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Scientific validation of an Ayurvedic herbal preparation Chandraprabha vatika

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ABSTRACT

Background: Chandraprabha Vatika (CV) is a widely used polyherbal Ayurvedic drug known for its therapeutic applications in urinary and metabolic disorders. This study investigates the phytochemical profile, antioxidant activity, antimicrobial activity, antibiofilm assay, SEM analysis, cytotoxicity, and HR-LCMS analysis of CV.
Methods: Ethanolic extract of CV(ECV) was prepared using the Soxhlet extraction method, followed by rotary evaporation and lyophilization. Preliminary phytochemical screening, antimicrobial, and antibiofilm studies were conducted, and the antioxidant activity was analyzed using DPPH, FRAP, and ABTS assays. The further cytotoxicity of ECV was tested against L929 and HEK293 cell lines, and SEM analysis was conducted, which led to the choice of HR-LCMS analysis for identifying its bioactive constituents.
Results: Phytochemical analysis confirmed the existence of flavonoids, phenolics, tannins, alkaloids, saponins, proteins, glycosides, carbohydrates, amino acids, and steroids. The phenolic content was 13.74 mg GAE/g extract, and the flavonoid content was 36.57 mg QE/g extract. The ethanolic extract exhibited antioxidant assay with IC_{50} values of 37.346 μg/ml (DPPH), 25.452 μg/ml (FRAP), and 48.05 μg/ml (ABTS). CV extract proved to be a potent antimicrobial agent, inhibiting Gram-positive and Gram-negative bacteria. It displayed varying MIC and MBC values, also showing excellent antibiofilm activity when cultured with MDR strains. Cytotoxicity assays confirmed that ECV was non-toxic to L929 and HEK-293 cells at all tested concentrations. SEM analysis confirmed significant cellular damage in gram positive and gram negative bacteria. HR-LCMS analysis revealed bioactive compounds that could be linked to the therapeutic properties of CV.
Conclusion: The findings validate ECV as a good ayurvedic preparation having myriad therapeutic applications, including antimicrobial and anti-oxidant activity without cytotoxity

1. Introduction

Plants contain biologically active phytochemicals, which have been demonstrated to have substantial medicinal potential (Brahma et al., 2024). Over the years, phytochemistry has gained momentum due to the escalating research interest in natural and green remedies for treating a wide spectrum of diseases (Jacob et al., 2024). These pharmacologically active substances, including terpenoids flavonoids, alkaloids, polyphenols and, saponins, which are the integral components of herbal formulations and contribute to various bioactivities such as immunomodulatory, anti-inflammatory, antioxidant, and antibacterial effects. These phytochemical constituents exert their pharmacological effects through multiple mechanisms, including reactive oxygen species (ROS) scavenging, modulation of inflammatory pathways, disruption of microbial cell membranes, and inhibition of bacterial biofilm formation (Konkeri et al., 2025; Swami et al., 2024).

In addition to these compounds, several traditional herbal formulations, such as Triphala, Ashwagandha Churna, Brahmi Vati, and Dashmoolarishta, remain widely utilized in India for their rejuvenating, adaptogenic, and therapeutic properties (Sahoo and Mahanta, 2022).

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Chandraprabha Vatika (CV), a traditional Ayurvedic medicine, is known for its therapeutic benefits in managing metabolic, urinary, and reproductive disorders, including inflammation, diabetes, and urinary tract infections (Christa et al., 2013). Correlating CV's historic uses with scientific evidence, particularly in contemporary therapeutic applications, requires understanding its phytochemical nature. The main ingredients of CV include Haritaki (*Terminalia chebula*), Amalaki (*Emblica officinalis*), *Shilajit, Vacha, Haridra, Musta, Bhunimba*, and *Guduchi* (*Tinospora cordifolia*), all of which enhance the formulation's overall therapeutic effectiveness. These components include strong bioactive substances like flavonoids, gallic acid, polyphenols, and tannins that have been researched for their potential to improve health (Charkha et al., 2020). The synergy between these ingredients makes CV a powerful herbal remedy, capable of modulating various physiological processes and protecting the body from oxidative stress and microbial infections (Dessai, 2012; Raj and Joseph, 2016).

Previous studies by Dongre & Majumdar (2024), and Sahoo & Mahanta (2022) on CV have primarily focused on its traditional applications and phytochemical composition, emphasizing its efficacy in managing metabolic and urinary disorders. While key bioactive compounds mentioned earlier are elucidated, the comprehensive analyses of the formulation using advanced analytical techniques remain limited. Furthermore, studies have not extensively correlated CV's phytochemical profile with its antimicrobial, antioxidant, and cytotoxic properties (Dongre and Majumdar, 2024; Sahoo and Mahanta, 2022; Tripathi et al., 2016). This study systematically investigates the phytochemical profile, antibacterial, antibiofilm, cytotoxic, and antioxidant properties of ECV, while integrating HR-LCMS analysis to comprehensively characterize its bioactive constituents. By correlating the phytochemical profile with antimicrobial, antibiofilm, SEM-based morphological observations, antioxidant capacity, and cytotoxic responses, the present work adopts a multi-analytical experimental framework for the scientific validation of CV. Such an integrated approach provides deeper insight into the relationship between its phytochemical composition and biological activities, thereby strengthening the scientific basis of this traditional Ayurvedic formulation and bridging the gap between traditional knowledge and modern pharmacological evaluation.

2. Materials and methods

2.1. Chandraprabha vatika

Chandraprabha Vatika (CV) was obtained from Kottakkal Arya Vaidya Sala, Tiruvalla, Kerala. (Batch No. 221557) To obtain the extract from CV, the Soxhlet extraction process was performed using a thimble loaded with approximately 30 g of the finely powdered CV and 250 ml of HPLC-grade ethanol. After extraction, the extract is concentrated through a rotary evaporator and then subjected to lyophilization and kept at 4 °C for subsequent analysis

2.2. Extraction yield

We used the calculation to show the yield of ECV as a percentage (Merlin et al., 2022).

$$Extraction\ YiELD = \frac{(Final\ extract\ weght)}{Initial\ sample\ weight} \times 100$$

2.3. Batch-to-batch comparative analysis of ECV

The polyherbal formulation, CV, was subjected to batch-to-batch comparative analysis using ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS). Two independent batches (Batch No. 221557 and 220242) were analyzed to evaluate the consistency of their phytochemical composition. The analysis was performed using a Shimadzu LC–MS 8045 system equipped with a C18 column (2.1 × 150 mm, 1.9 μm particle size). Chromatographic separation was carried out using a gradient mobile phase consisting of 0.1% formic acid in water and methanol, following the solvent program presented in Table S6 (the detailed gradient program is provided in the Supplementary Materials). The mass spectrometer was operated under the following conditions: nebulizing gas flow of 2 L/min, interface temperature of 300 °C, and system pressure ranging from 0 to 1326 kgf/cm^2. Data acquisition was performed over an m/z range of 100–2000 at a flow rate of 0.3 mL/min, with an injection volume of 10 μL. The obtained chromatographic and mass spectral data were processed and analyzed using LabSolutions software to compare the retention time and mass profiles of the detected compounds across the two batches (Rahman et al., 2025).

2.4. Phytochemical screening test

Standard qualitative phytochemical tests were conducted to evaluate the presence of different bioactive components (Al-Ogaili et al., 2023).

2.4.1. Qualitative phytochemical screening

2.4.1.1. Test for alkaloids. Hager's test was conducted using 2 ml of the ECV, which was treated with 4 - 5 drops of Hager's reagent. Simultaneously, Wagner's test was also done. ECV(3 ml) and Wagner's reagent (3 drops) were treated together, similar to the protocol followed by (Al-Ogaili et al., 2023).

2.4.1.2. Test for steroids. 3 ml of conc. Sulfuric acid, and 2 ml of chloroform was mixed with 5 ml of the ECV. Subsequently the mixture was heated for five minutes (Malviya et al., 2021).

2.4.1.3. Test for saponins. A few drops of sodium carbonate (Na_2CO_3) were added to 5 ml of the ECV and shaken vigorously. (Al-Ogaili et al., 2023).

2.4.1.4. Tests for flavonoids. ECV extract(2 ml) was mixed with 2–3 drops of NaOH and a few drops of HCl (Sasikala et al., 2018).

2.4.1.5. Test for phenolic and tannins. To detect phenolics, the gelatin test involves mixing ECV with a 1% gelatin solution containing 10% NaCl. The lead tetra-acetate test involves combining ECV with a 0.5 ml solution. The iodine test involves adding 4–5 drop of iodine solution to 2 ml of extract. The ferric chloride test for tannins involves adding 5% neutral ferric chloride solution to 1 ml of extract and observing color changes (Sasikala et al., 2018).

2.4.1.6. Test for carbohydrates. The presence of carbohydrates in ECV is determined through qualitative tests like Molisch, Benedict's, and Fehling's, which involve adding an alcoholic α-naphthol solution (Malviya et al., 2021).

2.4.1.7. Test for glycosides. In the Keller-Killiani test, ECV (2 ml) was combined with a solution of 0.5 ml (containing 2–3 drops of ferric chloride and glacial acetic acid). According to the protocol of Al-Ogaili et al., 2023, conc. H_2SO_4 (1 ml) was carefully added along the inner walls of the test tube.

2.4.1.8. Test for protein. Millon's and Biuret tests detect protein in the ECV. Millon's test mixes the ECV with a specific reagent. Biuret test adds sodium hydroxide and copper sulfate to the extract (Malviya et al., 2021).

2.4.1.9. Test for amino acid. 5 drops of nitric acid are mixed with 2 ml of ECV for the Xanthoproteic test (Shinde et al., 2021).

2.4.2. Quantitative phytochemical screening

2.4.2.1. Estimation of the total phenolic content (TPC). TPC of ECV was determined using the Folin–Ciocalteu reagent assay. The results were expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g extract). (Al-Ogaili et al., 2023); (Atanassova et al., 2011).

2.4.2.2. Estimation of total flavonoid content (TFC). TFC of ECV was estimated using the aluminum chloride colorimetric method, and the results were expressed as milligrams of quercetin equivalents per gram of dried extract (mg QE/g extract) (Atanassova et al., 2011).

2.5. Antioxidant assay

2.5.1. DPPH scavenging assay

The radical scavenging potential of the ECV against stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals was evaluated. Various concentrations of the extract (6.25–100 µg/ml) were allowed to react with a 0.1 mM ethanolic DPPH solution. After vigorous mixing, the reaction mixtures were kept in darkness for 30 min. The absorbance measured at 520 nm using a UV-spectrophotometer. A control was prepared using ethanol and DPPH, while gallic acid served as the standard (Merlin et al., 2022; Plyduang et al., 2021). The percentage of DPPH scavenging was calculated using the formula:

$$DPPH\ Scavenging\ activity\ (\%) = \frac{(Acontrol - Asample)}{Acontrol} \times 100.$$

The IC_{50} value, representing the concentration required to achieve 50% antioxidant activity, was determined from the scavenging activity data.

2.5.2. Ferric reducing antioxidant power (FRAP)

The reducing capacity of the ECV was determined using the FRAP assay. A standard curve was prepared using different concentrations of ascorbic acid (6.25 - 100 µg/ml). For the test, 2.85 ml of the FRAP working reagent was combined with each sample or standard. The mixtures were incubated in the dark for 30 min, after which the absorbance was read at 593 nm. Ethanol was used as a blank. The antioxidant capacity based on reducing power was expressed as micrograms of ascorbic acid per milliliter (µg /ml) (Ahmed et al., 2015; Al-Mansoub et al., 2014).

2.5.3. ABTS radical scavenging assay

The ability of the extract to scavenge the $ABTS^{\cdot+}$ radical cation was also investigated. A stock solution of the ECV (10 mg/ml) was prepared and serially diluted (6.25–100 µg/ml), labeled T1 to T5. Each dilution was mixed with a 7 mM ABTS solution and left to stand in the dark for 15 min. Ascorbic acid (10 mM) was used as the standard reference. The decrease in absorbance was monitored at 734 nm. All analyses were conducted in triplicate to ensure result reliability (Yoon et al., 2024). The percentage of ABTS radical scavenging was determined as follows:

$$\text{Radical scaveging activity}\ (\%) = \frac{(Acontrol\ OD - Asample\ OD)}{Acontrol\ OD} \times 100$$

All antioxidant assays were performed in triplicate, and the results were expressed as mean ± standard deviation. The antioxidant activity of the extract was compared with standard antioxidants (gallic acid for DPPH and ascorbic acid for FRAP and ABTS) used as reference compounds.

2.6. Cytotoxic activity (MTT assay)

The potential cytotoxicity of the ECV was assessed on two cell lines: L929 mouse fibroblast cells and HEK-293 human embryonic kidney cells using the MTT colorimetric assay. Cells were seeded into 96-well plates at a density of 200 µl per well and allowed to adhere overnight in an incubator set at 37 °C with a 5% CO_2 atmosphere. The test compound was added at varying concentrations, from 0.01 to 100 µg/ml, and the microplates were incubated for an additional 24 h under the same conditions. The ECV extract was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and subsequently diluted with the culture medium to obtain the required concentrations. The final concentration of DMSO was maintained below 0.1% to avoid solvent-induced cytotoxicity, and a solvent control containing the same concentration of DMSO without extract was included in the experiment. After incubation, the spent medium was removed, and MTT reagent (0.5 mg/ml) was added to each well. Plates were covered to protect them from light and incubated for 3 h. The resulting formazan crystals were dissolved by gentle shaking using an appropriate solvent. The assay included three controls: a medium control (medium without cells) to eliminate background interference, a negative control (untreated cells) to determine baseline viability, and a positive control (cells treated with ~12 µM doxorubicin) to validate assay sensitivity. Absorbance was measured at 570 nm using an ELISA plate reader. The selection of L929 and HEK-293 cell lines was based on their relevance for evaluating general cytocompatibility and kidney-associated cellular responses, thereby enhancing the biological and translational relevance of the safety assessment in relation to urinary tract disorders. The assay was designed to evaluate the cytocompatibility of the extract on normal mammalian cells; therefore, normal cell lines were selected. (Khammaneejan et al., 2020; Saharkhiz et al., 2023)

2.7. Antibiotic susceptibility and multiple antibiotic resistance (MAR) analysis of bacterial strains

The study included multidrug-resistant strains of *Escherichia coli, Pseudomonas aeruginosa,Acinetobacter baumannii, Klebsiella pneumoniae, Acinetobacter baumannii, MRSA* (*Methicillin Resistance Staphylococcus aureus*), and *Enterococcus faecalis.* These strains were incubated for 24 h at 37 °C after being inoculated on nutrient agar slants. The Kirby-Bauer disc diffusion method was used to evaluate antibiotic resistance. The antibiotic discs that were examined included Ampicillin, Penicillin, Methicillin, Cefazolin, Cefuroxime, Levofloxacin, Ciprofloxacin, Nalidixic Acid, Norfloxacin, Nitrofurantoin, Imipenem, Amikacin, Tetracycline, Colistin, Cefepime, Fosfomycin, Erythromycin, Ofloxacin, Gentamicin, Chloramphenicol, and Bacitracin. Bacterial cultures adjusted to 0.5 McFarland standard in nutrient broth were evenly spread on Mueller-Hinton agar plates using sterile swabs (Sharma and Godatwar, 2022). The Multiple Antibiotic Resistance Percentage (MAR %) of the pathogen was calculated using the formula:

$$MAR\ \% = \frac{No\ of\ antibiotics\ to\ which\ the\ isolate\ resisted}{Total\ number\ of\ antibiotics\ tested}\ x\ 100$$

2.8. Antimicrobial activity

The antibacterial activity of ECV against bacterial pathogens isolated from urinary tract infections was examined using the agar well diffusion technique. Different concentrations of ECV (10%, 20%, and 30% w/v) were prepared in dimethyl sulfoxide (DMSO) as the solvent. In this assay, DMSO served as the negative control, and a 1% w/v azithromycin was used as the positive control (Sharma and Godatwar, 2022).

2.9. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A microtiter plate-based resazurin assay was conducted to determine the MIC and MBC of the ECV. Briefly, 100 µl of Mueller-Hinton broth was aliquoted into each well of a 96-well plate. An initial concentration of ECV (200 mg/ml) was added to the first well and subjected to a two-fold

serial dilution across the plate. A 20 µl suspension of the test bacteria (adjusted to 1×10^8 CFU/ml) was then introduced into each well. The plates were incubated at 37 °C for 24 h. Following incubation, 50 µl of resazurin dye solution was added to each well. A visual color change from blue to pink indicated bacterial growth, and the MIC was recorded as the lowest concentration of the extract that prevented this color change, indicating no bacterial growth. For MBC determination, a small volume from each well showing no growth in the MIC test was streaked onto fresh Mueller-Hinton agar plates. These plates were incubated at 37 °C for 24 h. The MBC was defined as the lowest concentration of the ECV that did not support any bacterial colonies (Adelakun et al., 2024; Mekky et al., 2021).

2.10. Biofilm formation in Congo red agar

This is a semiquantitative assessment method. The CRA medium was prepared by mixing 100 ml of distilled water with Congo Red dye (0.16 g), agar (3 g), sucrose (10 g), and brain heart infusion broth (7.4 g). The Congo Red stain was autoclaved separately at 121 °C for 15 min and then added to the autoclaved brain-heart infusion agar at 55 °C along with sucrose. Test organisms were inoculated onto five CRA plates and incubated at 37 °C for 24 h, and the formation of black colonies indicated biofilm production (Baidya et al., 2021; Freeman et al., 1989; Hassan et al., 2011).

2.11. Antibiofilm assay

ECV was evaluated at concentrations ranging from 12.5 to 200 mg/ml in test tubes containing sterile nutrient broth, bacterial suspension, and ECV. The negative control was sterile nutrient broth without extract, while the positive control contained bacterial culture and DMSO for biofilm formation. Tubes were incubated for 3 days at 37 °C, rinsed with phosphate-buffered saline (PBS), air-dried, and assessed for biofilm formation using crystal violet staining and rinsing. Bound dye was dissolved in 1% acetic acid, and OD at 595 nm (Cahyakirana et al., 2024). The ECV demonstrated antibiofilm activity, as indicated by a reduction in OD values compared to the positive control. The percentage inhibition of biofilm formation or disruption was elucidated by:

$$\textit{Inhibition Percentage}(\%) = \frac{(\textit{Control OD} - \textit{Test OD})}{\textit{Control OD}} \times 100$$

2.12. Scanning electron microscopy (SEM) analysis

The antibacterial activity of ECV against *MRSA* and *E. coli* was analyzed using scanning electron microscopy (SEM) to observe structural changes in bacterial morphology. Fresh cultures of *MRSA* and *E. coli* were grown separately in sterile Mueller-Hinton broth (MHB) and adjusted to 0.5 McFarland standard. Each 100 ml culture was treated with 2 mL of ECV at a concentration of 200 mg/mL and incubated at 37 °C for 24 hr. Untreated bacterial cultures were maintained as controls. After incubation, the cultures were centrifuged at 10,000 × g for 10 min, and the pellets were washed twice with sterile Milli-Q water. A 10 µl aliquot from each suspension was placed on a sterile glass slide and air-dried. The dried samples were sputter-coated with a thin layer of gold to enhance conductivity and imaging resolution. SEM examination was conducted using a JEOL JSM-6390 scanning electron microscope equipped with a tungsten filament electron source. Imaging was carried out under high vacuum in both secondary electron (SE) and back-scattered electron (BSE) modes at an accelerating voltage of 10 kV. Images were captured at magnifications ranging from 500 × to 3300 × to assess morphological alterations in the bacterial cells (Asokan et al., 2025). SEM analysis was performed qualitatively to evaluate morphological changes in bacterial cells. Multiple images were captured from different areas of each sample at various magnifications, with untreated cells used as controls for comparison.

2.13. Bioactive compounds identification by HR-LCMS analysis

HR-LCMS was performed on the ECV using the Agilent 1200 LC System at IIT SAIF, Mumbai, India, to identify bioactive components. The system operated with high mass accuracy (<1 ppm), a resolution of 40,000 FWHM, and a mass range of 50–3200 amu. The instrument exhibited high sensitivity, with a reserpine concentration of 1 pg yielding an S/N ratio of 100:1. Ionization was conducted using APCI and API with ESI in both positive and negative modes. Mass analysis was performed via infusion in MS and MS/MS modes using a UHPLC-PDA mass spectrometer (Asokan et al., 2025; Badraoui et al., 2020).Compound identification was carried out based on accurate mass measurements and MS/MS fragmentation data acquired in Auto MS/MS mode, followed by database matching using METLIN, HMDB, and the Mass-Hunter library.

2.14. Data analysis

The data were expressed as mean ± SD, with experiments performed in triplicate. Pearson's correlation and one-way ANOVA followed by Tukey's test were used for statistical analysis using Microsoft Excel and SPSS software.

3. Result

3.1. Yield of extraction

The extraction yield of ECV was 12%, obtained using ethanol as the extraction solvent. The yield was calculated from an initial plant material weight of 125 g, which yielded 15 g of dried extract after lyophilization and evaporation of the sample.

3.2. Batch-to-batch comparative analysis (LC–MS)

The LC–MS analysis revealed a highly comparable phytochemical profile between the two analyzed batches (CV1 Batch No. 221557 and CV2 Batch No.220242). The chromatographic patterns exhibited similar peak distributions, retention times, and mass spectral characteristics across both batches. Minor variations in peak intensity were observed (Fig. 1), which may be attributed to natural variability in herbal raw materials; however, these differences did not significantly affect the overall chemical profile. The consistency in the major peaks and their corresponding m/z values confirms the reproducibility of the formulation. These findings demonstrate that ECV possesses a stable and consistent phytochemical composition, thereby supporting its reliability for subsequent biological and pharmacological evaluations.

3.3. Qualitative phytochemical screening

The presence of various phytochemical constituents in the ECV was evaluated through preliminary qualitative phytochemical screening. The analysis indicated the presence of several bioactive compounds, including flavonoids, alkaloids, glycosides, phenolic compounds, tannins, saponins, triterpenoids, carbohydrates, amino acids, and steroids, with varying degrees of abundance. Among these, alkaloids, glycosides, saponins, and steroids were found to be highly abundant, while flavonoids, triterpenoids, and carbohydrates were detected in moderate amounts. In contrast, certain constituents, such as proteins and starch, were either weakly detected or absent. These findings suggest that ECV contains a diverse range of phytochemicals that may contribute to its biological activities. The detailed results of the qualitative phytochemical screening are provided in Supplementary Data (Table S1).

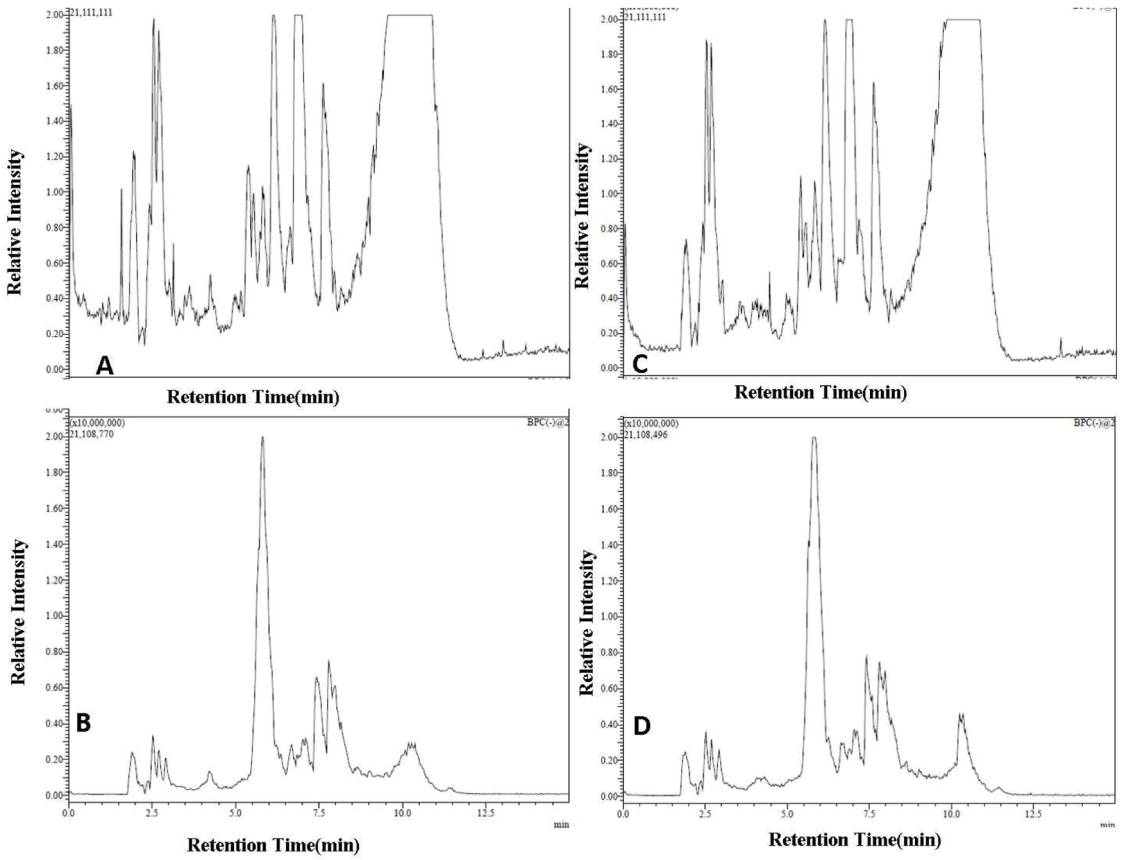

Fig. 1. LC–MS base peak chromatograms (BPC) of ECV recorded in positive and negative ionization modes: (A) CV1 (Batch No. 221557, positive mode), (B) CV1 (Batch No. 221557, negative mode), (C) CV2 (Batch No. 220242, positive mode), and (D) CV2 (Batch No. 220242, negative mode). The chromatographic profiles revealed multiple peaks corresponding to diverse phytoconstituents, with high similarity in retention time and peak distribution between both batches, indicating batch-to-batch consistency.

3.4. Estimation of total phenolic content (TPC) and total flavonoid content (TFC)

TPC) and TFC) of the ECV were determined using standard calibration curves. The TPC was estimated at 13.74 mg GAE/g extract, while the TFC was 36.57 mg QE/ g extract, indicating the presence of phenolic and flavonoid compounds that may contribute to the biological activity of the extract. The corresponding calibration curves are provided in the Supplementary Information (Figures S1 and S2).

3.5. ROS scavenging assays

The antioxidant potential of ECV was evaluated using DPPH, FRAP, and ABTS assays (Table X). ECV exhibited moderate ROS scavenging activity with IC_{50} values of 37.34 ± 2.12 µg/ml (DPPH), 25.452 ± 1.23 µg/ml (FRAP), and 48.05 ± 2.44 µg/ml (ABTS). However, the activity was lower than that of the reference antioxidants, gallic acid (IC_{50} = 23.987 ± 1.97 µg/ml) and ascorbic acid (IC_{50} = 14.729 ± 1.55 µg/ml for FRAP and 25.056 ± 0.96 µg/ml for ABTS). The corresponding graphical representations of these assays are provided in the Supplementary Materials (Figures S3–S5). These results indicate that ECV possesses appreciable antioxidant activity across multiple assay systems (Table 1).

Table 1
IC_{50} values of ECV and standard antioxidants in DPPH, FRAP, and ABTS ROS scavenging assays.

No	Sample	DPPH assay	FRAP assay	ABTS assay
1	ECV IC_{50}	37.34± 2.12 µg/ml	25.452 ± 1.23µg/ml	48.05 ± 2.44 µg/ml
2	Standard IC_{50}	23.987 ± 1.97µg/ml (Gallic acid)	14.729 ± 1.55µg/ml (Ascorbic acid)	25.056 ± 0.96 µg/ml (Ascorbic acid)

3.6. Cytotoxic activity (MTT assay)

The cytotoxic potential of ECV was evaluated on L929 and HEK-293 cell lines using the MTT assay after 24 h (Fig. 2–5). ECV maintained high cell viability across all tested concentrations in both cell lines, indicating good biocompatibility and negligible cytotoxicity. Although a slight concentration-dependent reduction in viability was observed, particularly in L929 cells, the effect remained minimal. Statistical analysis (one-way ANOVA followed by Tukey's test) indicated significant differences compared to control ($p < 0.05$); however, these changes were not biologically relevant. Morphological observations revealed no detectable alterations in cell structure. Detailed statistical data are provided in the Supplementary Materials (Tables S3–S4), with significance levels indicated in the corresponding Figs. 3 and 4.

3.7. Antibiotic susceptibility and multiple antibiotic resistance (MAR) analysis of bacterial strains

Table 2 presents the antibiotic resistance profiles of six different bacterial species, which showed differing resistance levels.

3.8. Antimicrobial activity

The antibacterial efficacy of ECV was measured with the agar well

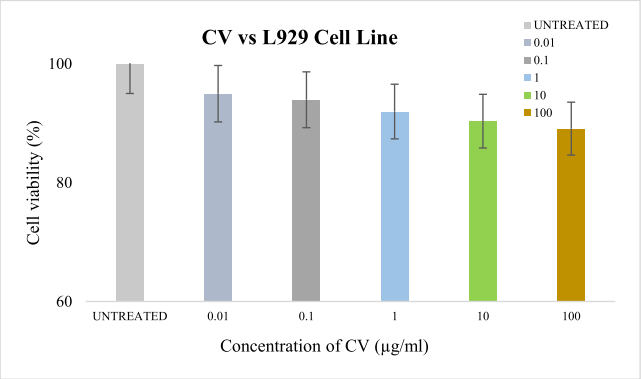

Fig. 2. Effect of ECV on L929 cell viability after 24 h, assessed by MTT assay. Values are expressed as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$ vs control).

diffusion test against multidrug-resistant (MDR) bacterial isolates from six uropathogenic strains. Fig. 6 shows the comparison of the ECV activity with a negative control, dimethyl sulfoxide (DMSO), and a positive control (azithromycin at 1% w/v). Table 3 presents the mean zones of inhibition in mm for all CV extracts against *E. coli, P. aeruginosa, MRSA, K. pneumoniae, Acinetobacter baumannii*, and *E. faecalis*, and the results for the controls. The antimicrobial activity of CV extract at various concentrations (10%, 20%, and 30% W/V) was compared with the positive control.

3.9. MIC and MBC

MIC of the ECV against MDR bacteria isolated from urinary tract infections (UTIs) was determined using a resazurin-based microtiter plate assay. The MIC values ranged from 50 to 100 mg/ml, while the MBC values ranged from 100 to 200 mg/ml against the tested pathogens. The detailed MIC and MBC values for each bacterial strain are presented in Table 4, and the microtiter plate assay results are shown in Fig. 7.

3.10. Biofilm formation

The results of the biofilm formation assay are presented in the Fig. 8, indicate that all tested bacterial strains were capable of forming biofilms.

3.11. Anti-biofilm activity of ECV against UTI pathogens

As shown in the Fig. 9, all tested bacterial strains exhibited biofilm formation. At the same time, treatment with ECV reduced biofilm density in a dose-dependent manner, with strong inhibition observed at 200 mg/ml.

3.11.1. Inhibition of MDR bacterial biofilm growth by ECV at different concentrations

The quantitative evaluation of biofilm inhibition was determined by measuring optical density (OD). As presented in Table 5 and Fig. 10, ECV significantly inhibited biofilm formation in all MDR bacterial strains in a concentration-dependent manner. A gradual decrease in OD values was observed with increasing concentrations of the extract (12.5–200 mg/ml), indicating reduced biofilm biomass. The highest antibiofilm activity was recorded at 200 mg/ml for all tested isolates, including *A. baumannii, P. aeruginosa, E. faecalis, K. pneumoniae, E. coli*, and *MRSA*. Statistical analysis using one-way ANOVA followed by Tukey's post hoc test confirmed that the reductions in biofilm formation at all tested concentrations were statistically significant compared with the positive control ($p < 0.05$).

3.11.2. Inhibition % of biofilm growth

Pearson's correlation analysis was performed to determine the relationship between ECV concentration (12.5–200 mg/ml) and the percentage of biofilm inhibition for each tested bacterial strain. The data in Table 6 demonstrates a clear dose-dependent antibacterial effect, with inhibition percentages increasing as the concentration rises. The high correlation coefficients indicate a strong relationship between concentration and bacterial inhibition. The correlation coefficients (r) ranged from 0.8626 to 0.9086 across the tested strains, indicating a strong positive correlation between ECV concentration and biofilm inhibition. *P*-values stay below 0.05, indicating that these results are statistically significant.

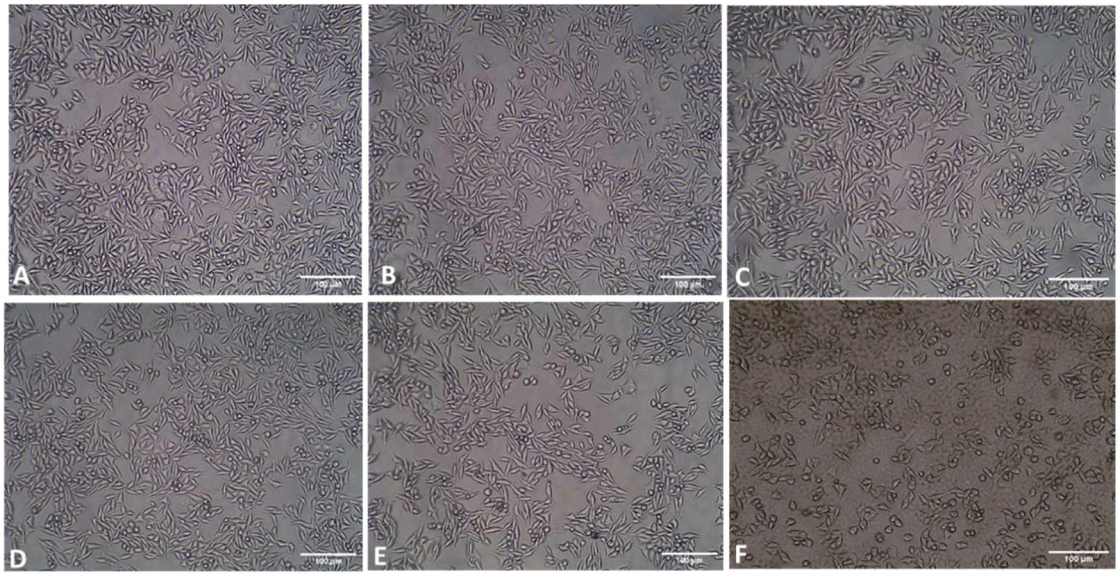

Fig. 3. Morphologic evaluation of L929 cells exposed to different concentrations. Observation conducted at 24 hr.

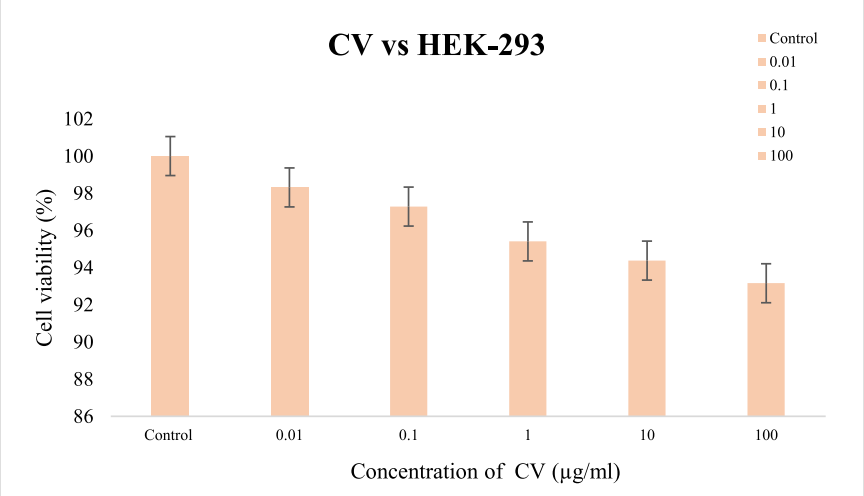

Fig. 4. Effect of ECV on HEK-293 cell viability after 24 h, assessed by MTT assay. Values are expressed as mean ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$ vs control).

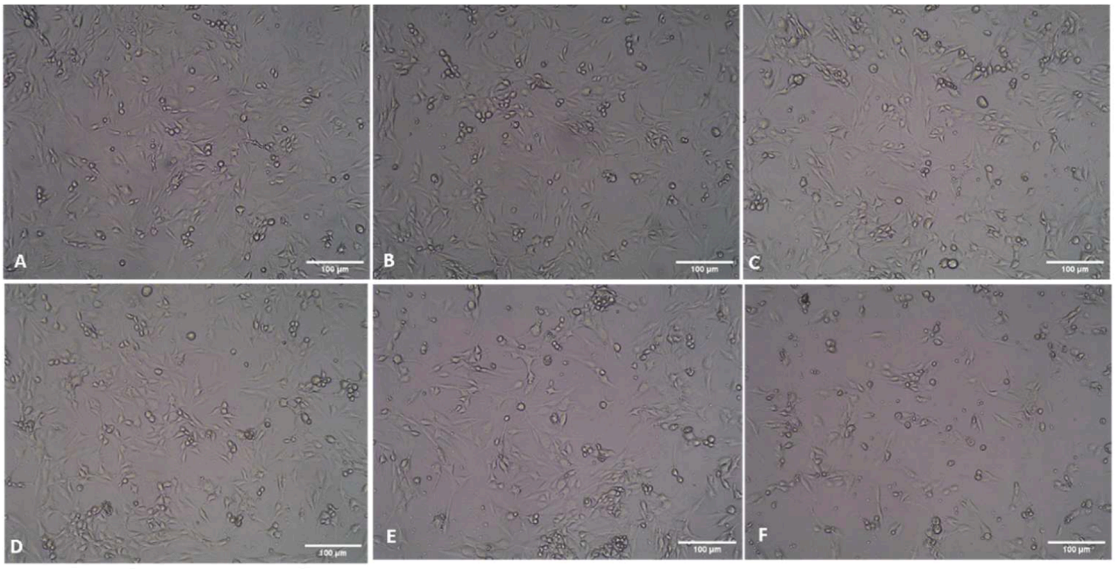

Fig. 5. Morphologic evaluation of L929 cells exposed to different concentrations. Observation conducted at 24 hr.

Table 2
Antibiotic susceptibility and multiple antibiotic resistance (MAR) analysis of bacterial strains.

Si . No	Name of bacteria	Total No. of antibiotics	Number of Antibiotics Resisted by Bacteria	Number of Antibiotics to Which Bacteria Are Sensitive	MAR %
1	*MRSA*	11	6	5	54.54%
2	*E. faecalis*	9	5	4	55.55%
3	*K. pneumoniae*	21	17	4	80.95%
4	*A. baumannii*	17	10	7	58.82%
5	*E. coli*	21	17	4	80.95%
6	*P. aeruginosa*	16	15	1	93.75%

3.12. SEM analysis

SEM analysis of untreated *MRSA* and *E. coli* showed intact, smooth cell surfaces with typical cocci and rod-shaped morphology, respectively. The cells appeared densely packed, indicating normal cell integrity and healthy growth. In contrast, *MRSA* and *E. coli* cells treated with ECV displayed clear morphological damage. The *MRSA* cells exhibited irregular shapes, collapsed surfaces, and evidence of cell lysis, indicating a severe disruption of the cell wall. The treated *E. coli* cells exhibited rough, wrinkled, and ruptured surfaces with a significant reduction in cell density compared to the control, as shown in Fig. 11. These structural alterations indicate that ECV causes the breakdown of bacterial cell walls and membrane integrity, leading to the leakage of cellular contents and eventual cell death. The SEM findings confirm the strong antibacterial action of ECV against both Gram-positive (*MRSA*) and Gram-negative (*E. coli*) bacteria.

3.13. HR-LCMS results

HR-LCMS analysis of the ECV identified 68 bioactive compounds. All identified compounds are listed in Table 7, along with their respective

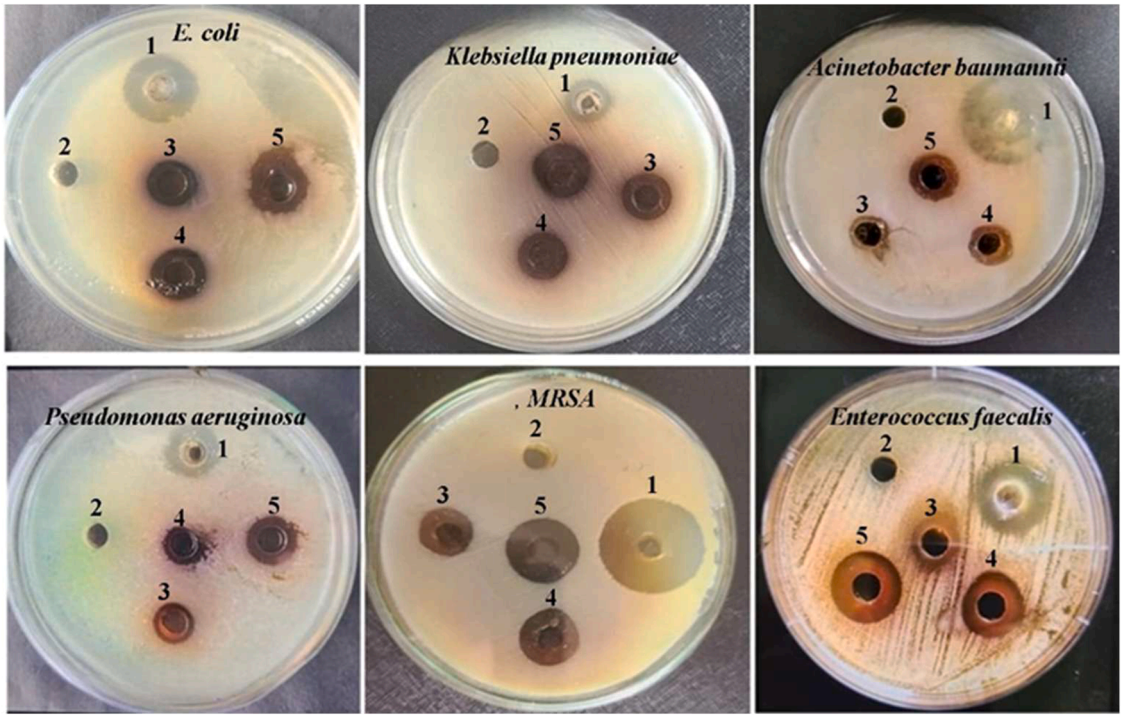

Fig. 6. Antimicrobial activity of CV against MDR bacteria.

molecular mass, retention time (RT), *m/z*, chemical formula, compound class, and reported biological activities, while the complete dataset of detected compounds is provided in the Supplementary Data (Table S5). The chromatograms obtained confirmed the presence of several bioactive constituents (Figs. 12 and 13). The compound identification was based on accurate mass measurements and database matching; therefore, the reported compounds are presented as putative (tentative) identifications.

All compound identifications in this study are classified as Metabolomics Standards Initiative (MSI) Level 2 (putatively annotated compounds), based on accurate mass measurements and MS/MS fragmentation pattern matching. MS/MS fragmentation data were acquired using Auto MS/MS mode and compared against available spectral databases such as METLIN, HMDB, and the MassHunter library. No authentic reference standards were used; therefore, Level 1 confirmation was not assigned. However, the combination of high-resolution mass accuracy and MS/MS spectral comparison provides a reliable level of confidence for compound annotation.

The detected metabolites belong to diverse phytochemical classes, including alkaloids, flavonoids, phenolics, triterpenoids, glycosides, and fatty acids, many of which are known to exhibit antimicrobial, antioxidant, and anti-inflammatory activities. The presence of these bioactive constituents provides chemical evidence supporting the biological activities observed in the present study.

Furthermore, HR-LCMS profiling offers a comprehensive insight into the phytochemical composition of ECV, thereby contributing to its scientific validation as a traditional herbal formulation. Detailed HR-LCMS analytical conditions, including instrument parameters, ionization settings, chromatographic gradient program, and method validation parameters used for metabolite profiling of ECV, are provided in the Supplementary Information (Table S2).

4. Discussion

A urinary tract infection (UTI)is a common microbial infection which can affect any component of the urinary system. It is one of the most common bacterial infections, affecting over 150 million people worldwide each year (Shrestha et al., 2023). UTIs occur at least four times more often in females than males (Öztürk and Murt 2020). Past literature surveys have shown that UTI-causing bacteria are now resistant to antibiotics, and antimicrobial resistance (AMR) poses a major global health challenge, leading to an estimated 1.27 million deaths directly and contributing to 4.95 million deaths each year, along with roughly 136 million hospital-associated drug-resistant infections worldwide. In India, resistance is among the highest worldwide, where >70% of *E. coli, Klebsiella pneumoniae*, and *Acinetobacter baumannii* isolates show resistance to fluoroquinolones and third-generation cephalosporins, and carbapenem resistance reaches up to 71% in *A. baumannii* and 56% in *K. pneumoniae.* (Antimicrobial and Network 2023; Lathakumari et al., 2025; Murray et al., 2022; Patel et al., 2024). Due to this reason, many people are turning towards herbal and traditional medicine treatments in recent times. (Al-Shahrani and Belali 2024; Mao et al., 2022). This growing interest in plant-based therapeutics has encouraged scientific investigations aimed at validating the pharmacological properties of traditional herbal formulations. Many of these formulations have proven effective, including Chandraprabha Vatika (CV), Gokshuradi Guggulu (GG), and Punarnavadi Mandoor (PM), well-known Ayurvedic preparations used for urinary tract and kidney-related disorders. Chandraprabha Vatika is an Ayurvedic herbal formulation in tablet form, widely used for various health conditions, particularly those related to the urinary and reproductive systems (Sahoo and Mahanta 2022). This medicine is traditionally reported to be used for conditions such as UTI, kidney stones, bladder infections, urinary incontinence, and reproductive disorders. However, despite its long-standing traditional use, comprehensive scientific validation of its phytochemical composition and biological activities remains limited The major ingredients of CV included *Guggulu (Commiphora mukul),Vacha (Acorus calamus) Guduchi (Tinospora cordifolia), Musta (Cyperus rotundus), Chandraprabha (Cinnamomum), Haridra (Curcuma longa), and Bhunimba (Andrographis paniculata)* (Dongre and Majumdar, 2024).

The LC–MS-based batch-to-batch analysis revealed a high degree of similarity between CV1 (Batch No. 221557) and CV2 (Batch No. 220242), with comparable retention times and peak distribution,

Table 3
Average inhibition zones of CV extract against MDR bacteria.

Name	Concentration 10% W/V (mm)	Concentration 20% W/V (mm)	Concentration 30% W/V(mm)	Positive control: Azithromycin 1%w/v (mm)	Negative control: DMSO 100% V/V
E. coli	15±0.002	18±0.001	21±0.003	22±0.001	0
K.pneumoniae	18±0.001	21±0.005	24±0.003	14±0.002	0
A. baumannii	16±0.006	18±0.002	19±0.001	20±0.002	0
P. aeruginosa	14±0.002	17±0.003	21±0.001	15±0.004	0
MRSA	13±0.001	15±0.002	19±0.005	25±0.003	0
E. fecalis	17 ± 0.002	19 ± 0.004	23 ± 0.003	32 ± 0.001	0

Table 4
Antibacterial activity of ECV: MIC and MBC values against UTI pathogens.

Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)
E. coli	50	100
Klebsiella pneumoniae	50	100
Acinetobacter baumannii	50	100
Pseudomonas aeruginosa	100	200
MRSA	100	200
Enterococcus faecalis	50	100

indicating consistent phytochemical composition (Fig. 1). Minor variations in peak intensity were observed, likely due to natural variability in raw materials; however, these did not affect the overall chromatographic profile. These observations indicate good reproducibility rather than absolute uniformity. These findings confirm the reproducibility and stability of ECV and support its reliability for subsequent pharmacological evaluations, consistent with previous reports on LC–MS-based standardization of herbal formulations (Muley et al., 2026).

Table S1 presents a qualitative phytochemical screening of ECV, depicting a number of bioactive (phenolic, alkaloids, saponins, glycosides, flavonoids, triterpenoids, carbohydrates, proteins, amino acids, tannins, steroids) compounds. The presence of these diverse phytochemicals suggests that the biological activity of the extract may arise from the synergistic interaction of multiple constituents. Although the alkaline reagent test indicated the presence of flavonoids, Shinoda's test showed a negative result, which may be attributed to differences in the sensitivity and specificity of qualitative assays, as Shinoda's test primarily detects specific flavonoid subclasses such as flavones and flavonols These findings show that these compounds are known for having many pharmacological activities that are consistent with findings of similar herbal formulations. Phenolic compounds, for instance, have been noted for their strong antioxidant properties, as described by (Amalraj et al., 2022). Such antioxidant effects are particularly relevant in infectious diseases, where oxidative stress contributes to tissue damage and disease progression. These compounds act mainly by scavenging reactive oxygen species and upregulating important antioxidant enzymes such as (SOD), catalase (CAT), and (GPx), while modulating signaling pathways like Nrf2–Keap1 and NF-κB, which regulate oxidative stress and inflammation (Ma et al., 2024) Alkaloids, frequently associated with antimicrobial and anti-inflammatory activities, were also present in significant amounts in ECV, supporting its traditional use in managing infections (Singh et al., 2023a). Similar antimicrobial mechanisms of plant-derived alkaloids have been widely reported in phytopharmacological studies. Their mechanism involves interfering with bacterial DNA replication, inhibiting topoisomerase enzymes, disrupting cell membrane integrity, and inhibiting quorum sensing, leading to the suppression of virulence gene expression (García et al., 2011) . Saponins, detected in ECV, contribute to its potential antimicrobial and anti-inflammatory effects, aligning with previous research on their role in enhancing immunity and reducing inflammation (Sharma et al., 2021). They act by binding to membrane sterols, causing cell lysis in microbes, and by modulating immune-related genes such as IL-1β, TNF-α, and NF-κB, thereby reducing inflammatory responses (Xue et al., 2020). The ROS scavenging potential of ECV is documented due to the presence of flavonoids, glycosides, and tannins, which have anti-oxidant ability to scavenge free radicals, lessen the effect of oxidative stress, and provide therapeutic effects (Chaudhary et al., 2023). Steroids, are also detected in ECV, and may contribute to their ROS scavenging properties, adding further evidence to their traditional therapeutic uses (Chaudhary et al., 2023). These phytochemical constituents collectively support the traditional Ayurvedic use of CV in the treatment of infections and inflammatory conditions, as many of these compounds are known to exhibit antimicrobial, antioxidant, and anti-inflammatory activities. These interpretations are supported by recent phytopharmacological studies (El-Saadony et al.,

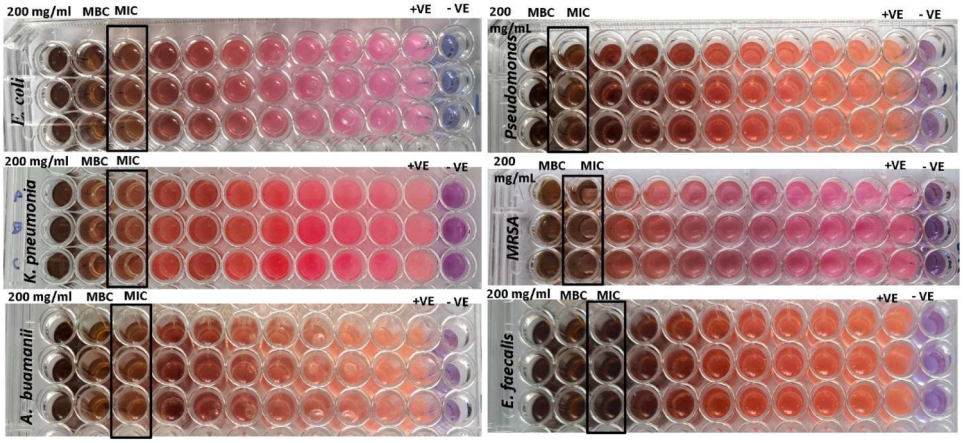

Fig. 7. MIC of CV against MDR bacteria.

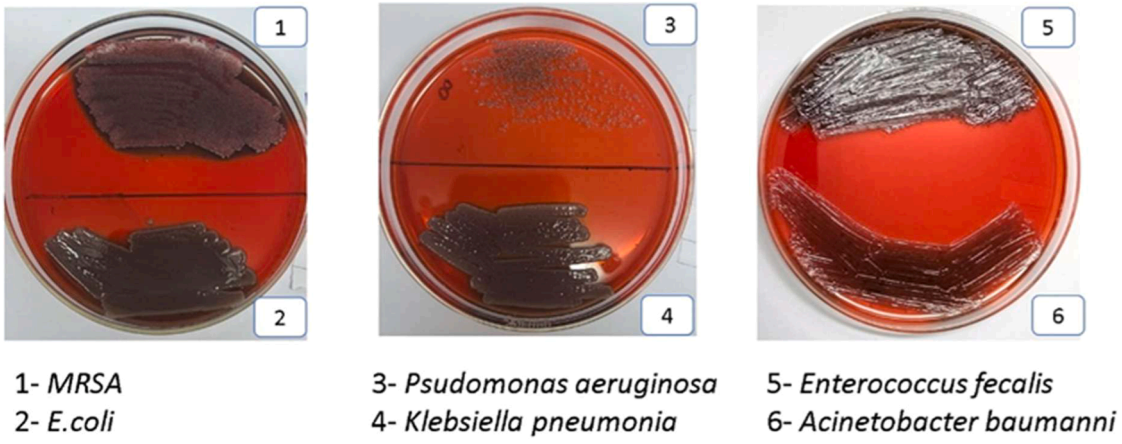

Fig. 8. Biofilm formation of MDR bacteria in Congo red agar.

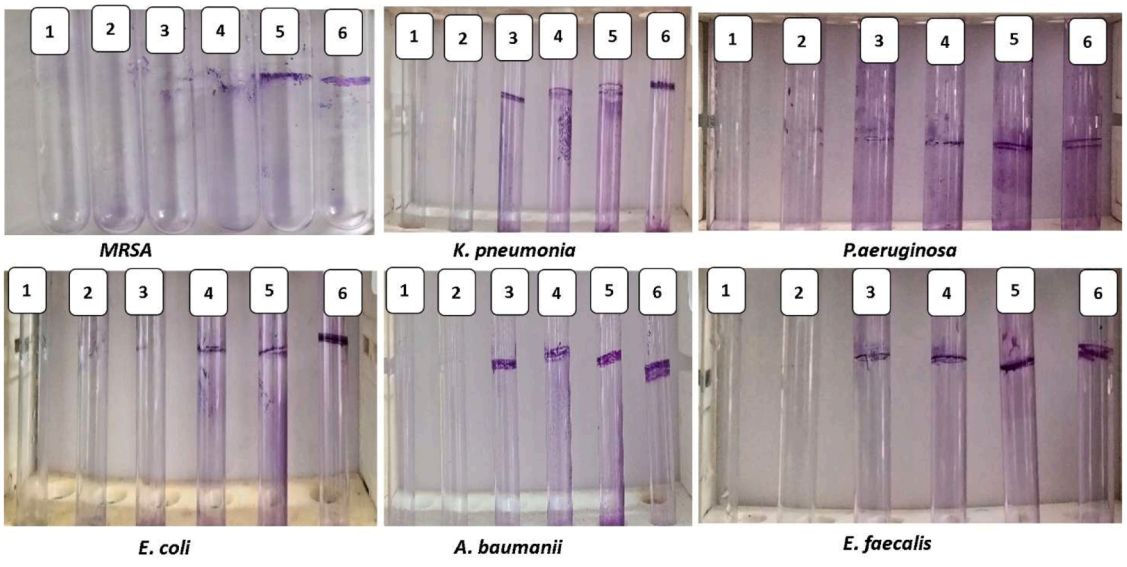

Fig. 9. Antibiofilm of ECV against MDR bacteria.

2025).

The concentration of TPC in the ECV was measured at 13.74 mg GAE/g extract (Figure S1). These values are expressed as mg equivalents per gram of extract for better comparison with existing literature. The

Table 5
Effect of different concentrations of ECV on biofilm formation of MDR bacteria expressed as mean absorbance (OD) ± SD.

Name of organism	Concentration mg/ml	N	Mean absorbance	SD	Tukey vs Control	*P*-value ANOVA	*F* value
A. baumanii	Positive control	3	1.986	0.002	—	< 0.001	281324.444
	12.5	3	1.810	0.001	p < 0.001		
	25	3	1.256	0.001	p < 0.001		
	50	3	1.079	0.004	p < 0.001		
	100	3	0.304	0.001	p < 0.001		
	200	3	0.212	0.001	p < 0.001		
P.aeruginosa	Positive control	3	1.118	0.003	—	< 0.001	112754.657
	12.5	3	0.794	0.002	p < 0.001		
	25	3	0.650	0.001	p < 0.001		
	50	3	0.540	0.001	p < 0.001		
	100	3	0.205	0.001	p < 0.001		
	200	3	0.161	0.001	p < 0.001		
E. faecalis	Positive control	3	1.639	0.001	—	< 0.001	322326.970
	12.5	3	1.509	0.001	p < 0.001		
	25	3	1.151	0.002	p < 0.001		
	50	3	0.816	0.002	p < 0.001		
	100	3	0.325	0.001	p < 0.001		
	200	3	0.205	0.002	p < 0.001		
K. pneumonia	Positive control	3	1.297	0.001	—	< 0.001	93651.904
	12.5	3	1.032	0.005	p < 0.001		
	25	3	0.778	0.003	p < 0.001		
	50	3	0.544	0.001	p < 0.001		
	100	3	0.234	0.004	p < 0.001		
	200	3	0.174	0.001	p < 0.001		
E. coli	Positive control	3	1.751	0.003	—	< 0.001	313736.924
	12.5	3	1.385	0.002	p < 0.001		
	25	3	1.106	0.001	p < 0.001		
	50	3	0.663	0.001	p < 0.001		
	100	3	0.343	0.001	p < 0.001		
	200	3	0.159	0.001	p < 0.001		
MRSA	Positive control	3	1.371	0.123	—	< 0.038	3.406
	12.5	3	1.419	0.001	p < 0.043		
	25	3	1.194	0.002	p < 0.038		
	50	3	0.864	0.005	p < 0.001		
	100	3	0.420	0.002	p < 0.001		
	200	3	0.189	0.005	p < 0.001		

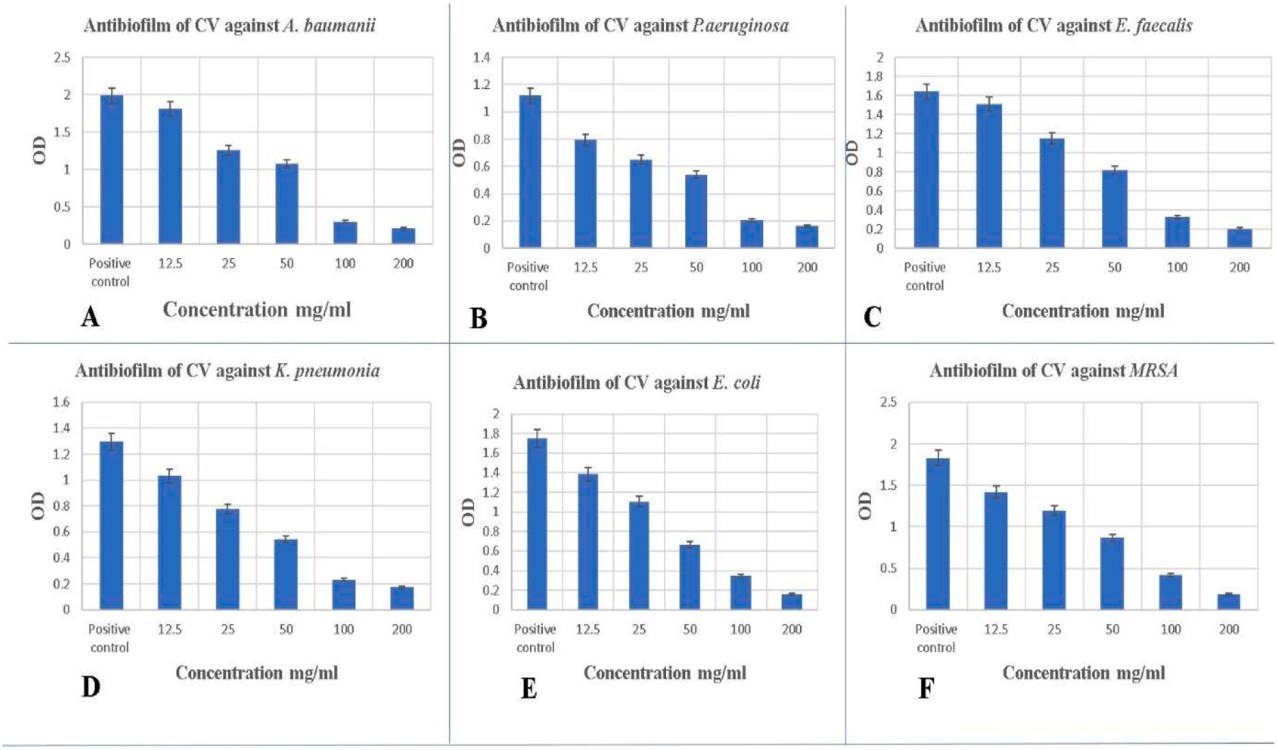

Fig. 10. Antibiofilm activity of ECV against multidrug-resistant bacteria. Biofilm inhibition at different concentrations (12.5–200 mg/ml) against (A) *A. baumannii,* (B) *P. aeruginosa,* (C) *E. faecalis*, (D)*K. pneumoniae*, (E) *E. coli*, and (F) *MRSA*. Positive control included for comparison.

Table 6
The Detection of inhibition % of biofilm growth.

s. no	Concentration mg/ml	*K. pneumonia* Inhibition %	*A. baumanii*	*MRSA*	*P. aeruginosa*	*E. coli*	*E. faecalis*
1	**Positive control**	0	0	0	0	0	0
2	**12.5**	20.446	8.843	22.384	28.992	20.879	7.930
3	**25**	40.046	36.734	34.688	41.865	36.810	29.768
4	**50**	58.052	45.662	52.716	51.698	62.105	50.203
5	**100**	81.967	84.661	76.995	81.615	80.376	80.154
6	**200**	86.591	89.293	89.755	85.548	90.902	87.474
7	**correlation coefficient**	0.8626	0.8927	0.9086	0.8640	0.8791	0.8997
8	***P value***	0.027	0.017	0.012	0.026	0.021	0.014

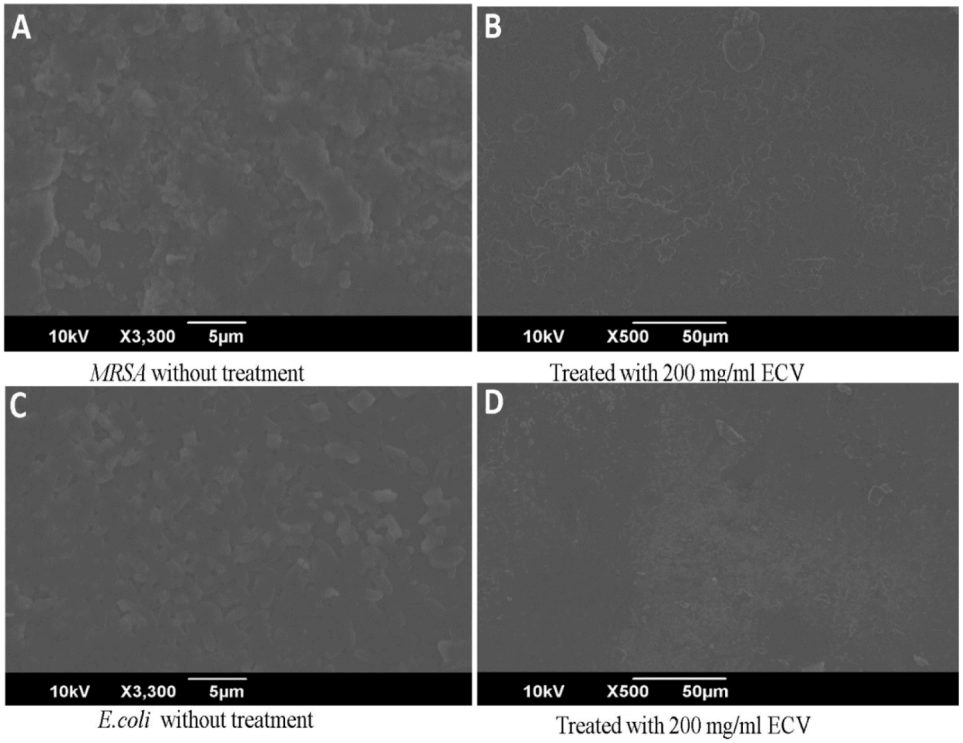

Fig. 11. SEM Analysis results showing the effect of ECV on *MRSA* and *E. coli*. (A) Untreated *MRSA*; (B) *MRSA* treated with 200 mg/ml ECV; (C) Untreated *E. coli*; (D) *E. coli* treated with 200 mg/ml ECV.

above value is in agreement with the previously conducted studies on polyherbal formulations, which reported TPC ranging from 10 to 25 57 mg QE/ g extract depending on the extraction and solvent used. (Keshavarz Mirzamohammadi et al., 2021). Variations in phytochemical content among studies may arise from differences in plant sources, extraction solvents, and analytical methods used.

The TFC of the ECV was determined to be 36.57 57 mg QE/ g extract (Figure S2). These results are consistent with previously reported values for similar formulations. (Hamad and Hartanti 2025; Kandibanda 2025) . The existence of flavonoids is significant in the extract or synthesis from the previous studies on the polyherbal formulations. Studies on Ayurvedic formulations such as *Triphala* have shown flavonoid content within similar ranges, further reinforcing these findings (Nguyen 2026; Peterson et al., 2017). Flavonoids are well known for their antioxidant and antimicrobial activities, which may contribute to the biological effects observed in the present study.

In Table 1 of the DPPH assay, the ECV exhibited significant antioxidant activity with an IC_{50} value of 37.346 μg/ml, indicating its efficiency in scavenging 50% of free radicals. In comparison, gallic acid, the reference standard, showed a significantly lower IC_{50} value of 23.987 μg/ml, confirming its stronger antioxidant potential relative to ECV. Although the activity of ECV was lower than the standard compound, the results nevertheless indicate a considerable free radical scavenging potential for a complex herbal extract. In addition, the data presented in Table 1 reveal the FRAP and ABTS assays, with ECV having IC_{50} values of 25.452 μg/ml and 48.05 μg/ml, respectively. Data generated from our study confirm the antioxidant activity of ECV once again, solidifying the possibility of ECV as a source of natural free radical scavenging compounds (Bapat et al., 2016; Kiss et al., 2025; Li et al., 2021; Wardani et al., 2025). However, this activity is moderate compared to standard antioxidants. Comparable antioxidant capacities have been reported in several plant-based formulations rich in phenolic and flavonoid compounds.

The MTT assay findings (Figs. 2–5) revealed that the test compounds exhibited no cytotoxic effects on L929 and HEK-293 cell lines after 24 h of incubation, indicating that ECV was non-toxic at all tested concentrations and suggesting its safety and biocompatibility for further applications. These findings should be interpreted cautiously as they are based on short-term in vitro exposure. The absence of cytotoxicity implies that the bioactive constituents of ECV do not interfere with normal cellular metabolism or viability, even at higher doses. Such results are crucial in validating the therapeutic safety of herbal formulations, as cytotoxicity is a key concern when developing natural products for biomedical use. These findings are consistent with previous reports on

Table 7
Putative identification of bioactive compounds in ECV by HR-LCMS showing retention time (RT), m/z values, molecular formula, and compound class:$(M+H)^+$ 59 (1–59) and $(M+H)^-$ 9(60–68).

S. No	Name of Compound	Class of compound	RT	*m/z*	Formula	Mass
1	Betaxolol	Flavonoids	6.43	330.2047	$C_{18}H_{29}NO_3$	307.2155
2	Capsaicin	Alkaloid	6.503	328.1883	$C_{18}H_{27}NO_3$	305.199
3	Ankorine	Alkaloid	6.557	358.1992	$C_{19}H_{29}NO_4$	335.2102
4	Mephedrone	Cathinone	7.21	200.107	$C_{11}H_{15}NO$	177.1177
5	ibopamine	Dopamine and epinine	7.375	330.1682	$C_{17}H_{25}NO_4$	307.1787
6	Pyraclostrobin	Strobilurin	7.393	410.0843	$C_{19}H_{18}ClN_3O_4$	387.0954
7	Harzianopyridone	a pyridone moiety and Alkaloids	7.873	304.1167	$C_{14}H_{19}N\ O_5$	281.1276
8	Piscerythramine	Alkaloids, Pyrrole Alkaloids, and Isoflavonoid	9.154	452.2042	$C_{26}\ H_{29}\ N\ O_6$	451.1965
9	4R,5R,6S-Trihydroxy-2-hydroxymethyl-2-cyclohexen-1-one 6-(2-hydroxy-6-methylbenzoate)	Hydroxycyclohexenone Esters and Polyhydroxyketones	9.236	331.0794	$C_{15}\ H_{16}\ O7$	308.0903
10	(*S*)-Edulinine	Alkaloids and Isoquinoline Alkaloids	10.294	314.1371	$C_{16}\ H_{21}\ N\ O_4$	291.1478
11	Quinidinone	Alkaloids and Quinoline Alkaloids	10.834	323.1741	$C_{20}\ H_{22}\ N_2\ O_2$	322.1666
12	Karakoline	Alkaloids, Pyrrolo[1,2-a]azepine Alkaloids	12.543	400.2466	$C_{22}\ H_{35}\ N\ O_4$	377.2572
13	P-Nitrophenyl stearate	Esters and Nitrophenyl Ester	14.065	428.2774	$C_{24}\ H_{39}\ N\ O_4$	405.2882
14	Symlandine	Alkaloids and Pyrrolizidine Alkaloids (PAs)	14.397	404.2038	$C_{20}\ H_{31}\ N\ O_6$	381.2145
15	LysoPE(20:0/0:0)	Lipids,Glycerophospholipids, and Lysophosphatidylethanolamines (LysoPEs)	14.738	532.3386	$C_{25}\ H_{52}\ N\ O_7\ P$	509.3492
16	Feruperine	Alkaloids and Pyrrolizidine Alkaloids (PAs)	15.055	288.1585	$C_{17}\ H_{21}\ N\ O_3$	287.1512
17	Piperine	piperidine alkaloid and Piperamide alkaloids	15.209	286.1441	$C_{17}\ H_{19}\ N\ O_3$	285.1366
18	Dipiperamide E	Piperamides	15.22	571.277	$C_{34}\ H_{38}\ N_2\ O_6$	570.2701
19	Promacyl	Promacyl backbone	15.365	300.1577	$C_{16}\ H_{23}\ N\ O_3$	277.1685
20	Annotinine	lycopodium alkaloids	15.458	298.1422	$C_{16}\ H_{21}\ N\ O_3$	275.153
21	Secodemethylclausenamide	Alkaloids	15.509	286.1428	$C_{17}\ H_{19}\ N\ O_3$	285.1356
22	Fluoxetine	Phenylpropylamine	15.522	310.1421	$C_{17}\ H_{18}\ F_3\ N\ O$	309.1347
23	Donepezil	Aromatic compound	15.686	402.2046	$C_{24}\ H_{29}\ N\ O_3$	379.2151
24	Dobutamine	Catecholamine	15.902	302.1737	$C_{18}\ H_{23}\ N\ O_3$	301.168
25	alpha-Amylcinnamyl isovalerate	Ester compound	16.451	311.1993	C19 H28 O2	288.21
26	Variotin	Polyketides and macrolides	16.534	314.1738	$C_{17}\ H_{25}\ N\ O_3$	291.1847
27	Cimifugin	Furanocoumarins	16.562	329.1001	$C_{16}\ H_{18}\ O_6$	306.1112
28	Neferine	Bisbenzylisoquinoline alkaloids	16.654	623.3055	$C_{38}\ H_{44}\ N_2\ O_6$	622.3122
29	Dihydroferuperine	Alkaloids, Isoquinoline alkaloids, and Phenethylisoquinoline	16.7	312.1582	$C_{17}\ H_{23}\ N\ O_3$	289.169
30	Rodiasine	Bisbenzylisoquinoline alkaloids	16.701	623.3072	$C_{38}\ H_{42}\ N2\ O_6$	622.3012
32	(6E)-Piperamide-C7:1	Amide alkaloids	16.897	324.1569	$C_{18}\ H_{23}\ N\ O_3$	301.1679
33	(2E,4E,8E)-Piperamide-C9:3	Piperamides	16.923	326.1739	$C_{20}\ H_{23}\ N\ O_3$	325.1667
34	Isothebaine	Opium alkaloid derivative	16.999	312.1584	$C_{19}\ H_{21}\ N\ O_3$	311.1513
35	10-Oxo-11-octadecen-13-olide	Fatty acid derivatives, Oxolides	17.144	316.1894	$C_{18}\ H_{30}\ O_3$	293.2037
36	(E,E)-2,4-Decadienoic isobutylamide	Amide derivatives of unsaturated fatty acids	17.411	224.2007	$C_{14}\ H_{25}\ N\ O$	223.1934
37	3a,17a-Dihydroxy-5b- androstane	Steroids, androstane derivatives	17.439	315.2306	$C_{19}\ H_{32}\ O_2$	292.2412
38	Geissospermine	Indole alkaloids	17.447	655.3697	$C_{40}\ H_{48}\ N_4\ O_3$	632.3802
39	Vernolepin	Terpenoids	17.631	299.0895	$C_{15}\ H_{16}\ O_5$	276.1003
40	[7]-Paradol	Phenolic	17.726	315.1939	$C_{18}\ H_{28}\ O_3$	292.2047
41	(E,E)-2,4-Decadienoic isobutylamide	fatty acid amide	17.805	224.2007	$C_{14}\ H_{25}\ N\ O$	223.1942
42	Pipercide	Alkaloid	18.036	356.2204	$C_{22}\ H_{29}\ N\ O_3$	355.213
43	Spiredine	Alkaloid	18.125	354.204	$C_{22}\ H_{27}\ N\ O_3$	353.1979
44	2-[Octahydro-4,7-dimethyl-1- oxocyclopenta[c]pyran-3- yl] nepetalactam	lactams, terpenoids, and pyran-based structures	18.175	332.2202	$C_{20}\ H_{29}\ N\ O_3$	331.2128
45	Dipiperamide E	Piperamide	18.614	517.2778	$C_{34}\ H_{38}\ N2\ O_6$	570.2703
46	Convallasaponin A	triterpenoid saponin	19.077	581.3628	$C_{32}\ H_{52}\ O_9$	580.3553
47	(3a,5b,7b,12a)-(1,3-dihydro-5- nitro-1,3-dioxo-2H-isoindol-2- yl)methyl ester-3,7,12- trihydroxy-Cholan	Cholan-derived molecule	19.544	613.3131	$C_{33}\ H_{44}\ N_2\ O_9$	612.3056
48	Calcipotriol	Steroid	19.787	413.3031	$C_{27}\ H_{40}\ O_3$	412.2957
49	Pipercyclobutanamide B	Piperamide	19.796	597.2924	$C_{36}\ H_{40}\ N_2\ O_6$	596.285
50	Dihydroretrofractamide B	Retrofractamide, Alkaloid	19.809	358.2365	$C_{22}\ H_{31}\ N\ O_3$	357.2291
51	Stenocereol	Triterpenoid	20.66	437.3391	$C_{28}\ H_{46}\ O_2$	414.3496
52	(22R,23R)-22,23-Dihydroxycampesterol	Phytosterol	20.67		$C_{28}\ H_{48}\ O_3$	432.3584
53	(E,E,E)-Sylvatine	Alkaloid	20.919	455.3485	$C_{24}\ H_{33}\ N\ O_3$	383.2436
54	Ajaconine	Alkaloid	20.991	384.2507	$C_{22}\ H_{33}\ N\ O_3$	359.247
55	Actinonin	Heterocyclic	21.62	382.2363	$C_{19}\ H_{35}\ N_3\ O_5$	385.2597
56	5-(12-Nonadecenyl)-1,3- benzenediol	Phenolic	21.791	397.3068	$C_{25}\ H_{42}\ O_2$	374.3182
57	Phytosphingosine-1-P	Lipid	21.851	398.2662	$C_{18}\ H_{40}\ N\ O_6\ P$	397.259

(*continued on next page*)

Table 7 (*continued*)

S. No	Name of Compound	Class of compound	RT	*m/z*	Formula	Mass
59	Fesoterodine	Diphenylmethanes	22.875	412.2827	$C_{26}H_{37}NO_3$	411.2753
60	Gallic acid	Phenolic acid	3.864	169.0149	$C_7H_6O_5$	170.0222
61	Methyl 2-furoate	Furan carboxylic acid ester	3.864	125.0248	$C_6H_6O_3$	126.032
62	Butylparaben	Phenolic compound	13.606	193.0876	$C_{11}H_{14}O_3$	194.0949
63	N-Undecylbenzenesulfonic acid	Sulfonic acid derivative	20.814	311.1702	$C_{17}H_{28}O_3S$	312.1774
64	VPGPR Enterostatin	Pentapeptide	21.63	523.3016	$C_{23}H_{40}N_8O_6$	524.3068
65	Sodium Tetradecyl Sulfate	Anionic surfactant	22.219	293.1807	$C_{14}H_{30}O_4S$	294.1878
66	4-Dodecylbenzene sulfonic acid	Organic sulfonic acid	22.291	325.1859	$C_{18}H_{30}O_3S$	326.1931
67	Hydrocortisone cypionate	Corticosteroid	23.29	485.286	$C_{29}H_{42}O_6$	486.2932
68	Hydroxyprogesterone caproate	Steroid	25.088	473.286	$C_{27}H_{40}O_4$	428.2877

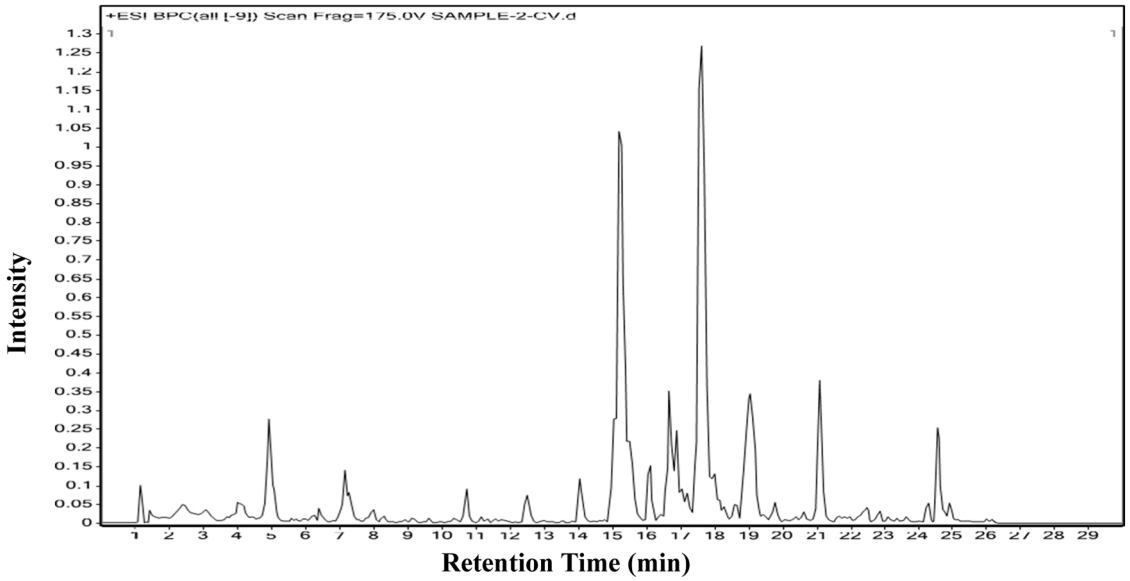

Fig. 12. Bioactive compounds identified in ECV using HR-LCMS in positive ion mode.

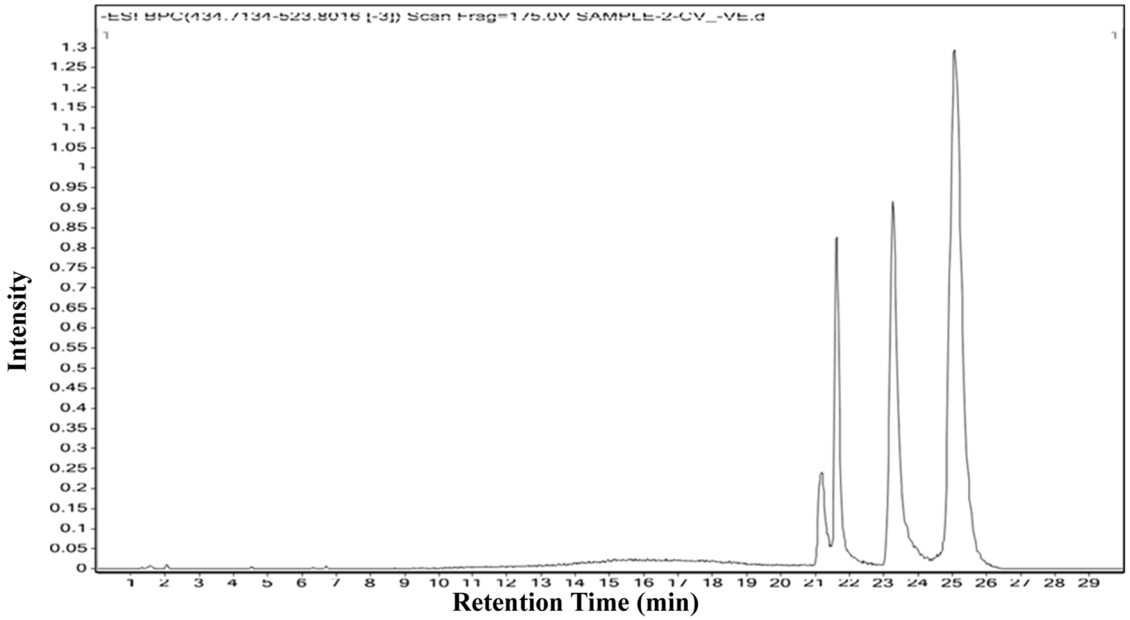

Fig. 13. Bioactive compounds identified in ECV using HR-LCMS in negative ion mode.

Ayurvedic preparations, which have shown minimal toxicity and high cellular compatibility, supporting their traditional use in long-term

therapy (Cannella et al., 2019; Khan et al., 2023; Merlin et al., 2022). However, it should be emphasized that these observations represent only preliminary evidence of biocompatibility based on short-term in vitro assessment. Furthermore, the present findings are limited to in vitro cytotoxicity evaluation after 24 h of exposure, and further in vivo and long-term toxicity studies are required to comprehensively confirm the safety profile of ECV.

Multiple Antibiotic Resistance (MAR) in Table 2 shows *E. coli, P. aeruginosa, E. faecalis, K. pneumoniae, A. baumannii*, and MRSA in this research are consistent with other studies. The high MAR indices observed in the present study highlight the urgent need for alternative therapeutic strategies to combat multidrug-resistant pathogens. The exact high MAR percentages were noted in *Klebsiella pneumoniae* and *E. coli* (80.95%), *Pseudomonas aeruginosa* (93.75%), and *Acinetobacter baumannii* (58.82%), which are in line with findings from (Abdelwahab et al., 2021; Jordana-Lluch et al., 2023; Kyriakidis et al., 2021). Furthermore, *Enterococcus faecalis* and *MRSA* demonstrated moderate resistance, similar to the findings of Malik et al. (2022). These observations reflect the widespread antibiotic resistance among clinical pathogens and emphasize the necessity for effective antimicrobial stewardship and alternative therapy strategies (Malik et al., 2022).

The results in Fig. 6 and Table 3 show the antibacterial activity of CV ethanol extracts against multidrug-resistant (MDR) uropathogenic bacteria, which aligns with previous studies highlighting its broad-spectrum antimicrobial potential. Such observations support the growing body of evidence indicating that plant-derived extracts may serve as complementary agents in antimicrobial therapy. There are many researches supporting the CV efficacy against various bacterial strains (Christa et al., 2013; Sharifi-Rad et al., 2021) and (Aladejana 2023). The bioactive compounds, such as alkaloids and flavonoids are considered responsible for the bio-activity CV. Nevertheless, the precise molecular mechanisms underlying the antibacterial action of ECV require further investigation. (Kong and Park, 2026; Singh et al., 2023b), emphasized the antimicrobial properties of Cinnamomum species, another component of CV. These findings validate CV's potential as a natural alternative to antibiotics against MDR infections, though further clinical studies are needed to confirm its therapeutic applications.

The MIC and MBC of ECV against MDR uropathogenic bacteria were established in this work. Although the MIC values obtained in this study are relatively higher than those typically observed for conventional antibiotics, such findings are common for crude plant extracts due to the complex mixture of bioactive constituents. The MIC against *E. coli, K. pneumoniae, A. baumannii*, and *E. faecalis* was 50 mg/ml, whereas against *P. aeruginosa* and *MRSA*, it was 100 mg/ml. The MBC was 100 mg/ml for the former and 200 mg/ml for the latter. These findings reflect the moderate bactericidal activity of CV. See Fig. 7 and Table 4. The findings are in agreement with earlier research, including (Elmaidomy et al., 2022; Thakur and Khushboo, 2024), and (Abdelwahab et al., 2021), which expressed similar MIC values of herbal extracts against MDR pathogens. These findings underscore the antibacterial potential of CV, and indicate moderate antimicrobial activity against the tested MDR bacteria, support its use as a therapeutic agent against MDR bacteria.

The results from this study, showing black coloration on Congo red agar for *E. coli, Enterococcus faecalis, P. aeruginosa, MRSA, K. pneumoniae*, and *A. baumannii*, indicate biofilm formation by these MDR pathogens see the Fig. 8. Biofilm formation enhances bacterial resistance to antibiotics and contributes to the persistence of infections. The research outcomes aligns with former studies conducted by (Assefa and Amare, 2022; Folliero et al., 2021), and (Senobar Tahaei et al., 2021), which observed similar biofilm formation in MDR strains of these bacteria. The presence of biofilms in MDR pathogens underscores the challenges in treating chronic infections and highlights the need for new therapeutic strategies targeting biofilm-associated bacteria.

Importantly, the present findings extend beyond descriptive biological activity by providing translational relevance. The observed antibiofilm activity against multidrug-resistant uropathogens highlights the potential of ECV as an alternative or adjunct therapeutic strategy in managing persistent UTIs, where conventional antibiotics often fail due to biofilm-associated resistance. Furthermore, integrating phytochemical profiling with biological assays enables clearer correlation between chemical composition and biological function, thereby strengthening the mechanistic understanding and scientific validation of this traditional formulation.

The study demonstrates that CV extract effectively inhibits MDR bacterial biofilm formation in a dose-dependent test, as indicated by decreasing OD values, see Fig. 9& 10 and Table 5. The inhibition of biofilm formation is particularly important, as biofilms play a crucial role in the persistence and recurrence of chronic infections. At low concentrations (12.5–25 mg/ml), moderate inhibition was observed, while at 50 mg/ml, a significant reduction in biofilm formation occurred. Higher concentrations (100–200 mg/ml) showed the strongest inhibitory effects, leading to near-complete biofilm suppression. The find outs are collinear with existing studies of (Mehrishi et al., 2020), and (Furquim dos Santos Cardoso et al., 2021), which reported similar concentration-dependent anti-biofilm effects of medicinal plant extracts against MDR pathogens. The results highlight the potential of CV extract as a natural anti-biofilm agent, offering a promising alternative for treating biofilm-associated infections caused by antibiotic-resistant bacteria. However, additional mechanistic studies are necessary to fully elucidate the pathways through which ECV interferes with biofilm development. In contrast, conventional antibiotics such as ciprofloxacin, amikacin, and imipenem have shown variable success in eradicating biofilms, often requiring higher doses or combination therapies due to the protective nature of biofilms. Previous studies have reported that standard antibiotics may exhibit limited penetration and reduced activity against biofilm-embedded bacteria, leading to persistent infections. (Prinzi and Rohde, 2023). Conversely, the phytochemical constituents of CV, including flavonoids, alkaloids, and phenolic compounds, might act synergistically to inhibit biofilm formation through several mechanisms, including the inhibition of bacterial adhesion, the disruption of quorum sensing, and the induction of oxidative stress. For example, it has been shown that natural phenolic chemicals inhibit the formation of biofilms by disrupting quorum sensing and other regulatory processes in bacteria (Buchmann et al., 2023).

SEM analysis of untreated *MRSA* and *E. coli* showed intact and smooth cell surfaces with typical cocci and rod-shaped morphology, indicating normal cell integrity, as seen in Figure 11.These observations are consistent with previous SEM-based antimicrobial studies. (AL-Mojahid et al., 2026) In contrast, cells treated with ECV exhibited clear morphological damage, including irregular shapes, collapsed and wrinkled surfaces, and reduced cell density. These structural alterations suggest that ECV disrupts bacterial cell wall and membrane integrity, leading to leakage of cellular contents and cell death (Asokan et al., 2025). The SEM observations provide visual evidence of bacterial cell damage and support the antibacterial activity of ECV, thereby contributing to the scientific validation of ECV.

HRLC-MS in Table 7 and Figs. 12 and 13 indicates that CV detected a wide range of active ingredients, including alkaloids, triterpenoids, flavonoids, phenolics, glycosides, steroids, fatty acids, and macrolides. The conventional application of CV in managing inflammatory diseases, metabolic disorders, and urinary tract infections is supported by these compounds' antibacterial, anti-inflammatory, antioxidant, and immunomodulatory qualities. These bioactive phytochemicals identified in the HR-LCMS analysis are known to exert their pharmacological effects through multiple mechanisms, including reactive oxygen species (ROS) scavenging, disruption of bacterial cell membranes, inhibition of quorum sensing, and interference with microbial biofilm formation (Dongre and Majumdar, 2024; Swami et al., 2024). In addition to phytochemical class-level identification, several putative bioactive compounds detected through HR-LCMS, including piperine, gallic acid, quinidinone, ajaconine, and neferine, were considered in relation to their reported pharmacological activities. These compounds are known

to exhibit antimicrobial, antioxidant, and antibiofilm properties through mechanisms such as disruption of bacterial membrane integrity, modulation of oxidative stress, inhibition of quorum sensing, and interference with biofilm development. The presence of these compounds provides a more compound-specific mechanistic insight into the observed biological activities of ECV.

These results are consistent with earlier research on CV's individual constituents. For example, studies on *Commiphora mukul (guggul),* one of the main constituents of CV, have reported triterpenoids, flavonoids, and alkaloids as the primary constituents responsible for its ROS scavenging and antioxidant activities (Rawat et al., 2025; Sarup et al., 2015). Similarly, interventions on *Cinnamomum species*, another component of CV, have reported the presence of phenolic and flavonoid compounds associated with antioxidant and antimicrobial activities (Sharifi-Rad et al., 2021). These similarities indicate that the therapeutic potential of CV may result from the synergistic interaction of its bioactive constituents, thereby validating its traditional medicinal applications. Notably, although ECV represents a complex polyherbal extract, a level of phytochemical specificity is achieved through HR-LCMS characterization, which defines a consistent profile of bioactive constituents. The therapeutic activity observed in this study can therefore be interpreted as "functional specificity," arising from the collective and synergistic action of multiple phytochemicals targeting microbial growth and biofilm formation. This multi-component interaction is a recognized feature of traditional herbal formulations. However, further fractionation and isolation studies are required to identify individual active compounds and refine pharmacological specificity. This represents an important direction for future research.

Importantly, the present study extends beyond mere phytochemical identification by establishing a mechanistic link between the detected bioactive classes and the observed biological activities. The predominant phytochemicals identified, particularly phenolics, flavonoids, and alkaloids, are well documented to exert antimicrobial and antibiofilm effects through multiple mechanisms, including disruption of bacterial cell membranes and modulation of oxidative stress. These mechanisms are consistent with the experimental findings of the present study, as evidenced by SEM observations demonstrating structural damage to bacterial cells and the significant inhibition of biofilm formation. Furthermore, these results provide a strong scientific rationale for subsequent in vivo investigations, particularly in established UTIs animal models, where both antimicrobial efficacy and antibiofilm activity of ECV can be evaluated under physiologically relevant conditions.

5. Conclusion

The current study clarifies the phytochemical composition and biological activity of CV, thereby providing scientific backing for its traditional use. The identification of diverse bioactive compounds, along with their antioxidant, antimicrobial, and non-toxic nature, underscores its potential for therapeutic applications. The observed antimicrobial and antibiofilm properties against multidrug-resistant bacteria further highlight its significance as a natural alternative for combating infections. Future studies focusing on mechanistic insights and clinical evaluations will be crucial in fully understanding the pharmacological potential of CV and its role in modern medicine.

CRediT authorship contribution statement

Faheem Q. AL-Mojahid: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Tijo Cherian:** Writing – review & editing. **Afaf A. Alsosowaa:** Methodology, Formal analysis. **N Sharmila Devi:** Writing – review & editing. **Ardra A P:** Formal analysis. **Namitha Vijay:** Formal analysis. **Sijo Asokan:** Formal analysis. **Teena Merlin:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Data curation, Conceptualization. **Willie J.G.M. Peijnenburg:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationshipsthat could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2026.101007.

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