compared to the control to identify the bacterial growth in each well. Hazy visual showed that the sample would not capable of inhibiting the bacterial growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that was not cultivated with bacteria (clear) (Mulqie, et. al., 2022).

MBC value determination

A total of 15 mL of MHA was melted to reach a temperature between 45 – 53˚C before being poured into a sterile petri dish and left to solidify. In a microplate well that shows clarity, a 5µL aliquot was pipetted, and a sterile cotton swab was used to streak the aliquot on sterile MHA media. Incubation was then conducted at 37˚C for 18 to 24 hours, and the MBC value was identified as the portion of the agar medium that did not exhibit any bacterial growth with discernible visual results (Mulqie, et. al., 2022).

Liquid chromatography with high resolution mass spectrophotometry (LC-HRMS)

Approximately 10 mg each of EAFFF and BFFF was dissolved with methanol and then filtered using a nylon filter membrane 0.2µm. Methanol is a solvent that effectively dissolves a wide range of semi-polar to polar compounds commonly found in plant extracts, such as flavonoids, tannins, and glycosides. The MS-grade quality methanol that has been widely accepted in metabolomics due to its low UV absorbance and volatility was used in this research (Truong, et, al., 2019). The sample filtrate (5 µL) was then injected into the instrument system. Thermo Scientific™ Orbitrap™ Exploris 240 HRMS (Bremen, Germany) with Optamax™ NG Heated Electrospray Ionization (H-ESI) was used for the MS analysis, and a Thermo Scientific™ Vanquish™ Horizon UHPLC with Binary Pump (Germering, Germany) fitted with a Thermo Scientific™ Accucore™ Phenyl Hexyl 100 mm length x 2.1 mm ID x 2.6 µm particle size (Lithuania) analytical column was used for the LC analysis.

The EAFFF and BFFF samples were evaluated using a solvent gradient of MS-grade water with 0.1% formic acid (A) and MS-grade

acetonitrile with 0.1% formic acid (B). In 16 minutes, the gradient gradually rose to 90% using 5% B for 4 minutes before it declined to 5% B in 25 minutes at flow rate of 0.3 mL/min to ensure optimal separation. Within the 25-minute timeframe, analysis was performed, including the measurement of the 40°C column temperature.

Full MS/dd-MS2 capture mode with positive and negative polarity toggling was used between the range of 70-800 m/z, and MS resolution of 60,000 FWHM. The charge states were 1, the intensity threshold was 5000, and the maximum injection time was 100 ms. The dd-MS2 resolution was 22,500 FWHM, the normalized collision energy was 30, 50, and 70, with mass tolerance of 5 ppm. Using spray voltages of +3500 V and –2500 V, the system employed nitrogen as the collision gas, with gas settings of 1 AU for sweep gas, 7 AU for auxiliary gas, and 35 AU for sheath gas. The vaporizer and ion transfer tube were maintained at 320°C and 300°C, respectively (Kusuma et al., 2025).

Data processing was conducted using the mzCloud Library, Thermo Scientific Compound Discoverer 3.3 (San Jose, USA), and data analysis software were used for data processing; Nature Chemical Biology; Nature Chemistry; Nature Communications; NPAtlas; Plant Metabolic Network; Planta Piloto de Química Fina. Universidad de Alcalá; PlantCyc; Natural Products/Medicines; Natural Toxins, Chemspider Database: ChEBI; ChEMBL; KEGG; Nature Chemical Metabolites Flavonoid Structure Database, Arita Lab 6549; Natural Products Atlas 2021_08.

Antibacterial compound analysis

LC-HRMS profiling generated chromatograms with retention time for each predicted compound. After removing duplicates and non-natural chemical entities, the remaining compounds were classified by chemical class, plant origin, and reported antibacterial properties (polyphenol, tannins, flavonoid, terpenoid, alkaloid, etc.) (Retnaningrum & Rahmawati, 2023). Four compounds in the EAFFF sample and five in the BFFF sample matched those previously reported for *P. macrocarpa*.

RESULTS AND DISCUSSION

The taxonomy of the plant material used in this study was verified at the School of Life Sciences and Technology, Institut Teknologi Bandung. The specimen was identified as *P. macrocarpa* (Scheff.) Boerl., a member of the *Thymelaeaceae* family commonly known in Indonesia as Mahkota Dewa. Details of the phytochemical screening and total phenolic content of the extract have been reported in our earlier work (Setyaningrum et al., 2025).

Water infusion was performed to the peel, flesh, and seed of the fruit as the initial extraction technique to ensure the preservation of bioactive constituents and to meet halal requirements. The crude aqueous extracts obtained from each part underwent the successive liquid–liquid partitioning with ethyl acetate and n-butanol to represent solvents of intermediate and high polarity (Table I).

Table I. Fractionation yield % result

Fraction	% Peel Yield	% Flesh of Fruit Yield	% Seeds Yield
Ethyl acetate	9.88	17.16	7.14
Water	30.56	57.84	35.16
n-butanol	16.05	6.86	12.64

Water fractions exhibited substantially higher yields compared to ethyl acetate and n-butanol fractions. The result shows the ability of highly polar protic solvents to solubilize a broader range of polar phytochemicals. However, a higher extraction yield does not necessarily correlate with higher concentrations of bioactive molecules, since some active compounds may be present. Therefore, extraction and fractionation strategies should balance yield optimization with the preservation of compound integrity, particularly for thermolabile and oxidation-sensitive metabolites.

Prior researchers found that the ethyl acetate fraction of *P. macrocarpa* typically contains flavonoids, alkaloids, tannins, saponins, terpenoids, and lignin, while the n-butanol fraction is enriched in alkaloids, steroids, saponins, and flavonoids (Rusdi et al., 2024). Whereas, water fractions contain alkaloids, steroids, saponins, flavonoids, and tannins (Sugiwati et al., 2009). Tannins are polyphenolic compounds that are well documented for their antimicrobial property, as they denature the bacterial cell proteins which compromises the cell wall integrity (Cushnie & Lamb, 2005). Similarly, flavonoids from *P. macrocarpa* inhibits the growth of *S. aureus* through the disruption of microbial membranes and inhibition of nucleic acid synthesis (Shamsudin et al., 2022).

Antibacterial activity screening with broth microdilution assay

The present research examined the antibacterial qualities of *P. macrocarpa* water

extracts and their derived fractions using the broth microdilution technique to measure the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) based on the Clinical and Laboratory Standards Institute (CLSI) M07 recommendations. The minimum inhibitory concentration (MIC) of an antibacterial agent that inhibit the microbe growth within in vitro conditions (Kowalska-Krochmal & Dudek-Wicher, 2021). Meanwhile, MBC showed the lowest concentration at which the antibacterial agent effectively kills the microorganisms (Parvekar et al., 2020).

The Z factor presents the viability and robustness of high-throughput screening in the evaluation of the screening quality (Pompilio, et al., 2025). The Z-factor assesses the separation between sample responses and positive controls. For the MIC and MBC measurements, the average Z-factor on the 96-well assay plate was 0.000. Individually, the Z-factor for MBC ranged from 1.99725 to 0.96836, while that for MIC ranged from 1.34021 to 1.00791. These values indicate that the assay produced normally distributed data (see Supplementary Data 2).

EAFFF demonstrated the strongest inhibitory and bactericidal effects against the tested bacterial strains among the samples. As presented in Table II, the MIC values for EAFFF were 12,500, 6,250, 3,125, and 12,500 ppm against *E. coli*, *S. aureus, S. epidermidis*, and *P. acnes*, respectively. The MBC values (Table II) were 12,500, 6,250, 6,250, and 25,000 ppm for the same strains, indicating that EAFFF was not only does not only inhibit the growth but also exerts the bactericidal effects. Gram-negative bacterium exhibited lower susceptibility to EAFFF than the Gram-positive bacteria *S. aureus* and *S. epidermidis*. The thick peptidoglycan coating found in Gram-positive bacteria's cell walls could increase the sensitivity and vulnerability to antibiotics. On the other hand, gram-negative bacteria possess more complex cell walls that feature an outer layer of lipoproteins as a formidable barrier against antibacterial compounds (Siregar et al., 2018). *P. acnes* is a gram-positive bacterium with similar MIC value and a higher MBC value than those Gram-positive and other Gram-negative bacteria in this study. This discrepancy can be explained by the bacterium's aerotolerant anaerobic nature, which enables it to survive more effectively in low-oxygen environments. As a result, higher concentrations of antibacterial agents are needed to eliminate it compared with other Gram-positive bacteria (Zahrah et al., 2018).

The polar secondary metabolites in EAFFF (saponins, alkaloids, flavonoids, and tannins) affect the antibacterial properties that have been found (Easmin et al., 2015). The extraction method maximizes the recovery of these polar bioactives, while infusion at 90°C for 15 minutes enhances solubility and accelerates the release of these metabolites, thereby improving extraction yield while preserving bioactivity (Leswana & Sianturi, 2024).

The semi-polar characteristics of ethyl acetate extracts a broader range of structurally diverse secondary metabolites compared to purely polar or nonpolar solvents (Berghuis, 2026). The ethyl acetate fraction contained high concentrations of bioactive antibacterial compounds such as flavonoids, alkaloids, tannins, polyphenols, and saponins. This chemical profile likely explains its strong antibacterial activity, reflected by the lowest MIC and MBC values among all extracts and fractions. The water fraction showed weak antibacterial effects due to the selective partitioning of secondary metabolites during fractionation, in which ethyl acetate preferentially extracts the more potent antibacterial constituents, leaving the water fraction depleted of these key bioactive molecules.

The intricacy of their cell wall architecture of gram-positive and gram-negative bacteria is a distinctive feature. Gram-negative bacteria possess a more complex cell envelope with thin peptidoglycan layer, an outer membrane, lipoproteins, and lipopolysaccharides which create a strong permeability barrier to reject antimicrobial drugs (Xu et al., 2026). Consequently, higher concentrations of antibacterial compounds are needed to achieve bacteriostatic or bactericidal effects. In this research, the gram-negative bacteria have greater MIC and MBC values than Gram-positive bacteria.

Due to the limited antibacterial qualities of the secondary metabolites found in *P. macrocarpa* seeds, the water extract and water fraction obtained from these seeds both showed poor antibacterial activity. The *P. macrocarpa* seeds are known to contain essential oils with strong antimicrobial qualities (Jinoni et al., 2024). Because volatile compounds are more efficiently extracted with non-polar methods, future research should explore approaches such as steam distillation or Soxhlet extraction with non-polar solvents.

Table II. The MIC and MBC of P. macrocarpa Extract and fraction against *E. coli, S. aureus, S. epidermidis, and P. acnes*.

Extract or Fraction	MIC (ppm)				MBC (ppm)			
	E. coli	*S. aureus*	*S. epidermidis*	*P. acnes*	*E. coli*	*S. aureus*	*S. epidermidis*	*P. acnes*
Water extract of peel	-	25,000	50,000	50,000	-	25,000	25,000	-
Water extract of flesh fruit	50,000	12,500	12,500	12,500	-	25,000	25,000	50,000
Water extract of seed	-	-	50,000	25,000	-	-	-	-
Ethyl acetate fraction of peel	50,000	6,250	6,250	50,000	50,000	12,500	12,500	50,000
Ethyl acetate fraction of flesh fruit	12,500	6,250	3,125	12,500	12,500	6,250	6,250	25,000
Ethyl acetate fraction of seed	25,000	-	50,000	50,000	-	-	-	-
Water fraction of peel	12,500	-	6,250	12,500	-	-	-	-
Water fraction of flesh fruit	25,000	50,000	12,500	25,000	-	-	-	-
Water fraction of seed	50,000	50,000	50,000	50,000	-	-	-	-
n-butanol fraction of peel	12,500	12,500	12,500	3,125	-	12,500	25,000	-
n-butanol fraction of flesh fruit	25,000	6,250	6,250	12,500	25,000	12,500	12,500	25,000
n-butanol fraction of seed	-	50,000	6,250	50,000	-	50,000	25,000	-

Table III. Metabolites in the EAFFF and BFFF sample of *P. macrocarpa* according to LC-HRMS

Identified Compound	Retention Time	Molecular Weight	Formula	Delta Mass (ppm)	EAFFF	BFFF
Eriodictyol	8.486 (EAFFF) 8.488 (BFFF)	289.07078	C15 H12 O6	0.46	+	+
(±)-Naringenin	6.791 (EAFFF) 6.787 (BFFF)	271.06139	C15 H12 O5	0.6	+	+
Apigenin	6.736 (EAFFF) 6.727 (BFFF)	269.04578	C15 H10 O5	0.57	+	+
Glycitein	8.432	285.07568	C16 H12 O5	-0.23	-	+
(L)-(-)-Carvone	6.11	133.1011	C10 H14 O	-0.36	-	+
Gallic acid	1.208	169.01431	C7 H6 O5	0.41	+	-

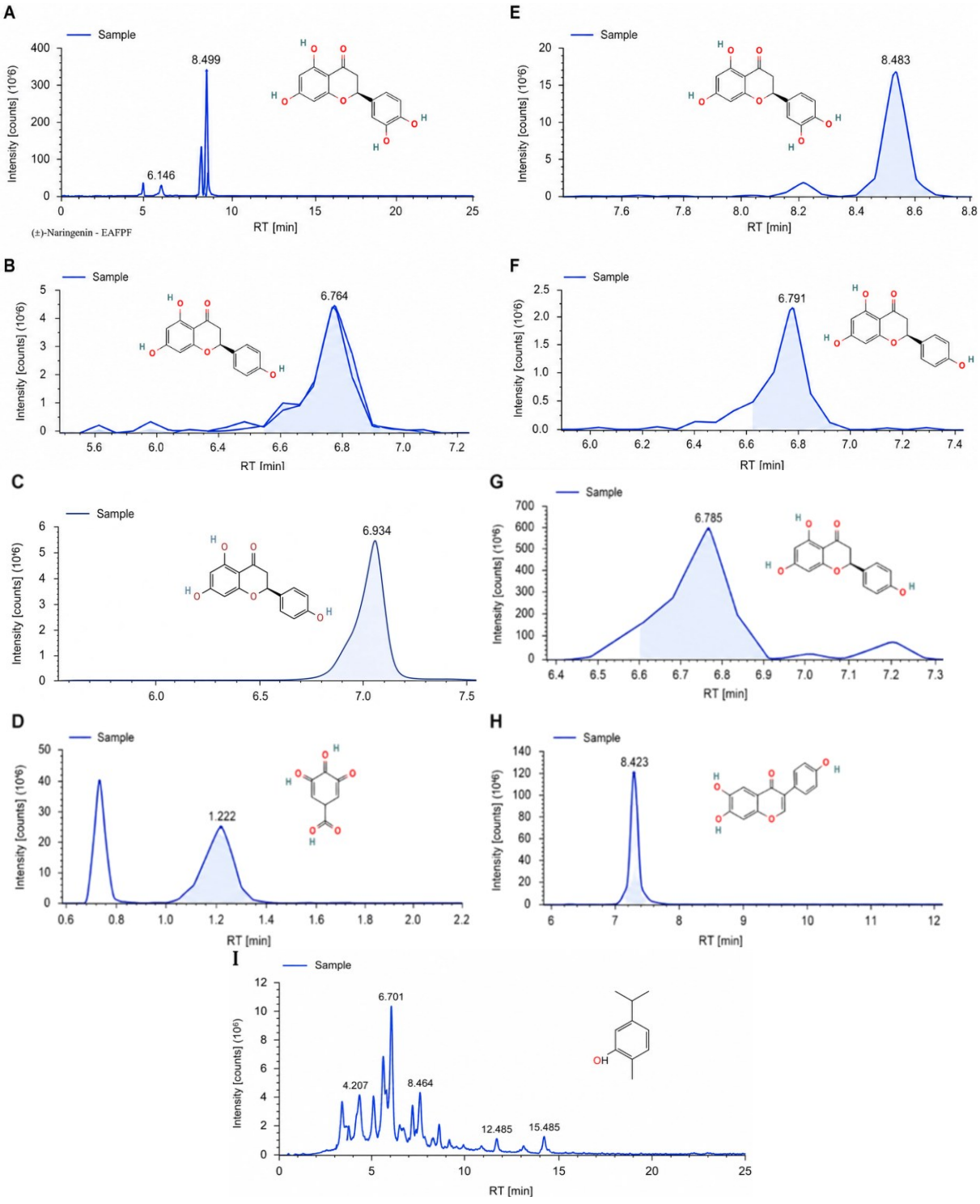


Figure 1. Chromatogram results of compounds: (A) Eriodictyol - EAFFF; (B) (±)-Naringenin - EAFFF; (C) Apigenin - EAFFF; (D) Gallic Acid – EAFFF; (E) Eriodictyol - BFFF; (F) (±)-Naringenin - BFFF; (G) Apigenin - BFFF; (H) Glycitein - BFFF; (I) (L)-(-)-Carvone – BFFF using LC-HRMS

Improving the recovery of these essential oils may markedly strengthen the antibacterial activity of seed-derived preparations and expand their potential therapeutic use.

Putative identification of the metabolites by LC-HRMS

In this study, EAFFF and BFFF samples were evaluated for their metabolites using Orbitrap High-Resolution Mass Spectrometer in conjunction with Ultra-High Performance Liquid Chromatography (UHPLC–Orbitrap HRMS). This technique allowed for the identification of 77 putative compounds in EAFFF and 110 in BFFF, based on exact mass measurements that were cross-referenced with mzCloud and additional databases (see Supplementary Data 1).

Compound identification relied on mass accuracy as a key criterion, with a mass error threshold of ±5 ppm (Arrebola-Liebanas et al., 2017). Database searches were conducted using a combination of mzCloud, the compound database mzVault, ChemSpider, Mass List (Arita 6549 flavonoids, Natural Product Atlas, etc., as described in the instrument parameters). Compounds with ±5 ppm mass accuracy was considered high-confidence identifications.

A detailed review of the chromatographic and mass spectral data shows qualitative and quantitative differences between the two fractions. Although the base peak chromatograms are similar, variations in peak intensities indicate differences in metabolite abundance, which may help explain the strong antibacterial activity of EAFFF in the MIC and MBC assays (see Supplementary Data 1). The metabolite classes (flavonoids, phenolics, isoflavones, and terpenoids) play established roles in antibacterial activity.

Six specific compounds displayed matching fragmentation patterns with entries in the database and relevant literature (Table III). EAFFF contains eriodictyol, (±)-naringenin, apigenin, and gallic acid (Maharani et al., 2021; Christina et al., 2021; Altaf et al., 2013), while BFFF contains eriodictyol, (±)-naringenin, apigenin, glycitein, and L-(-)-carvone (Maharani et al., 2021; Christina et al., 2021). Eriodictyol ($C_{15}H_{12}O_6$; m/z 289.07078 $[M-H]^-$) was present in both EAFFF and BFFF (Table III), with nearly identical retention times (8.499 and 8.483 min, respectively, Figure 1A and 1E). Its MS/MS spectrum revealed seven fragment ions at m/z: 289.07089, m/z: 274.04739, m/z: 246.05243, m/z: 245.04448, m/z: 229.04970, m/z: 217.04967, and m/z: 180.04170 for EAFFF (Figure 2A). Whereas eight characteristic fragment ions at m/z: 289.07043, m/z: 274.04697, m/z: 257.04401, m/z: 246.05200, m/z: 217.04930, m/z: 180.04153, m/z: 167.03346, and m/z: 151.03888 for BFFF (Figure 2E). Eriodictyol also inhibits biofilm formation and sortase A activity in *S. aureus*. The membrane-bound cysteine transpeptidase Sortase A (SrtA) is the catalysist of surface protein covalent attachment to the *S. aureus* cell wall. Eriodictyol also exhibits good therapeutic benefits in mice with pneumonia from *S. aureus* and reduces invasion of A549 cells, which affects *S. aureus* adhesion (Wang et al., 2021).

Another flavonoid derivative, (±)-Naringenin ($C_{15}H_{12}O_5$; m/z 271.06139 $[M-H]^-$) (see Supplementary Data 1) were also present in both fractions, with retention times of 6.764 and 6.791 min (Figure 1B and 1F). To create ten fragment ions at m/z: 271.06122, m/z: 229.05080, m/z: 177.01938, m/z: 151.00371, m/z: 137.02969, m/z: 119.05026, m/z: 107.01385, m/z: 93.03457, m/z: 83.05383, and m/z: 65.00330 for EAFFF, whereas eight fragment ions at m/z: 271.06122, m/z: 229.05080, m/z: 177.01933, m/z: 151.00371, m/z: 133.02969, m/z: 93.03467, m/z: 83,01383, and m/z: 65.00330 for BFFF, this molecule disintegrates due to an ion collision (Figure 2B and 2F). Its fragmentation pattern matched reference spectra, and it shares similar antibacterial mechanisms with eriodictyol.

Apigenin ($C_{15}H_{10}O_5$; m/z 270.05278 and detected ion species $[M+H]^-$) had comparable retention times in EAFFF (6.934 min) and BFFF (6.785 min) (Figure 1C and 1G). Its MS/MS spectrum revealed nine fragment ions at 268.03717, m/z: 269.04559, m/z: 241.05064, m/z: 225.05571, m/z: 201.05579, m/z: 151.00363, m/z: 117.03458, m/z: 107.01387, and m/z: 65.00330 for EAFFF. The MS/MS fragments of BFFF sample detected at m/z: 269.04559, m/z: 151.00345, m/z: 149.02428, m/z: 117.03458, m/z: 107.01366, and m/z: 65.00332 were consistent with reference data (Figure 2C and 2G). Apigenin disrupts the bacterial membranes and inhibits essential enzymes. With MIC values of 31-125 and 13-50 ppm, apigenin, a flavonoid molecule, exhibits antibacterial action against *S. aureus* and 11 additional oral bacterial species. By inhibiting biofilm formation and reducing hemolysis, apigenin effectively suppresses *S. aureus*. Despite their strong antibacterial and antibiofilm activities, the biological efficacy of polyphenols is often limited by their poor water solubility and instability in complex biological environments (Xu et al., 2025).

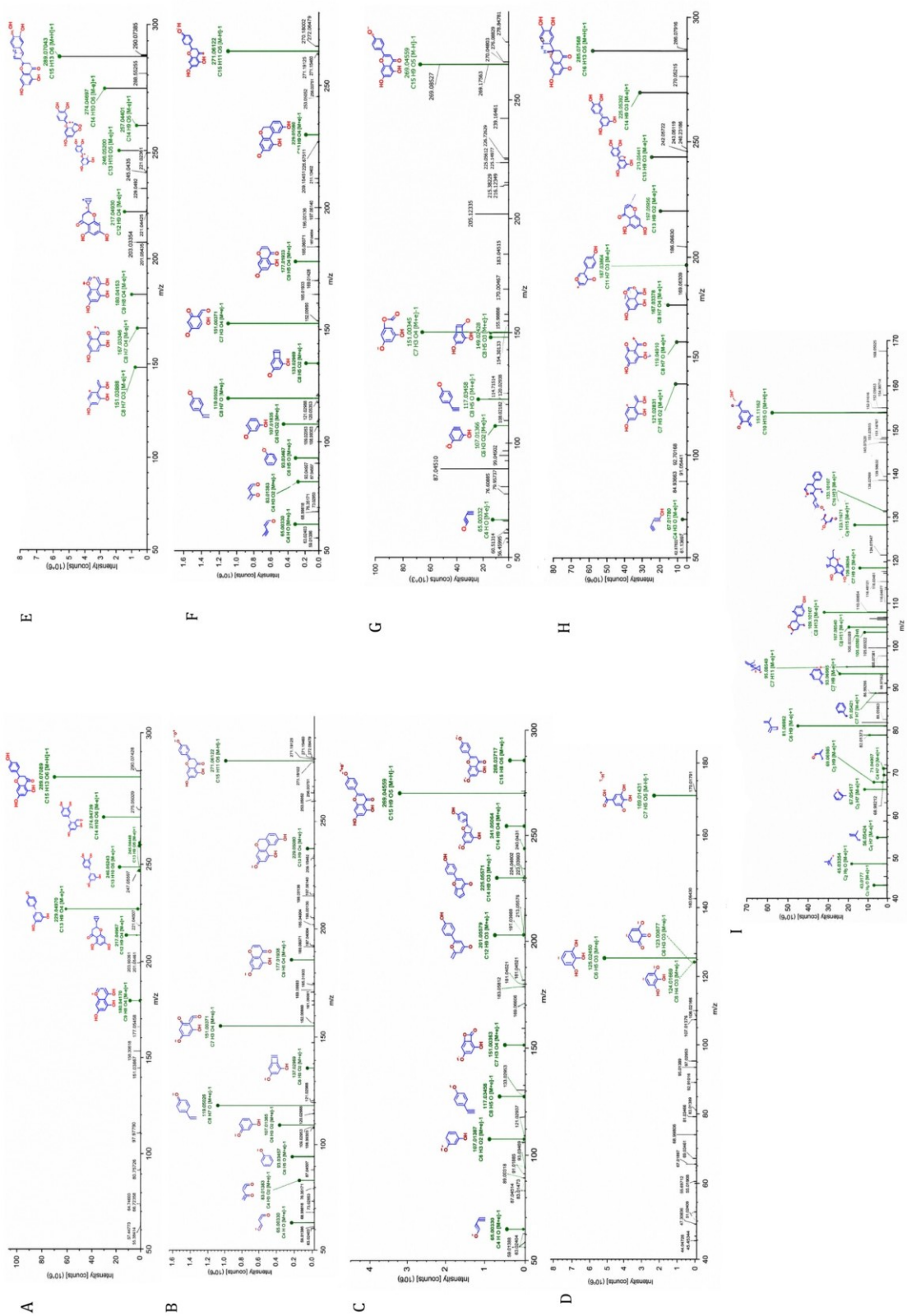


Figure 2. Spectrum results of compounds: (A) Eriodictyol - EAFFF; (B) (±)-Naringenin - EAFFF; (C) Apigenin - EAFFF; (D) Gallic Acid – EAFFF; (E) Eriodictyol - BFFF; (F) (±)-Naringenin - BFFF; (G) Apigenin - BFFF; (H) Glycitein - BFFF; (I) (L)-(-)-Carvone – BFFF using LC-HRMS

Polyphenols have a well-established therapeutic role in reducing inflammation and tissue damage associated with bacterial and viral infections. Bacterial infections trigger the production of reactive oxygen species (ROS) that stimulates an immune response and leads to inflammation and oxidative stress (OS). Elevated OS disrupts cellular metabolism and increases susceptibility to further infection. Flavonoids modulate the human body's defenses against OS by chelated metal ions and scavenging free radicals. Many antibacterial medications kill the bacteria by activating ROS pathways. Only a small quantity of ROS has been shown to support the microbes' signalling systems (Rudrapal et al., 2022).

Gallic acid ($C_7H_6O_5$) was found only in EAFFF eluted at 1.222 min with four diagnostic fragment ions at m/z: 169.01431, m/z: 123.00877, m/z: 125.02450, and m/z: 124.01669 and detected ion species $[M-H]^-$ (Figure 1D and 2D). As a hydrolyzable tannin subunit, gallic acid acts synergistically with flavonoids in increasing membrane permeability in Gram-positive bacteria (Xie et al., 2015). Through iron chelation, the inhibition of the fatty acid biosynthesis pathway, cell wall synthesis suppression, and cell membrane rupture, tannins supress the bacterial growth. Tannins may also operate as quorum sensing inhibitors that decrease the production of a number of virulence factors, including biofilm formation, enzymes, adhesins, motility, and toxins (Farha et al., 2020; Villanueva et al., 2023).

Glycitein ($C_{16}H_{12}O_5$; m/z 285.0756) that was detected only in BFFF (retention time 8.423 min, Figure 1H) is an isoflavone with mild antibacterial and antifungal properties (Mughal et al., 2025). The HRMS spectrum revealed nine ionized fragments at m/z values: 285.07568, m/z: 225.05392, m/z: 213.05441, m/z: 197.05956, m/z: 187.03864, m/z: 167.03378, m/z: 119.04910, m/z: 121.02831, and m/z: 67.01780 with detected ion species $[M-H]^-$ (Figure 2H). Since glycitein compromises the integrity of microbial cell walls, the cells are unable to protect their unique morphology. The production of certain phytochemicals can lead to microbial cell death by damaging cell structures and cell walls, ultimately causing leakage (Adeosun et al., 2022). In addition, early in vitro and in silico studies have reported that glycitein exhibits anti-SARS-CoV activity (Rudrapal et al., 2022).

L-(–)-Carvone is a monoterpenoid identified in BFFF (retention time 6.110 min, Figure 1I] that has bacteriostatic and bactericidal properties against isolates of *Salmonella* spp., *P. aeruginosa, Klebsiela pneumoniae, Acinetobacter baumanocae, E. coli, S. aureus, Enterobacter aerogenes,* and *K. oxytoca* (Sayout et al., 2020). Carvone prevents the biofilm formation and adhesion of several bacteria, including *E. coli* and *P. aeruginosa* (Pina et al., 2022). Monoterpenoid is also recognized as a precursor that damages bacterial biomembranes, disrupts lipids in the membranes, affects bacterial protein production and motility, and causes reactive oxygen species (ROS) to be produced in treated cells (Marchese et al., 2017; Gyrdymova & Rubtsova, 2021). The antibacterial property of terpenoids interrupts membrane, while cell wall production inhibits bacterial growth. These compounds may create strong polymer bonds with porins in the outer membrane of bacterial cell walls. When the porin is compromised, the permeability of the bacterial cell wall decreases and deprives the bacterial cells of nutrients, eventually stopping their growth (Wiart et al., 2023; Rosalinda et al., 2025). The HRMS spectrum detected 16 ionized fragments at the following m/z values: 151.11162, m/z: 133.10107, m/z: 123.11671, m/z: 129.08054, m/z: 109.10107, m/z: 107.08540, m/z: 95.08549, m/z: 93.06985, m/z: 91.05421, m/z: 81.06982, m/z: 79.04907, m/z: 69.06985, m/z: 67.05417, m/z: 56.05424, m/z: 45.03354, and m/z: 43.0177 with detected ion species $[M+H]^+$ (Figure 2I). These findings align with carvone's antibacterial properties and potentials for the development of novel treatments against bacterial illnesses (Pina et al., 2022).

LC-HRMS profiling showed that both EAFFF and BFFF contain bioactive secondary metabolites such as flavonoids, phenolics, and terpenoids with known antibacterial functions. The higher abundance and diversity of semi-polar compounds in EAFFF correspond to its stronger MIC and MBC activity, while unique metabolites in BFFF provide additional antimicrobial effects. These results offer a clear chemical basis for the observed bioactivity and highlight *P. macrocarpa* as a promising source of multifunctional antibacterial agents. Integrating metabolomic profiling with antibacterial assays also provides a useful foundation for future structure–activity studies and the development of halal-compliant, plant-based therapeutics.

CONCLUSION

The findings of this study present the comprehensive integration of antibacterial activity assessment and LC-HRMS-based metabolite

profiling of *P. macrocarpa* fruit extracts and fractions. With a MIC value as low as 3.125 ppm against *S. epidermidis* and an MBC value of 6,250 ppm against both *S. aureus* and *S. epidermidis*, EAFFF had the highest antibacterial action of all tested substances. EAFFF and BFFF contain various bioactive compounds, including flavonoids (eriodictyol, (±)-naringenin, and apigenin), phenolics (gallic acid), isoflavones (glycitein), and terpenoids (L-(−)-carvone), many of which have documented antimicrobial mechanisms such as membrane disruption, enzyme inhibition, and interference with biofilm formation. This study establishes a clear link between chemical composition and antibacterial potency, providing a solid foundation for isolating and characterizing the active constituents of *P. macrocarpa*. The use of a halal-compliant, sustainable water-based extraction method further supports its potential for developing natural antibacterial agents. Collectively, these results enhance current phytochemical and pharmacological insights into *P. macrocarpa* and underscore its promise for future structure–activity investigations and the development of novel antibacterial therapeutics.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest.

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