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Integrated Phytochemical Characterisation and LC-MS/MS Profiling of *Portulaca oleracea* with Antioxidant and Antimicrobial Activity Assessment

SARITA DIWAKAR[1], RASHMI VERMA[1], SANJOY KUMAR BERA[2], ANANYA DAS MAHAPATRA[3], MILAN HAIT[1] and NAND KUMAR KASHYAP[1,4,*]

[1]Department of Chemistry, Dr. C.V. Raman University, Kota, Bilaspur-495113, India
[2]Department of Chemistry, Dr. C.V. Raman University, Vaishali-844114, India
[3]Department of Biotechnology, Brainware University, Barasat, Kolkata-700125, India
[4]Government Higher Secondary School Hasuwa, Kasdol, Balodabazar-Bhatapara-493344, India

*Corresponding author: E-mail: nandk79@gmail.com

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The present study evaluates the phytochemical profile, antioxidant activity and antimicrobial potential of the ethanolic extract of *Portulaca oleracea*, revealing a high abundance of flavonoids, phenolic acids, tannins and cardiac glycosides, with weak terpenoid presence and absence of alkaloids, confirming ethanol as an effective solvent for polyphenol extraction. Quantitative analysis demonstrated significantly higher total phenolic and flavonoid contents compared to previously reported studies, reflecting the superior polyphenolic richness of the extract. LC-MS/MS profiling enabled the annotation of diverse bioactive constituents, including phenolic acids, flavonoids, flavonoid glycosides and high-molecular-weight polyphenols such as procyanidin oligomers and tannins, with consistent mzCloud and ChemSpider FISh scores supporting confident compound identification. The extract exhibited strong antioxidant activity, particularly in the DPPH free radical scavenging assay, with lower IC_{50} values than earlier reports, while $ABTS^+$ assay results indicated comparable but assay-dependent efficacy. Furthermore, the extract showed moderate broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, as evidenced by measurable zones of inhibition and minimum inhibitory concentrations. One-way ANOVA followed by Tukey's HSD post hoc analysis revealed no significant variation among the samples ($p > 0.05$), indicating a high degree of consistency and supporting the standardisation of *P. oleracea* extracts.

Keywords: *P. oleracae*, Phytochemical, LC-MS profile, Antioxidant activity, Antimicrobial potential.

INTRODUCTION

Phytochemical characterisation is essential in medicinal plant research as it enables the identification of bioactive secondary metabolites responsible for therapeutic effects. Medicinal plants contain diverse compounds including phenolics, flavonoids, alkaloids, terpenoids, tannins and glycosides, which contribute to a wide range of pharmacological activities [1,2]. Comprehensive phytochemical analysis not only validates traditional medicinal uses but also supports the discovery of novel compounds with pharmaceutical and nutraceutical potential [3]. The liquid chromatography–tandem mass spectrometry (LC-MS/MS) has become a powerful tool for profiling complex plant extracts, allowing accurate identification of metabolites and structural characterisation through fragmentation patterns. When integrated with phytochemical screening and biological assays, LC-MS/MS provides valuable insight into the relationship between chemical composition and biological activity [4].

Plant-derived phenolics and flavonoids are recognized for their antioxidant properties, including free-radical scavenging and metal-chelating activities, which help mitigate oxidative stress. In addition, the growing threat of antimicrobial resistance has intensified the search for alternative antimicrobial agents from natural sources [5,6]. Thus, medicinal plants offer considerable potential in this regard due to their chemical diversity and multiple mechanisms of action. *Portulaca oleracea* has attracted attention due to its traditional use as an antimicrobial remedy [7]; however, reported activities of its ethanolic extracts vary considerably depending on factors such as geographical origin, extraction procedures, target microorganisms and experimental conditions.

P. oleracea L. (family: Portulacaceae), commonly known as purslane, is a cosmopolitan, succulent herb extensively distributed throughout tropical, subtropical and temperate regions, including Asia, Africa and the Mediterranean basin. The plant is widely consumed as a leafy vegetable and has been deeply integrated into traditional medical systems such as Ayurveda, traditional Chinese medicine and various folk remedies, where it is prescribed for the treatment of gastrointestinal disorders, skin ailments, fever, diabetes and inflammatory conditions. Its long-standing dietary and medicinal use underscores its safety and therapeutic relevance [7]. Extensive ethnomedicinal documentation attributes a broad spectrum of pharmacological activities to *P. oleracea*, for example, anti-inflammatory, antimicrobial, antidiabetic, hepatoprotective, neuroprotective and wound-healing effects [8]. These diverse bioactivities have been primarily linked to its complex and abundant phytochemical composition. Previous investigations have reported the presence of various phytochemicals and essential vitamins, many of which are recognised for their antioxidant and antimicrobial potential [9]. In particular, flavonoids and phenolic compounds are known to modulate oxidative stress and inflammatory pathways, while alkaloids and terpenoids contribute to antimicrobial and cytoprotective effects. Despite increasing scientific interest in *P. oleracea* and its recognised medicinal value, detailed chemical characterisation of its bioactive constituents remains incomplete. The study was designed based on the hypothesis that the ethanolic extract of *P. oleracea* contains a chemically diverse pool of polyphenolic bioactive metabolites that contribute significantly to its antioxidant and antimicrobial activities. Although several previous studies have reported the general medicinal importance of *P. oleracea*, comprehensive LC-MS/MS-based metabolite profiling integrated with phytochemical quantification and biological activity evaluation remains limited. Therefore, the present work aimed to characterise the phytochemical composition of the ethanolic extract and correlate its metabolite profile with antioxidant and antibacterial potential.

The novelty of this study lies in the comprehensive characterisation of the ethanolic extract of *P. oleracea* through the combined use of qualitative phytochemical screening, quantitative phenolic and flavonoid analysis and high-resolution LC-MS/MS profiling. The analysis revealed a diverse range of bioactive metabolites including flavonoids, flavonoid glycosides, phenolic acids, procyanidins, and tannin derivatives, which were associated with strong antioxidant and moderate antibacterial activities. The extract also exhibited relatively high phenolic and flavonoid contents along with enhanced DPPH radical-scavenging activity compared with previous reports. These findings provide detailed metabolomic evidence supporting the potential of *P. oleracea* as a valuable natural source of antioxidant and antimicrobial compounds.

EXPERIMENTAL

Collection of plant materials: *Portulaca oleracea* plant was collected from Hariharpur area (21.99º N, latitude, 81.55º E, longitude, of 288 m), in Mungeli District, India. The plant was authenticated by the Botanical Survey of India (BSI), Central Regional Centre, Prayagraj, India. A voucher specimen was deposited at BSI under reference number 2706250077517 (27th June 2025) and an authentication certificate was issued under letter number BSI/CRC/Tech/2025-26/288, dated 2nd July 2025.The aerial plant parts including flowers, stem and leaves were cleaned with tap water.

Sample preparation: The leaves of *P. oleracea* were shade-dried at 29 ± 1 ºC. The dried plant material was then powdered manually using a mortar and pestle, sieved to obtain a uniform particle size and stored in airtight plastic bags to prevent exposure to moisture and contamination until further use.

Extract preparation: The Soxhelt extract technique used to making extract. Approximately 50 g of powdered sample was packed into a thimble made of Whatman filter paper and placed in a Soxhlet extraction apparatus. Extraction was carried out using ethanol as the extraction solvent in a round-bottom flask at the solvent's boiling temperature for 6-8 h until the solvent in the siphon tube became colourless. After completion of extraction, the obtained extract was filtered and concentrated under reduced pressure using a rotary evaporator at 40-45 ºC to remove the solvent. The concentrated crude extract was further dried to obtain a semisolid mass and stored in airtight containers at 4 ºC until further analyses.

Phytochemical screening: Phytochemical screening was carried out following established standard methods reported earlier [10,11].

Flavonoids (Shinoda test): Approximately 1 mL of plant extract was mixed with a small quantity of magnesium turnings, followed by the careful addition of a few drops of conc. HCl. The development of a pink, crimson or reddish colouration indicated the presence of flavonoids, due to the reduction of flavonoid compounds by magnesium in an acidic medium

Phenol ($FeCl_3$ test): A few drops of freshly prepared 5% $FeCl_3$ solution were added to 1 mL of the plant extract. The formation of a deep blue, green or black coloration confirmed the presence of phenolic compounds, resulting from complex formation between phenols and ferric ions.

Terpenoids (Liebermann-Buchard): About 1 mL of the extract was mixed with 2 mL of chloroform, followed by the careful addition of 1 mL of conc. H_2SO_4 along the sides of the test tube. The appearance of a reddish-brown, violet or green colouration at the interface indicated the presence of terpenoids and triterpenes.

Alkaloids (Dragendorff test): A 1 mL of extract was acidified with 2-3 drops of dilute HCl and filtered. To the filtrate, a few drops of Dragendorff's reagent were added. The formation of an orange or reddish-brown precipitate confirmed the presence of alkaloids.

Tannin (gelatine test): To 1 mL of the extract, 1 mL of 1% gelatin solution containing sodium chloride was added. The appearance of a white precipitate indicated the presence of tannins, due to the formation of insoluble tannin-protein complexes.

Cardiac glycoside (Keller-Killiani test): A 1 mL of the extract was treated with 1 mL of glacial acetic acid containing a trace amount of $FeCl_3$. This mixture was carefully underlayered with conc. H_2SO_4. The formation of a brown ring at the interface, along with bluish-green colouration in the acetic

acid layer, indicated the presence of cardiac glycosides, particularly deoxy-sugars characteristic of cardenolides.

Quantitative phytochemical analysis

Total phenolic content (TPC): The TPC of *P. oleracea* ethanolic extract was estimated using the Folin-Ciocalteu colorimetric technique, as reported by Molole *et al.* [12] with several modifications. In briefly, standard gallic acid solution was prepared by dissolving 10mg of gallic acid into 10 mL of methyl alcohol, resulting in a concentration of 1 mg/mL. Standard solutions of gallic acid in methanol have been made at various concentrations of 20, 40, 60, 80 and 100 µg/mL. The final volume of all concentrations was 10 mL, which was achieved by adding 5 mL of 10% Folin-Ciocalteu reagent and 4mL of 7% Na_2CO_3. The obtained Indigo blue-coloured solution was well agitated and then incubated for 30 min at 45 ºC in a water bath. After that, the absorbance was obtained by measuring it against a blank at 760 nm. The FCR reagent oxidizes the phenols in plant extracts, transforming their colour into a dark blue colour that a UV-Vis spectrophotometer then detects. The TPC was expressed as mg/GAE/g, with methanol used as blank. All measurements were performed in triplicate and the calibration curve was constructed using the mean absorbance values of gallic acid standards. Total phenolic content concentration was determined by using eqn. 1:

$$\text{Total phenolic content (\%)} = \frac{C_C \times V_S}{W_S} \times 100 \qquad (1)$$

where C_C is the concentration of control solution; V_S is the volume of sample extract in mL; W_S is the weight of sample extract in gram.

Total flavonoid content: The total flavonoid content of *P. oleracea* ethanolic extracts was assessed using the $AlCl_3$ colorimetric examination and previously described assay Shraim *et al.* [13] with slightly modifications. In brief, a stock solution of quercetin (4 mg/mL) was prepared by dissolving 4 g of quercetin in 1 mL of methanol. The standard solution was serially diluted to produce concentrations of 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL and 1 mg/mL. A 1 mL of quercetin at each concentration was added into the test tube containing 4 mL of distilled water. Simultaneously, 0.3 mL of 5% $NaNO_2$ was introduced to the test tube, followed by the addition of 0.3 mL of 10% $AlCl_3$ after a 5 min interval. Subsequently, 2 mL of 1 M NaOH was incorporated into the mixture after 6 min. The combined volume was adjusted to 10 mL by promptly adding 4.4 mL of distilled water. The total flavonoid content was expressed as mg/QE/g, with methanol used as the blank. All measurements were performed in triplicate, and the calibration curve was constructed using the mean absorbance values of quercetin standards. Total flavonoid content concentration was determined by using eqn. 2:

$$\text{Total flavonoid content (\%)} = \frac{C_C \times V_S}{W_S} \times 100 \qquad (2)$$

where C_C is the concentration of control solution; V_S is the volume of sample extract in mL; W_S is the weight of sample extract in gram.

LC-MS/MS analysis: High-resolution mass spectrometry (HR-MS/MS) analysis was carried out in the ethanolic extract of *P. oleracea* according to the reported methodology by Haron *et al.* [14] using a Q Exactive HF Orbitrap mass spectrometer (Thermo-Fisher Scientific, USA). Prior to mass detection, chromatographic separation of metabolites was achieved using Dionex UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo-Fisher Scientific, USA) equipped with a Syncronis C18 column (2.1 × 100 mm, 1.7 µm particle size). The column oven temperature was maintained at 55 ºC, while the mobile phase flow rate was set at 0.45 mL/min. Instrumental conditions, including gradient elution parameters and system calibration, were adopted from the protocol described by Teoh *et al.* [4]. The mobile phase consisted of solvent A (0.1% formic acid in HPLC-grade water) and solvent B (0.1% formic acid in acetonitrile). The gradient program was initiated at 0.5% solvent B for 1 min, followed by a linear increase to 95.5% solvent B over 15 min and subsequently held at this composition for 4 min. A sample injection volume of 2 µL was used for all analyses. After each run, the column was re-equilibrated under initial conditions for 2 min to ensure reproducibility. Raw data acquisition and processing were performed using Compound Discoverer software version 3.3 SP1 (Thermo-Fisher Scientific), with slight modifications to the standard natural products workflow. The processing steps included blank subtraction, retention time correction, peak detection, elemental composition prediction, spectral library comparison and fragment ion search (FISh)-based scoring. Compound annotation was primarily achieved through MS/MS spectral matching against the mzCloud database. Features that did not yield confident matches were further investigated using the ChemSpider database [15], with identification supported by FISh scores exceeding 50 to enhance annotation reliability.

Antioxidant activity

DPPH-FRS activity: The antioxidant potential of *P. oleracea* ethanolic extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, following the method described by Hussen & Endalew [16] with slight modifications. A stock solution of DPPH (0.2 mol/L) was freshly prepared in methanol and protected from light to prevent degradation. Various concentrations of *P. oleracea* extracts (20, 40, 60, 80 and 100 µg/mL) were prepared in ethanol. For each concentration, 200 µL of the DPPH solution was added to the extract samples, resulting in a total reaction volume adjusted to 1 mL. The reaction mixtures were vortexed gently to ensure uniform mixing and then incubated in the dark for 30 min at 35 ± 1 ºC to prevent light-induced decomposition of DPPH radicals. Ascorbic acid, prepared using the same procedure and concentration range, was employed as the standard antioxidant reference, while 100% methanol served as the blank control. After incubation, the absorbance of each reaction mixture was measured at 517 nm using a UV-visible spectrophotometer (model: Orion Aqua Mate 8100, Thermo-Scientific). The percentage of DPPH radical scavenging activity was calculated based on the reduction in absorbance relative to the control. The concentration of extract required to scavenge 50% of the DPPH radicals (IC_{50}) was determined by plotting the percentage inhibition against extract concentration and applying linear regression analysis. A lower IC_{50}

value corresponds to a higher free radical scavenging efficiency, indicating stronger antioxidant potential of the extract. The concentration of DPPH-FRS activity and the IC_{50} were determined using eqn. 3:

$$\text{DPPH-FRS inhibition (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \quad (3)$$

where $Abs_{control}$ is the absorbance of control solution; and Abs_{sample} is the absorbance of sample solution.

ABTS radical cation assay: Free radical scavenging activity of ethanolic extract of *P. oleracea* was determined by $ABTS^+$ radical cation decolorisation assay as described by Chaves *et al.* [17]. $ABTS^+$ cation radical was obtained by the reaction between 7 mM ABTS in water and 2.45 mM $K_2S_2O_8$ (1:1), stored in the dark at room temperature for 12-16 h before use. After the addition of 5 µL of plant extract to 3.995 mL of diluted $ABTS^+$ solution, the absorbance was measured at 30 min after the initial mixing. $ABTS^+$ solution was then diluted with methanol to obtain an absorbance of 0.700 at 734 nm UV-visible spectrophotometer. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Trolox was used as control. The concentration of $ABTS^+$ radical cation activity and the IC_{50} were determined using eqn. 4:

$$ABTS^+ \text{ inhibition (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \quad (4)$$

where $Abs_{control}$ is the absorbance of control solution; and Abs_{sample} is the absorbance of sample solution.

Antimicrobial activity: The antimicrobial activity of ethanolic extract of *P. oleracea* was evaluated using the agar disc diffusion method, following the slight modified procedure [18,19].

Preparation of microbial cultures: Pure cultures of test microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *etc.*) were obtained from standard microbial culture collections. Each strain was sub-cultured on nutrient agar slants and incubated at 37 ± 1 ºC for 24 h to ensure active growth. A loopful of the fresh culture was then transferred into nutrient broth and incubated for 4-6 h until the turbidity matched the 0.5 McFarland standard (equivalent to approximately 1×10^8 CFU/mL).

Preparation of media: Sterile Mueller-Hinton Agar (MHA) medium was prepared according to the manufacturer's instructions, sterilised by autoclaving at 121 ºC for 15 min and poured into sterile Petri dishes (~ 20 mL per plate). The plates were allowed to solidify under aseptic conditions.

Inoculation of test organisms: Once the agar solidified, each plate was uniformly inoculated with the standardised microbial suspension using a sterile cotton swab, ensuring even distribution over the entire surface to form a bacterial lawn. The plates were allowed to dry for 5 min at room temperature to allow proper absorption of inoculum.

Preparation and application of discs: Sterile Whatman No. 1 filter paper discs (6 mm diameter) were impregnated with known concentrations of the plant extract (*e.g.* 25, 50, 75 and 100 µg/disc) and dried to remove residual solvent. The impregnated discs were then gently placed on the surface of the inoculated agar plates using sterile forceps, ensuring firm contact with the agar. Ciprofloxacin discs were used as the positive control, while DMSO-impregnated discs served as the negative control to verify the absence of solvent-related antimicrobial effects. All plates were incubated in an inverted position at 37 ± 1 ºC for 24 h under aerobic conditions. For fungal strains (if included), Sabouraud Dextrose Agar (SDA) plates were used and incubated at 28 ± 2 ºC for 48 h.

After incubation, the antimicrobial activity was assessed by measuring the diameter of the zone of inhibition (ZOI) around each disc using a transparent millimeter scale. The results were expressed in millimeters (mm) as the mean of triplicate readings. Larger inhibition zones indicated stronger antimicrobial activity of the extract against the tested microorganisms.

Statistical analysis: All the experimental data were expressed as mean ± standard deviation (SD) based on triplicate measurements (n = 3). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA) to evaluate significant differences. A probability level of $p < 0.05$ was considered statistically significant. All statistical analyzes were conducted using SPSS software (Version 26, IBM Corp., USA).

RESULTS AND DISCUSSION

Phytochemical analysis: The preliminary phytochemical screening of the ethanol extract of *P. oleracea* revealed the presence of diverse secondary metabolites (Table-1). Strong positive reactions were observed for flavonoids, phenolic acids, tannins and cardiac glycosides indicating their high abundance in the ethanol extract. These findings suggest that ethanol is an efficient solvent for extracting polar bioactive compounds, particularly polyphenols, from *P. oleracea*. Flavonoids and phenolic acids showed higher intensity in the present study compared to the report of Khursheed & Jain [20], which may be attributed to variations in extraction protocol, plant maturity, geographical origin, or environmental conditions.

The detected phytochemicals, particularly phenolics, flavonoids, tannins and cardiac glycosides, are known to contri-

TABLE-1
QUALITATIVE PHYTOCHEMICAL ANALYTICAL DATA OF THE ETHANOLIC EXTRACT OF *P. oleracea* AND COMPARISON WITH LITERATURE DATA

Phytochemical	Applied test	Present work	Khursheed & Jain [20]
Flavonoids	Shinoda	Abundance	Medium
Phenolic acid	Ferric chloride	Abundance	Medium
Terpenoids	Liebermann-Buchard	Lower quantity	Medium
Alkaloids	Dragendorff	Absent	Lower quantity
Tannins	Gelatin	Abundance	Absent
Cardiac glycoside	Keller-Killiani	Abundance	Medium

bute to antioxidant, anti-inflammatory and antimicrobial activities. Tannins were identified in the present study but were not reported previously, which may reflect differences in plant source or extraction conditions. Terpenoids were detected in low amounts, whereas alkaloids were absent, differing from earlier findings. Such variations are commonly attributed to differences in extraction methods, solvent systems and environmental factors. The presence of cardiac glycosides further supports the pharmacological potential of the extract. These results indicate that *P. oleracea* is a rich source of bioactive polyphenolic compounds and support its observed antioxidant and antimicrobial properties, while highlighting the need for further quantitative and metabolomic studies.

Quantitative analysis: The quantitative phytochemical analysis of *P. oleracea* ethanol extract revealed a high abundance of phenolic and flavonoid compounds (Table-2). The total phenolic content (TPC) recorded in the present study was 37.19 ± 0.81 mg GAE/g, which is markedly higher than the values reported by Khursheed & Jain [20] and Fernandez-Poyatos *et al.* [21]. This increased phenolic concentration indicates a strong enrichment of redox-active compounds, which are known to play a central role in antioxidant and stress-mitigating activities. Similarly, the TFC of the ethanol extract was found to be 28.65 ± 0.65 mg DE/g, exceeding the values previously reported by Fernandez-Poyatos *et al.* [21] and slightly higher than those of Khursheed & Jain [20]. The higher flavonoid yield further confirms the efficiency of ethanol as an extraction solvent for polyphenolic constituents in *P. oleracea*. The differences observed between studies may be attributed to variations in extraction conditions, plant growth stage, solvent composition, geographical origin and analytical methods. The higher TPC and TFC values obtained in the present study were consistent with the strong antioxidant and antimicrobial activities observed, indicating the contribution of phenolics and flavonoids to the biological activity of *P. oleracea*.

TABLE-2
QUANTITATIVE DATA OF *P. oleracea* ETHANOLIC EXTRACT COMPARISION WITH REPORTED DATA

Solvent	This work	Khursheed & Jain [20]	Fernandez-Poyatos *et al.* [21]
Total phenolic content (mg/GAE/g)			
Ethanol	37.19 ± 0.81	21.75 ± 0.21	23 ± 0.95
Total flavonoid content (mg/DE/g)			
Ethanol	28.65 ± 0.65	25.12 ± 0.11	19 ± 0.79

LC-MS analysis: LC-MS/MS analysis of the ethanolic extract of *P. oleophera* identified nineteen phytochemical constituents (Table-3). The LC-MS/MS spectrum of the ethanolic extract of *P. oleracea* demonstrates several characteristic precursor and fragment ions that support annotation of polyphenolic. The spectrum shows a dominant base peak at *m/z* 219.000 confirmed the presence of a highly abundant acetylated phenolic derivative. Additional prominent ions observed at *m/z* 133.000, 168.000, 191.000, 263.050, 291.050, 335.050, 377.100, 404.150, 453.150, 518.150, 581.250, 632.300, 706.250, 719.300, 777.300 and 795.200 correspond to phenolic acids, flavonoids, glycosylated flavonoids, procyanidin oligomers and tannin-like polyphenolic constituents (Fig. 1). The sequential mass differences observed among higher molecular weight ions further suggest glycosylation and oligo-

TABLE-3
LIST OF IDENTIFIED PHYTOCHEMICAL CONSTITUENTS OF ETHANOLIC EXTRACT OF *P. oleracae* BY LC-MS

S. No.	Candidate mass (*m/z*)	Major MS^2 ions (*m/z*)	m.f.	Proposed compound name	Compound class	mzCloud match score (%)	ChemSpider FISh score
1	133	115, 105	$C_8H_5O_2^+$	Hydroxybenzyl cation	Phenolic	80	0.65
2	168	150, 122	$C_8H_8O_4$	Vanillic acid	Aromatic acid	85	0.68
3	191	173, 149	$C_7H_{11}O_6$	Quinic acid	Organic acid	90	0.72
4	219	191, 177, 161	$C_{12}H_{11}O_4$	Acetylated phenolic acid	Phenolic	95	0.82
5	263	219, 191	$C_{15}H_{11}O_5$	Apigenin	Flavonoid	92	0.78
6	291.05	263, 219	$C_{15}H_{11}O_6$	Kaempferol	Flavonoid aglycone	94	0.81
7	335.05	291, 263	$C_{16}H_{15}O_8$	Feruloyl hexose	Glycosylated phenolic	88	0.75
8	377	335, 291	$C_{18}H_{17}O_9$	Quercetin-O-pentoside	Flavonoid glycoside	90	0.79
9	404.15	377, 335	$C_{19}H_{20}O_{10}$	Quercetin-O-acetyl glycoside	Flavonoid	86	0.74
10	453.15	404, 377	$C_{21}H_{25}O_{11}$	Quercetin-3-O-glucoside (isoquercitrin)	Polyphenolic	85	0.71
11	518.15	453, 404	$C_{24}H_{30}O_{13}$	Diglycosylated flavonoid	Polyphenolic	82	0.69
12	539.25	518, 453	$C_{25}H_{31}O_{14}$	Rutinose-linked flavonoid	Glycosylated polyphenol	80	0.67
13	581.25	539, 518	$C_{27}H_{33}O_{15}$	Flavonoid dimer (procyanidin B-type)	Polyphenolic oligomer	78	0.64
14	632.3	581, 539	$C_{29}H_{36}O_{17}$	Procyanidin dimer glycoside	Dimeric phenolic compound	75	0.62
15	646.25	632, 581	$C_{30}H_{38}O_{17}$	Sodium-adducted procyanidin	Phenolic ion	71	0.6
16	706.25	646, 632	$C_{32}H_{42}O_{19}$	Trimeric Flavonoid	Poly phenol	70	0.58
17	719.3	706, 646	$C_{33}H_{43}O_{19}$	Flavonoid trimeric	Polyphenolic	68	0.56
18	777.3	719, 706	$C_{36}H_{49}O_{21}$	Polymerised proanthocyanidin	Polymerised phenolic	65	0.54
19	795.2	777, 719	$C_{37}H_{47}O_{22}$	Tannin	Phenolic	62	0.52

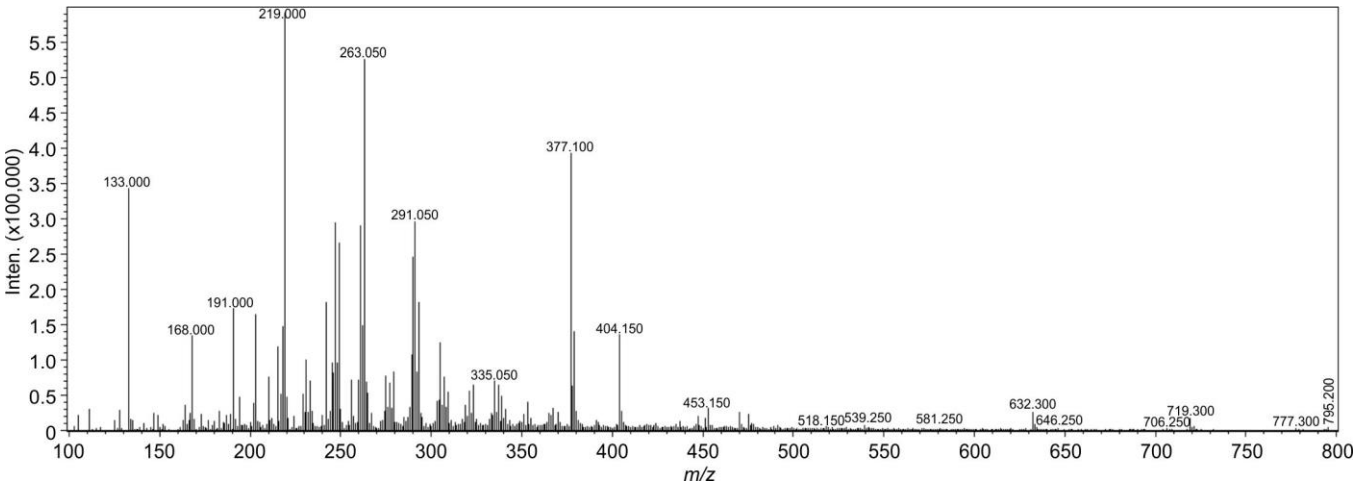

Fig. 1. LC-MS spectrum of ethanol extract of *P. oleracae* (positive ion mode)

merisation patterns commonly associated with flavonoid conjugates and condensed tannins. Compound annotation was achieved based on accurate mass measurements, characteristic MS^2 fragmentation patterns and spectral similarity scores obtained from mzCloud and ChemSpider FISh databases. The identified metabolites predominantly belonged to phenolic acids, flavonoids, flavonoid glycosides and oligomeric/polymerised polyphenols demonstrating that the ethanol extract is rich in bioactive secondary metabolites. Low molecular weight ions detected at *m/z* 133, 168 and 191 were assigned to hydroxybenzyl cation, vanillic acid and quinic acid, respectively.

The high mzCloud match scores (80-90%) and diagnostic fragment ions support the reliability of these assignments. The presence of quinic acid further suggests the activation of the shikimate pathway, which is central to the biosynthesis of phenolic acids and flavonoids. The most intense ion was observed at *m/z* 219, corresponding to an acetylated phenolic acid, which appeared as the base peak and showed the highest mzCloud match score (95%) and FISh score (0.82). The compounds are well documented for their strong free radical scavenging ability, treatment of inflammation, infections and oxidative stress related disorders. Mid-range *m/z* values (263-377) were dominated by flavonoid aglycones and glycosides such as apigenin (*m/z* 263), kaempferol (*m/z* 291.05), feruloyl hexose (*m/z* 335.05) and quercetin-O-pentoside (*m/z* 377). These compounds exhibited characteristic fragmentation involving neutral losses of sugar moieties and phenolic substituents, confirming their flavonoid nature. Flavonoids like apigenin, kaempferol and quercetin derivatives are extensively reported to possess antioxidant, anti-inflammatory, hepatoprotective and anticancer activities, which aligns with the ethnopharmacological use of *P. oleophera* in traditional medicine [22]. Higher molecular weight ions in the range of *m/z* 404.15-539.25 were identified as acylated, mono- and diglycosylated flavonoids such as quercetin-O-acetyl glycoside, isoquercitrin and rutinose-linked flavonoids

Compounds detected at *m/z* ≥581 were assigned to polyphenolic oligomers and polymerised phenolics, namely procyanidin B-type dimers**,** procyanidin glycosides, trimeric flavonoids and tannin derivatives. Although these high molecular weight compounds showed comparatively lower mzCloud and FISh scores, their classification was supported by consistent fragmentation behaviour typical of condensed tannins and proanthocyanidins. These compounds are known for their strong antioxidant capacity, metal-chelating properties and antimicrobial effects and are often implicated in the wound-healing and anti-diarrheal applications. LC-MS/MS profile of the ethanolic extract of *P. oleophera* reveals a chemically diverse phenolic-rich composition, with flavonoids and their conjugates as the dominant constituents, followed by oligomeric and polymerised polyphenols. The high abundance of these compounds may contribute to the medicinal properties of *P. oleracea* and supports its potential use in pharmaceutical applications.

Antioxidant activity: The antioxidant potential of the ethanolic extract of *P. oleracea* was evaluated using DPPH free radical scavenging (DPPH-FRS) and $ABTS^+$ cation radical assays and the results were compared with previously reported studies. The extract demonstrated considerable antioxidant potential in both assays (Table-4). In the DPPH assay, the ethanolic extract exhibited an IC_{50} value of 1.59 ± 0.28 mg/GAE/g, which was lower than the values reported by Fernandez-Poyatos *et al.* [21] (3.9 ± 0.43 mg/GAE/g) and Khursheed & Jain [20] (2.44 ± 0.14 mg/GAE/g), indicating comparatively stronger free radical scavenging activity. Similarly, in the $ABTS^+$ radical cation assay, the ethanolic extract showed an IC_{50} value of 3.14 ± 0.21 mg/TE/g. Although the activity was comparatively lower than reported findings by Fernandez-Poyatos *et al.* [21] (1.02 ± 0.37 mg/TE/g) and Khursheed & Jain [20] (1.52 ± 0.005 mg/TE/g), the extract still exhibited substantial antioxidant efficacy. The results demonstrated that the ethanolic extract possesses considerable radical-scavenging activity and may serve as a natural antioxidant source. This activity is likely related to the presence of phenolics, flavonoids and other reducing compounds extracted efficiently by ethanol. The lower IC_{50} value obtained in the DPPH assay compared with previous reports suggests a higher free-radical neutralizing capacity of the extract. Differences from earlier studies may be attributed to variations in geographical origin, plant maturity, extraction procedures, solvent systems and phytochemical composition. Although the extract showed a

TABLE-4
COMPARISON OF ANTIOXIDANT ACTIVITY DATA OF *P. oleracea* ETHANOLIC EXTRACT WITH SIMILAR FINDINGS

Plant extract	Present work	Fernandez-Poyatos *et al.* [21]	Khursheed & Jain [20]
DPPH-FRS assay IC_{50} (mg/GAE/g)			
Ethanol	1.59 ± 0.28	3.9 ± 0.43	2.44 ± 0.14
$ABTS^{+}$ radical cation assay IC_{50} (mg/TE/g)			
Ethanol	3.14 ± 0.21	1.02 ± 0.37	1.52 ± 0.005

TE = Trolax equivalent

higher IC_{50} value in the ABTS assay, reflecting the differences in scavenging activity toward different radical species, appreciable antioxidant activity was observed in both DPPH and ABTS models, supporting its potential therapeutic value.

Antimicrobial activity: The antimicrobial activity of the ethanolic extract of *P. oleracea* was evaluated against Gram-positive (*S. aureus*, *B. subtilis*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria and compared with previous studies. The extract showed antibacterial activity against all tested strains, with the highest inhibition observed for *S. aureus* and *B. subtilis* (ZOI = 13 mm), followed by *E. coli* (12 ± 0.21 mm) and *P. aeruginosa* (11 ± 0.28 mm) (Table-5). MIC values ranged from 2.9 to 5.3 mL. Although the antimicrobial activity was lower than that reported by Khursheed & Jain [20] and Sheikh *et al.* [23], the extract demonstrated consistent activity against clinically relevant pathogens. Higher susceptibility of Gram-positive bacteria compared with Gram-negative bacteria may be related to the absence of the outer lipopolysaccharide membrane that limits phytochemical penetration in Gram-negative species. The results confirm the broad spectrum antibacterial potential of *P. oleracea* and support its traditional medicinal use.

Statistical analysis

One-way ANOVA test: One-way analysis of variance (ANOVA) was performed to determine whether the differences in the zones of inhibition (ZOI) produced by the ethanolic extract of *P. oleracea* against the tested microorganisms were statistically significant. Significant differences were observed across zone of inhibition at the 95% confidence level ($p < 0.05$). The corresponding F- and *p*-values are shown in Table-6, while Tukey's HSD post-hoc comparisons are provided in Table-7. The ANOVA results revealed a between-group sum of squares of 61.6691 with 2 degrees of freedom (DF), resulting in a mean square value of 30.8345. The within-group (error) sum of squares was 1263.9539 with 17 DF and a mean square value of 74.3502. The calculated F-statistic was 0.4147 with a corresponding *p*-value of 0.667. Since the *p*-value was greater than the significance threshold of 0.05 ($p > 0.05$), the differences observed among the group means were statistically non-significant. This indicates that there was no significant variation in the antimicrobial activity of the ethanolic extract among the tested bacterial strains based on the measured ZOI values. The relatively low F-value further suggests that the variability within the groups was higher than the variability between the groups. Therefore, the antimicrobial effects exhibited by the extract against different microorganisms were comparatively uniform.

Tukey HSD post-hoc comparison: Post hoc multiple comparison analysis was performed following one-way ANOVA to evaluate pairwise differences among the tested groups. The comparison between x1 and x2 showed a mean difference of 4.2408 with a standard error (SE) of 3.2928 and a Q-value of 1.2879. The 95% confidence interval (CI) ranged from -7.7054 to 16.1871, while the *p*-value was 0.6411, indicating a statis-

TABLE-5
COMPARISON OF ANTIMICROBIAL ACTIVITY DATA OF *P. oleracea* ETHANOLIC EXTRACT WITH SIMILAR FINDINGS

Test organism	Present finding		Khursheed & Jain [20]		Sheikh *et al.* [23]	
	ZOI (mm)	MIC (µg/mL)	ZOI (mm)	MIC (µg/mL)	ZOI (mm)	MIC (µg/mL)
S. aureus	13 ± 0.22	5.3	21.5 ± 0.58	0.05	16 ± 0.577	No activity
B. subtilis	13 ± 0.39	4.8	23.5 ± 0.16	0.07	No activity	No activity
E. coli	12 ± 0.21	3.6	16.7 ± 0.58	0.14	15 ± 0.577	No activity
P. aeruginosa	11 ± 0.28	2.9	No activity	No activity	16 ± 0.577	No activity

TABLE-6
ONE-WAY ANOVA ANALYSIS FOR ZONE OF INHIBITION (ZOI) of *P. oleracea* ETHANOLIC EXTRACT

Source	Degree of freedom	Sum of square	Mean of square	Fisher's value	P-value
Group (between groups)	2	61.6691	30.8345	0.4147	0.667
Error (within group)	17	1263.9539	74.3502		
Total	19	1325.6229	69.7696		

TABLE-7
TUKEY HSD POST-HOC TEST ANTIMICROBIAL ACTIVITY DATA OF *P. oleracea*

Pair	Differences	SE	Q	LCI	UCI	CM	*p*-value
x1-x2	4.2408	3.2928	1.2879	-7.7054	16.1871	11.9463	0.6411
x1-x3	1.8593	3.2928	0.5647	-10.0869	13.8056	11.9463	0.9163
x2-x3	2.3815	3.3815	0.6765	-10.3896	151526	12.7711	0.8823

SE = standard error, CI = confidence interval, LCI = lower confidence interval, UCI = upper confidence interval, CM = critical mean, HSD = honestly significant difference

tically non-significant difference between the two groups. Similarly, the comparison between x1 and x3 revealed a mean difference of 1.8593 with an SE of 3.2928 and a Q-value of 0.5647. The lower and upper confidence interval values ranged from -10.0869 to 13.8056, respectively. The obtained *p*-value of 0.9163 demonstrated that the difference between x1 and x3 was also statistically non-significant. For the comparison between x2 and x3, the mean difference was 2.3815 with an SE of 3.3815 and a Q-value of 0.6765. The confidence interval extended from -10.3896 to 12.7711 and the *p*-value was 0.8823, further confirming the absence of significant variation between these groups. Thus, all comparisons yielded *p*-values higher than 0.05, with confidence intervals crossing zero, confirming the absence of statistically significant differences among the tested groups. These results agree with the ANOVA findings and indicate comparable antimicrobial activity of the ethanolic extract of *P. oleracea* across the studied microorganisms.

Conclusion

The ethanolic extract of *P. oleracea* was found to be rich in flavonoids, phenolic acids, tannins, and cardiac glycosides, demonstrating the effectiveness of ethanol for extracting bioactive constituents. The extract exhibited relatively high total phenolic and flavonoid contents and showed strong antioxidant activity, particularly in the DPPH assay, confirming its free radical scavenging potential. LC-MS/MS analysis identified a diverse range of polyphenolic compounds, including flavonoids, phenolic acids, glycosides and oligomeric phenolics, supporting the observed biological activities. The extract also displayed moderate antibacterial activity against both Gram-positive and Gram-negative bacteria, with measurable ZOI and MIC values. Statistical analysis using one-way ANOVA and Tukey's HSD test revealed no significant differences ($p > 0.05$) among the tested groups, indicating consistent antimicrobial performance. The presence of multiple bioactive phytochemicals suggests potential applications in nutraceutical, functional food and phytopharmaceutical formulations. Future studies should focus on bioactivity-guided isolation of active compounds, mechanistic investigations, toxicity assessment, and comparative solvent extraction studies to further establish the therapeutic potential of *P. oleracea*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

DECLARATION OF AI-ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the authors used an AI-assisted tool(s) to improve the language. The authors reviewed and edited the content and take full responsibility for the published work.

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