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ORIGINAL RESEARCH

Bioactive profiling and therapeutic potential of soxhlet-extracted *Murraya koenigii*: UHPLC-orbitrap-MS/MS characterization, antioxidant, antibacterial activities and histopathological amelioration in *Labeo rohita* fingerlings

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Abstract Curry leaves (*Murraya koenigii*) are valued for culinary and medicinal uses due to their rich phytochemical content. The paper analyzed Soxhlet-extracted curry leaf ethanolic extract (CLEE) using ultra-high-performance liquid chromatography with Orbitrap mass spectrometry (UHPLC-Orbitrap-MS/MS). In total, 79 compounds were detected: 14 phenolics (chlorogenic acid, gallic acid etc), 14 alkaloids (mahanimbin, murrayanine etc), 12 flavonoids (quercetin, kaempferol etc), 21 fatty acids, 11 terpenoids (curcumene etc), one saponin (bruceoside A), one carotenoid (echinenone), five vitamins (B3, B4, A, E, vitamin C derivative etc). These exhibited observable spikes throughout the entire ion chromatogram and occurred in both positive and negative ionization modes (mass errors up to 4.49 ppm). The results showed antioxidant assay, total phenolic contents of 163.112 ± 1.182 mg/g gallic acid equivalents, flavonoid contents of 56.167 ± 0.541 mg/g quercetin equivalents, tannin contents of 3.974 ± 0.169 mg/ g gallic acid equivalents, and strong free radical scavenging of 73.391 ± 0.84% (DPPH) and 69.176 ± 1.40% (DMSO). A concentration-dependent antibacterial effect against *Aeromonas hydrophila* was observed at 5.5 ± 0.29 mm at 200 mg/mL and 7.5 ± 0.29 mm at 250 mg/mL, respectively, compared with gentamicin (11 ± 0.58 mm at 10 mg/disc). Histopathological evaluation revealed that the group treated with CLEE exhibited minimal liver damage (vacuolation, erythrocyte infiltration, multinucleated cells, karyorrhexis, and vacuolated melano-macrophage centers) and caused less renal injury (glomerular shrinkage, melanomacrophage centers, tubular degeneration, and occlusion) compared to the positive control. In comparison, the CLEE-treated group showed reduced glomerular and tubular damage, characterized by well-compacted nephrons and the absence of melanomacrophage centers. The antioxidant and moderate antibacterial activities, as well as the histopathological improvements, attributed to CLEE (*M. koenigii*) show promising potential within the scope of pharmaceuticals and nutraceuticals in aquaculture for fish health management.

Keywords *Murraya koenigii* . UHPLC-ORBITRAP-MS/MS . Phytochemicals . Antioxidants . Phenolic compounds . Flavonoids

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Introduction

Curry leaf (*Murraya koenigii*) is a small, fragrant shrub native to the Indian subcontinent, and an irreplaceable part of South Indian culture, cuisine, and traditional healing practices. Throughout India, curry leaves are cherished due to their rather nutty, citrussy aroma that suits classic dishes such as sambar, rasam, chutneys and curries in some states, including Tamil Nadu, Karnataka, Andhra Pradesh, Telangana and Kerala Abeysinghe et al. 2021a). Whereas, outside of the kitchen, they are constituents of Ayurvedic and Siddha medicine, where they are renowned as antioxidants, anti-inflammatory substances, antimicrobials, and digestive aids and are utilized in treating such conditions as digestive disorders and diabetes and in improving the health of the hair and skin (Ganesan et al. 2013). These various properties of their medicinal and culinary value are due to an abundance of phytochemicals such as carbazole alkaloids, flavonoids, and essential oils (Balakrishnan et al. 2020).

To effectively isolate and analyse such bioactive compounds, appropriate extraction techniques are essential. Among these, the Soxhlet extraction technique is a widely used method in phytochemical studies as it is precise and efficient in extracting the bioactive compounds found in curry leaves. The application of this method involves constant solvent recirculation to recover compounds such as alkaloids, flavonoids, and essential oils, achieving high yields with a small amount of solvent, which is suitable for heat-stable phytochemicals (De Castro and Priego-Capote 2010). Soxhlet extraction demonstrates better efficiency in isolating antioxidants and antimicrobial components than simple techniques, such as maceration (Jensen 2007).

Advanced analytical tools are crucial for decoding the complex phytochemicals in plant extracts. This study uses the Thermo Scientific Orbitrap Eclipse Tribrid Mass Spectrometer, combined with the UltiMate 3000 RSLCnano or Vanquish UHPLC system and a heated Electrospray Ionization (H-ESI) source, to deliver high-resolution, accurate-mass (HRAM) profiling of curry leaf ethanolic extract (CLEE). It includes resolutions up to 1,000,000 FWHM at 200 m/z and sub-ppm mass accuracy, making such setups excel in measuring and detecting polyphenols, alkaloids, flavonoids, and terpenoids, which are essential to discovering a drug and developing a functional food. The UHPLC platforms offer high-quality chromatographic separation, and the H-ESI source enables more metabolite ionization, which is supported by more advanced data acquisition routes such as Real-Time Search and AcquireX, that is, extensive metabolomics (Sarkar et al. 2025). The method has also been used in quality control and biomarker detection of herbal medicines and nutraceuticals.

Curry leaves can contain high levels of carbazole alkaloids (e.g., mahanimbine, koenimbine), flavonoids (e.g., quercetin, myricetin), and phenolic acids (e.g., gallic acid), and therefore have been shown to have powerful antioxidant, antimicrobial, anticancer, and antidiabetic properties (Ghasemzadeh et al. 2014; Balakrishnan et al. 2020). They are also useful compounds in fish welfare through their benefit as natural antimicrobial compounds or immunostimulants, without the need to include synthetic antibiotics in aquaculture (Reverter et al. 2014). They have shown a good total phenolic content and DPPH radical scavenging activity that favors the formulation of health-boosting foods (Ghasemzadeh et al. 2014). Also, substances such as girinimbine, linalool-containing essential oils have pharmaceutical value (Rajendran et al. 2014). UHPLC-Orbitrap-MS/MS fingerprinting, coupled with a combination of antioxidant assays (DPPH, DMSO), indicates interrelationships between antioxidant activities and certain phytochemicals in this study.

The species Aeromonas hydrophila represents a significant pathogen in freshwater aquaculture, leading to high levels of disease and economic losses worldwide (Pattanayak et al. 2020). The common interventions, such as the use of antibiotics, vaccines, probiotics, and biosecurity controls, have limitations as antibiotic resistance emerges and due to the genetic variability of *A. hydrophila* strains (Sherif and Kassab 2023). Resistance to a variety of available antibiotics, including tetracyclines (e.g., oxytetracycline), fluoroquinolones (e.g., ciprofloxacin), sulfonamides, trimethoprim, beta-lactams (e.g., ampicillin), and macrolides (e.g., erythromycin). This emerging resistance presents the extreme importance of seeking alternative disease management interventions in aquaculture. Bioactive compounds in plants, such as those based on *M. koenigii*, represent a promising strategy, as the plants contain a variety of phytochemicals with antioxidant and antibacterial properties.

The proposed study is relevant in trying to understand the knowledge gap of a thorough profiling of

curry leaves (*M. koenigii*), based on ultra-high-performance liquid chromatography in combination with Orbitrap tandem mass spectrometry (UHPLC-Orbitrap-MS/MS), in evaluating its potential application as a natural antimicrobial and antioxidant agent towards control of infection of *A. hydrophila* in aquaculture fish farms.

Materials and methods

Collection and authentication of curry leave

The fresh organic curry leaves (*M. koenigii*) were collected from the Fisheries College and Research Institute campus, Thoothukudi. Taxonomic authentication and verification (taxonomic voucher No.M30062303k) of the plant had been done in the Central Council of Research in Siddha, Anna Government Hospital Campus, Aumbakkam, Chennai-600106. The leaves were thoroughly washed in running water and then left to dry in the air in a shaded place for seven days. After being dried, the leaves were ground into a fine powder using an electric blender (Bajaj GX-1, Bajaj Electricals Ltd., India; operated at 1120 rpm). The powder was then stored in a plastic container at ambient temperature until use.

Soxhlet extraction of curry leaves

Curry leaves 'ethanolic extract was prepared using a Soxhlet extraction apparatus (Techno Instruments Company, Model SOX-6, Code: 12030, Bangalore, India). During extraction, the powdered leaves of the curry plant (20 g) were extracted with 250 mL of absolute ethanol (99.9%). A Soxhlet thimble was covered with a cotton pad, which was filled with the leaf powder up to the siphon tube. Above the thimble, a condenser was connected, and at the bottom, a 500 mL round-bottom flask. Extraction was started by heating the flask on a heating mantle in a temperature range of 75-80°C. This was repeated until the solvent inside the siphon tube turned clear. The solvent of extraction was subsequently removed from the Soxhlet apparatus, cooled, and transferred to a rotary evaporator (Roteva-Equidron, Model EV11, Medica Instrument Mfg. Co., Mumbai, India). The extract was filtered, and the flask was clipped to the evaporator using a plastic clip. The filtrate was condensed at 50°C under reduced pressure until it formed a semi-liquid. The extract was later lyophilized in a freeze-dryer (Lark Innovative Fine Teknowledge, Tamil Nadu, India, Model: Penguin Classic, Cold Trap Lowest Temperature: -80°C) for 24 hrs. The dry extracts obtained were kept in -20 °C until further study.

UHPLC-orbitrap-MS/MS characterization of CLEE (ultra-high performance liquid chromatography coupled with orbitrap mass spectrometry/mass spectrometry)

Reagents and chemicals

Methanol (MeOH), LC/MS Grade (Fisher Chemical, A456-1), Water, LC/MS Grade (Fisher Chemical,



Fig. 1 Powdered curry leaves

W6-1) and Formic acid, LC/MS Grade (Fisher Chemical, A117-50) were obtained from Fisher Scientific Co. (Loughborough, UK). Deionized water was used. All other chemicals were of analytical grade.

Sample preparation

1 mL of chilled 80% aqueous methanol containing 0.1% formic acid (extraction buffer) was added to the CLEE and homogenized. Then, the homogenized solution was centrifuged at 16,000 × g for 15 minutes at 4°C to remove the debris from the extract. The supernatant was collected and filtered through a 0.22 µ nylon syringe filter. The filtrate was collected in HPLC vials and kept undisturbed at -20°C until LC/MS analysis.

Instrumentation and chromatographic conditions

Mass spectrometry (MS) was performed using an Orbitrap Eclipse Tribrid Mass Spectrometer, with either UltiMate 3000 RSLCnano UHPLC or Vanquish UHPLC system (Thermo Fisher Scientific). A heated electrospray ionization (H-ESI) source was fitted on the system (Thermo Scientific). The chemical constituents of the plant extract solution were separated by chromatography on UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with ACQUITY UHPLC HSS T3 column (2.1 × 100 mm, 1.8 µm; Waters Corporation, Milford, CT, USA). The mobile stage included 0.1% formic acid in water (A), and 0.1% formic acid in methanol (B), and the gradient elution profile was as shown below: 0.5-50% B (0-5 min), 50-98% B (5-6 min), 98% B (6-12 min), 98-0.5% B (12-13 min), 0.5% B (13-13.1 min), and 0.5-0% B (Flow rate, column temperature and injection volume were kept at 0.35 mL/min, 45 o C respectively. The heated electrospray ionization (H-ESI) source was operated with the following parameters: vaporizer temperature, 400 °C; capillary temperature, 300 °C; spray voltage, 3400 V; sheath gas flow rate, 40 L/min; auxiliary gas flow rate, 5 L/min; and sweep gas flow rate, 0 L/min. The mass spectra of the high resolutions were obtained in both positive and negative modes. The quadrupole-Orbitrap analyses were performed in full MS/dd-MS2 (TopN) and full MS mode. The complete MS mode used a mass scan of 100 1000 m/z, an Orbitrap resolution of 60,000, a maximum ion injection time (IT) of 50 ms, and a target of 3×10^6. dd-MS2 was run in Top5 mode with the following configureurations: Orbitrap resolution of 15,000, maximum IT of 22 ms, AGC target of 3 x 10(Polshettiwar et al. 2007) 6, loop count of 5, isolation window of 1.5 m / z; stepped normalized collision energy (NCE) of 20, 35, and 50.

Data preprocessing and analysis

The data processing was done using the Compound Discoverer 3.3 software. A pre-existing workflow template titled ‘Untargeted Metabolomics using Online Databases, mzLogic, and Molecular Networks’ was used to identify the differences between samples was used to extract information on the retention time (RT), accurate molecular weight (m/z), and MS/MS fragment ions from the raw data obtained by UHPLC-Or-

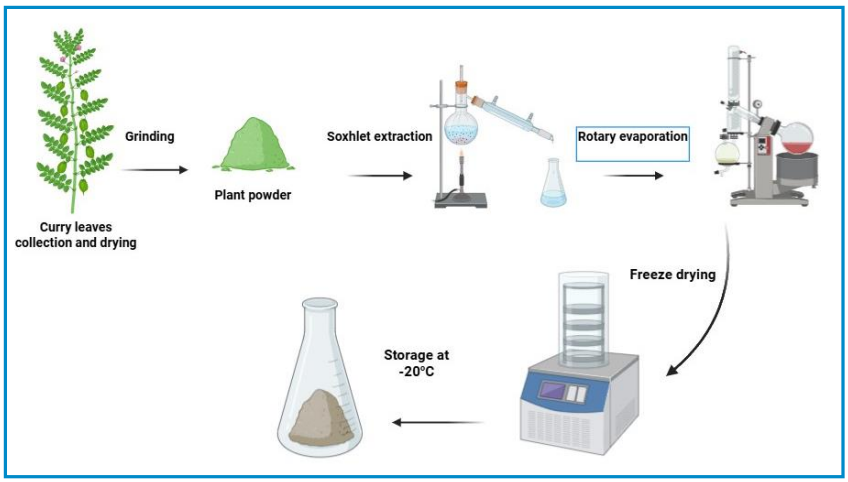


Fig. 2 Flowchart of preparation of ethanolic extract of curry leaves using the Soxhlet extraction method

bitrap-MS/MS analysis.

Phytochemical analysis and antioxidant estimation of the extract

The phytochemical properties, including total phenolic content, total flavonoid content, total tannin content, and antioxidant properties, such as DPPH (1,1-diphenyl-2-picrylhydrazyl) and alkaline DMSO radical scavenging activity, were estimated. The curry leaf ethanolic extract (CLEE) was analyzed for total phenolic content as the method described by Siddhuraju and Becker (2003), total flavonoid content (Chang et al. 2002), total tannin content (Polshettiwar et al. 2007), DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity (Brand-Williams et al. 1995), and alkaline DMSO radical scavenging activity (Ruch et al. 1989).

Bacterial strain and culture preparation

A. hydrophila bacterial suspension was made by inoculating a loop of colonies in Tryptic Soya Broth (TSB) (HiMedia, India) and incubating overnight at 37 °C. The OD600 was used to measure the log-phase bacterial suspension, which was then centrifuged at 8832 × g for 10 min using the Sigma 3K18 centrifuge (Rotor No. 12158). The pellet was then washed three times in Phosphate-Buffered Saline (PBS, pH 7.4). A standard plate count technique was used to determine the overall bacterial count using tryptic soy agar (TSA) plates to which the bacteria were added, incubated, and then counted. The results were expressed as CFU mL^{-1}. Following optimal growth, these young cultures were used as a test pathogen for the disc diffusion antibacterial activity assay.

Disc diffusion method

The antibacterial activity of CLEE against *A. hydrophila* was assessed by the Kirby-Bauer disc diffusion test (Bauer et al. 1966; Wayne 2012). The optical density was presented in bacterial culture with a UV spectrophotometer (Hitachi Instrument, India), at wavelengths OD 600 addressing a concentration of 10^7 CFU mL^{-1} (Anjur et al. 2021; Narsale et al. 2024). The bacterial suspension was diluted until the optical density of the sample corresponded to that of the standard. In MHA plates, bacterial suspension was gently discharged uniformly by using a clean cotton swab (100 µl). Stock solution: To 1 g of CLEE extract, 4 ml of ethanol was added (250 mg/ml). A gradient of their concentrations of 200, 150 and 100 mg/ml was prepared by serial dilutes: 3.2 ml of stock and 0.8 ml of ethanol were mixed to make 200 mg/ml, 3 ml of stock and 1 ml of ethanol were mixed to produce 150 mg/ml and 2 ml of stock and 2 ml of ethanol to produce 100 mg/ml. Whatman No. 1 filter paper was used to sterilize each of the solutions. Discs of sterile 6 mm filter paper (Hi media, India) were then saturated with 40 µL of each concentration, yielding disc loadings of 4 mg (100 mg/ml), 6 mg (150 mg/ml), 8 mg (200 mg/ml) and 10 mg (250 mg/ml). These discs were air-dried under a laminar flow hood within the laminar flow chamber for 2-3 minutes, and then placed in their designated positions on the inoculated MHA plates using sterile forceps. A 5 mg gentamicin disc was labeled as a positive control, and an ethanol disc was considered a negative control. The incubation of the plates was performed overnight at 37 °C (24 hours), and the diameter of the inhibition zone was measured using a Vernier caliper in millimeters (mm) and recorded.

Experimental set-up and experimental animal

The current study was performed at the Department of Fish Pathology and Health Management, Fisheries College and Research Institute, Tuticorin-628008. Nine (9) 100 L glass tanks with 65 L freshwater (30 ± 1°C, pH 7.4 ± 0.2), aerated using medium-sized carbon sponge filters. Rohu fingerlings from a local fish farm were acquired and acclimatized for 7 days. In total, 150 rohu fingerlings (average 8.2 cm length, 8.4 g weight) were divided into three treatments with three replicates (20 fish each) in a completely randomized design. Then, fish were transferred (15 fish/glass tank). Tanks were routinely cleaned by siphoning, with partial water replacement to remove waste. Nine (9) 100 L glass tanks with 65 L freshwater (30 ± 1°C, pH 7.4 ± 0.2), aerated using medium-sized carbon sponge filters. Rohu fingerlings from a local fish farm were

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Feed preparation and feeding trial

A commercial pellet feed (8 mm) (Growfin, Growel, Andhra Pradesh, India) containing 30% crude protein, 5% crude fat, 5.5% crude fiber, and 12% moisture was obtained. Soxhlet-derived curry leaf ethanolic extract (1 g/kg) was diluted in 80% ethanol, sprayed onto the pellets, and dried at 35°C for 12 hrs. The pellets were then coated with 4% aqueous gelatin (5:40 v/w) as a binder. The experimental diet consisted of 1000 g of Growel feed, 5 g of gelatin, 125 ml of distilled water, and 1 g of curry leaf ethanolic extract (CLEE). The pellets were air-dried at 25°C for 6 hrs and stored at 4°C in air-tight plastic containers. The negative control and bacterial control groups were fed the commercial pellet feed, while the CLEE group received feed supplemented with 1 g CLEE/kg, as per the previous study (Sahu et al. 2008).

Bacterial challenge study

Once the 28 days of feeding were completed, 0.1 mL of bacterial suspension of 3.8×10^6 CFU mL^{-1} (LD_{50} dose) of *A. hydrophila* was injected intraperitoneally into the individual fish. The experimental control fish was injected with PBS (100 µl/fish) only. The fish injected were put under observation after 2 weeks, and the dead fish were removed. The kidneys and liver organs were also collected for a histopathology study.

Histopathological changes

Histological analysis was performed according to the standard protocol outlined by Roberts (2012). Kidney and liver tissues from rohu fingerlings were collected at 7 days post-infection (dpi) after sedation with clove oil and percussive stunning to minimize stress. Tissues were immediately fixed in Bouin's solution (5% glacial acetic acid, 9% formaldehyde, 0.9% picric acid) for 24 hrs. Samples were processed using an Automatic Tissue Processor (Thermo Scientific, Shandon Citadel 2000) and embedded in paraffin with a Histocenter (ThermoScientific, Shandon Histocentre 3). Paraffin blocks were sectioned at 5–6 µm thickness using a Rotary Microtome (Leica, RM2245). Sections were stained with hematoxylin–eosin and mounted with Dibutylphthalate Polystyrene Xylene (DPX). Prepared slides were examined under a Leica DM750 light microscope (Leica Microsystems, Wetzlar, Germany) at 40x magnification.

Results

UHPLC-ORBITRAP-MS/MS analysis comprehensively characterizes the phytochemical composition of

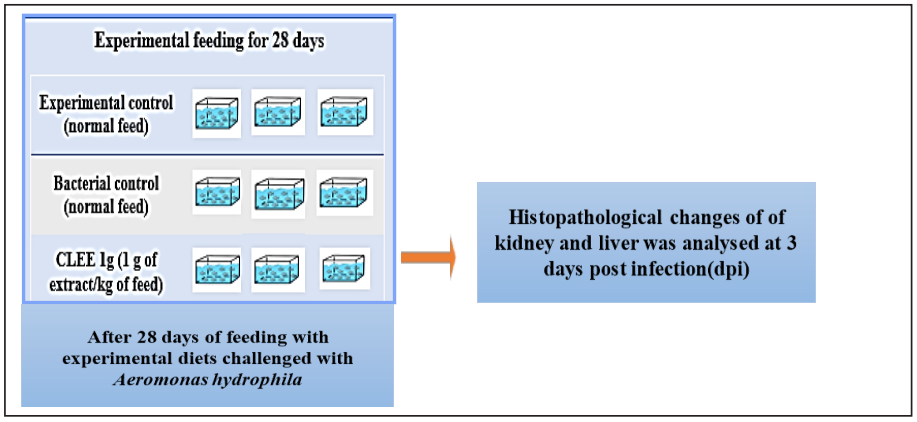


Fig. 3 Experimental feeding trial

Soxhlet-extracted CLEE. A total of 79 compounds were identified, including 14 phenolic compounds (phenolic acids, monophenols, and glycosides), 14 alkaloids (carbazole, piperidine, and isoquinoline derivatives), 12 flavonoids (flavonols, flavanones, and glycosides), 21 fatty acids (saturated, unsaturated, and hydroxy derivatives), 11 terpenoids (sesquiterpenes, triterpenes, and diterpenes), one saponin (bruceoside A), one carotenoid (echinenone), and five vitamins (B3, B4, A, E, and a vitamin C derivative).

Phenolic compounds

UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 15 phenolic compounds, including phenolic acids, phenolic aldehydes, monophenols, and complex phenolic derivatives. Compounds were detected in positive (ESI^+) and negative (ESI^-) ionization modes, with retention times (RT) ranging from 1.193 to 11.554 minutes. Table 1 summarizes the molecular formulas, ionization modes, theoretical and observed m/z values, mass errors (ppm), and retention times of phenolic compounds identified in Soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS

Phenolic acids

UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 15 phenolic compounds, including phenolic acids, phenolic aldehydes, monophenols, and complex phenolic derivatives. Compounds were detected in positive (ESI^+) and negative (ESI^-) ionization modes, with retention times (RT) ranging from 1.193 to 11.554 minutes. Table 1 summarizes the molecular formulas, ionization modes, theoretical and observed m/z values, mass errors (ppm), and retention times of phenolic compounds identified in Soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS.

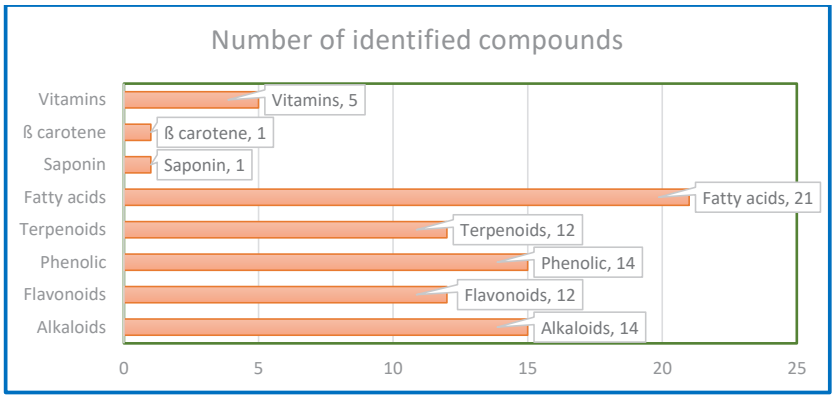


Fig. 4 Number of identified compounds by UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted curry leaves

Table 1 Phenolic compounds identified in soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS

Compound name	Molecular formula	RT (min)	Ion mode	Theoretical m/z	Observed m/z	Mass error (ppm)
Chlorogenic acid	C16H18O9	5.779	$[M-H]^-$	354.09511	353.08807	0.09
Phloroglucinol	C6H6O3	8.141	$[M+H]^+$	126.03165	127.03893	-0.31
Vanillin	C8H8O3	4.918	$[M-H]^-$	152.04748	151.04021	0.92
Guaiacol	C7H8O2	1.221	$[M-H]^-$	124.05272	123.04544	2.32
10-Gingerol	C21H34O4	10.227	$[M+H]^+$	350.24535	351.25262	-1.03
(E)-p-Coumaric acid	C9H8O3	6.121	$[M-H]^-$	164.04746	163.04018	0.71
Gallic acid	C7H6O5	1.193	$[M-H]^-$	170.02164	169.01436	0.69
Caffeic acid 3-glucoside	C15H18O9	1.283	$[M-H]^-$	342.09528	341.08800	0.58
5-Caffeoylshikimic acid	C16H16O8	7.597	$[M-H]^-$	336.08476	335.07748	0.72
Sinapinic acid	C11H12O5	8.313	$[M-H]^-$	224.06865	223.06137	0.78
Copalliferol B	C42H32O9	11.554	$[M+H]^+$	680.20629	681.21356	2.43
Cynarine	C25H24O12	9.055	$[M-H]^-$	516.12684	515.11957	0.13
Cinnamic acid derivative	C21H36O3Si2	10.936	$[M+H]^+$	392.22200	393.22928	4.34
Lusitanicoside	C21H30O10	8.969	$[M-H]^-$	442.18367	441.17639	-0.52

Phenolic acids

Eight phenolic acids were present, 7 of which were hydroxycinnamic acids and derivatives (chlorogenic acid, (E)-p-coumaric acid, caffeic acid 3-glucoside, 5-caffeoylshikimic acid, sinapinic acid, cinnamic acid derivative), and one hydroxybenzoic acid (gallic acid). Chlorogenic acid (Compound 1) was seen to have a high abundance as it had a major peak in the total ion chromatogram (TIC). Compounds 8 (caffeic acid 3-glucoside) and 12 (cynarine) were identified as low in mass error (0.58 and 0.13 ppm, respectively), confirming accurate identification.

Non-phenolic acid phenolics

There were four non-phenolic acid phenolics: phloroglucinol (polyphenol), vanillin (phenolic aldehyde), guaiacol and 10-gingerol (gingerol derivative). Phloroglucinol (Compound 2) and 10-gingerol (Compound 5) were found in ESI + mode whereas vanillin and guaiacol were found in ESI - mode. Mass error was between -1.03 and 2.32 ppm, which concurs with credible identification.

Complex phenolics

Complex phenolics were two compounds, copalliferol B (Compound 11) and lusitanicoside (Compound 14). The greatest mass error of 2.43 ppm was recorded with copalliferol B, which is a complex molecule. The TIC showed different peak values with high signals of chlorogenic acid, gallic acid, and sinapinic acid indicating high concentration of the substance in the extract. The existence of both ESI+ and ESI- modes provided total polar and semi-polar phenolics detection. Accuracy was high on a mass scale (less than 4.34 ppm), and did not compromise annotation of compounds with any confidence.

Alkaloids

UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE identified 14 alkaloid compounds, including carbazole alkaloids, piperidine alkaloids, pyridine alkaloids, isoquinoline alkaloids, and other alkaloid derivatives. Compounds were detected in positive (ESI^+) and negative (ESI^-) ionization modes, with retention times (RT) ranging from 1.052 to 11.569 minutes. Table 2 summarizes the molecular formulas, ionization modes, theoretical and observed m/z values, mass errors (ppm), and retention times.

Carbazole alkaloids

The following 3 carbazole alkaloids were identified: mahanimbin (Compound 1), murrayanine (Compound

Table 2 Alkaloids identified in soxhlet-extracted curry leaves via UHPLC-ORBITRAP-MS/MS

Compound name	Molecular formula	RT (min)	Ion mode	Theoretical *m/z*	Observed *m/z*	Mass error (ppm)
Mahanimbine	C23H25NO	11.569	$[M+H]^+$	331.19353	332.20080	-0.25
1-Piperideine	C5H9N	1.052	$[M+H]^+$	83.07359	84.08086	1.06
Guvacine	C6H9NO2	1.212	$[M+H]^+$	127.06336	128.07063	0.22
Murrayanine	C14H11NO2	9.597	$[M+H]^+$	225.07869	226.08595	-1.30
Arecoline	C8H13NO2	1.125	$[M+H]^+$	155.09459	156.10189	-0.24
Cryptopleurine	C24H27NO3	10.066	$[M+H]^+$	377.19911	378.20639	0.05
Ethaverine	C24H29NO4	10.123	$[M–H]^-$	395.20977	394.20248	0.29
Evocarpine	C23H33NO	9.120	$[M+H]^+$	340.26315	339.25588	-1.00
Conanine	C22H37N	9.526	$[M+H]^+$	315.29241	316.29968	-0.62
Dioncopeltine A	C23H25NO4	11.317	$[M+H]^+$	379.17876	360.16092	1.06
4'-O-Methylnorbelladine	C16H19NO3	9.363	$[M+H]^+$	273.13643	274.14371	-0.23
(−)-Strychnine	C21H22N2O2	7.386	$[M+H]^+$	334.16816	335.17544	0.10
Cammaconine	C23H37NO5	8.740	$[M+H]^+$	407.26709	390.26382	-0.19
Codeinone	C18H19NO3	8.967	$[M–H]^-$	297.13655	296.12927	0.19

4), and evocarpine (Compound 8). A high abundance was observed in mahanimbin in the total ion chromatogram (TIC). The correct identification is demonstrated by its low mass error (-0.25 ppm) in ESI+ mode. Murrayanine and evocarpine had mass errors of -1.30 and -1.00 ppm, respectively, which favour sound annotation.

Piperidine and pyridine alkaloids

There were 4 piperidine or pyridine alkaloids, namely 1-piperidine (Compound 2), guvacine (Compound 3), and arecoline (Compound 5). These compounds were identified in ESI+ mode, and mass deviations were between -0.24 and 1.06 ppm. Guvacine and arecoline showed high TIC signals which indicated high concentrations in the extract.

Isoquinoline alkaloids

The isoquinoline alkaloids were two compounds: cryptopleurine (Compound 6) and ethaverine (Compound 7). The mass error of cryptopleurine was very low (0.05 ppm) on the ESI+ mode and ethaverine was identified on ESI- mode with a mass error at 0.29 ppm, which was very high.

Other alkaloids

Other alkaloid derivatives were classified as six compounds: conanine (Compound 9), dioncopeltine A (Compound 10), 4'-O-methylnorbelladine (Compound 11), (−)-strychnine (Compound 12), cammaconine (Compound 13), and codeinone (Compound 14). These compounds were of differing structures with a mass error of -0.19 to 1.06 ppm. Codeinone was detected in ESI minus mode and the rest in ESI plus mode. The TIC showed strong peak intensities for (−)-strychnine and 4'-O-methylnorbelladine, indicating their relative abundance. Varying peak intensities were observed in the TIC with mahanimbin, guvacine and (−)-strychnine having strong signals attributable to high concentrations of these compounds in the extract. The polar and semi-polar alkaloids were thoroughly detected by the use of both ESI + and ESI - modes. The accuracy was high (mass) and the error was 1.30 ppm, which is sufficient to be sure of the compound being annotated.

Flavonoids

UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 12 flavonoid compounds, including flavonols, flavones, flavanones, and their glycosylated derivatives. Compounds were detected in positive (ESI^+) and negative (ESI^-) ionization modes, with retention times (RT) ranging from 8.029 to 10.306 minutes. Table 3 summarizes the molecular formulas, ionization modes, theoretical and observed m/z values, mass errors (ppm), and retention times.

Table 3 flavonoids identified in soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS

Compound name	Molecular formula	RT (min)	Ion mode	Theoretical *m/z*	Observed *m/z*	Mass error (ppm)
Hyperoside (quercetin-3-O-galactoside)	C21H20O12	8.565	$[M+H]^+$	464.09541	465.10236	-0.15
6"-O-Acetylisoquercitrin	C23H22O13	8.729	$[M+H]^+$	506.10598	507.11311	-0.12
Quercetin	C15H10O7	8.400	$[M+H]^+$	302.04256	303.04984	-0.30
12-Oxo phytodienoic acid	C18H28O3	10.306	$[M+H]^+$	292.20375	293.21101	-0.33
Kaempferol	C15H10O6	9.267	$[M–H]^-$	286.04774	285.04047	0.02
Trifolin (kaempferol 3-galactoside)	C21H20O11	8.768	$[M+H]^+$	448.10055	449.10768	-0.02
Isorhamnetin	C16H12O7	8.792	$[M+H]^+$	316.05800	317.06528	-0.95
Isorhamnetin 3-glucoside	C22H22O12	8.394	$[M–H]^-$	478.11162	477.10435	1.04
rac 8-Prenylnaringenin	C20H20O5	8.029	$[M+H]^+$	340.13091	341.13818	-0.49
Rutin	C27H30O16	8.699	$[M–H]^-$	610.15449	609.14722	1.82
Tiliroside	C30H26O13	9.018	$[M–H]^-$	594.13762	593.13034	0.46
Gossypin	C21H20O13	8.228	$[M–H]^-$	480.09058	479.08330	0.39

Flavonols

UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 12 flavonoid compounds, including flavonols, flavones, flavanones, and their glycosylated derivatives. Compounds were detected in positive (ESI^+) and negative (ESI^-) ionization modes, with retention times (RT) ranging from 8.029 to 10.306 minutes. Supplementary Table 3 summarizes the molecular formulas, ionization modes, theoretical and observed m/z values, mass errors (ppm), and retention times.

Flavonols

It was found that seven compounds were flavonols or flavonol glycosides: hyperoside (Compound 1), 6”-O-acetylisoquercitrin (Compound 2), quercetin (Compound 3), kaempferol (Compound 5), trifolin (Compound 6), isorhamnetin (Compound 7), and isorhamnetin 3-glucoside (Compound 8). Quercetin and kaempferol had high abundance as evidenced by strong peaks in the total ion chromatogram (TIC). Hyperoside and trifolin contained low mass errors (-0.15 and -0.02 ppm, respectively), indicating proper identification. The mass error in isorhamnetin 3-glucoside was somewhat larger (1.04 ppm), but that still did not exceed acceptable limits in high-resolution MS.

Flavanones

A total of one compound was detected as a flavanone, rac 8-prenylnaringenin (Compound 9). It was identified in ESI+ mode with a mass error of -0.49 ppm, corresponding to a reliable annotation. It exhibited a moderate TIC signal, indicating a low concentration compared to flavonols.

Flavonoid glycosides

Three of the compounds were flavonoid glycosides: rutin (Compound 10), tiliroside (Compound 11), and gossypin (Compound 12). They were identified in the ESI - mode and their mass errors are between 0.39 and 1.82 ppm. Rutin had the maximum error in mass (1.82 ppm), likely due to its complex structure as a diglycoside, and was also identified by its typical fragmentation patterns in MS/MS.
The TIC showed clear peak intensities, indicating high concentration of quercetin, kaempferol and hyperoside in the extract. Since both ESI + and ESI - modes were used, polar and semi-polar flavonoids were thoroughly detected. The mass accuracy was also good with errors at 1.82ppm and below and provided the confidence to annotate the compounds.

Fatty acids

The UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 21 fatty acids and related compounds, encompassing saturated, unsaturated, oxygenated, and furan fatty acids. These compounds were detected in both positive ($[M+H]^+$) and negative ($[M–H]^-$) ionization modes, with retention times (RT) ranging from 1.207 to 12.382 minutes. Table 4 summarizes the molecular formulas, ionization modes, theoretical and observed *m/z* values, mass errors (ppm), and retention times for the identified fatty acids.

Saturated fatty acids

The UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 21 fatty acids and related compounds, encompassing saturated, unsaturated, oxygenated, and furan fatty acids. These compounds were detected in both positive ($[M+H]^+$) and negative ($[M–H]^-$) ionization modes, with retention times (RT) ranging from 1.207 to 12.382 minutes. Supplementary Table 4 summarizes the molecular formulas, ionization modes, theoretical and observed *m/z* values, mass errors (ppm), and retention times for the identified fatty acids.

Saturated fatty acids

Three compounds were identified as saturated fatty acids: palmitic acid (Compound 12), myristic acid (Compound 21), and juniperic acid (Compound 20). The total ion chromatogram (TIC) displayed a sharp peak for palmitic acid, indicating its abundance. It is accurately identified by its low mass error (0.19 ppm) in the ESI mode. Maximum mass errors were 0.86 and 0.48 ppm in myristic and juniperic acids, respectively, which justify the use of reliable annotation.

Unsaturated fatty acids

There were five unsaturated fatty acids, namely, linolenelaidic acid (Compound 1), eicosapentaenoic acid (Compound 2), (E)-parinaric acid (Compound 7), oleic acid (Compound 15), and ricinoleic acid (Compound 18). The TIC signals of Linolenelaidic acid and oleic acid were high which indicated high concentrations. The mass error fell between -0.10 and 0.25 ppm, a high standard of accuracy in analysis.

Hydroxy fatty acids

There were six hydroxy fatty acids (13(S)-HOTrE (Compound 3), (±)13-HODE (Compound 6), corchorifatty acid F (Compound 4), aleuritic acid (Compound 16), 9,10-dihydroxystearic acid (Compound 17), and 2-hydroxy lignoceric acid (Compound 19). These compounds were mainly identified in ESI -mode with mass errors of between 0.17 and 1.01 ppm which supports correct identification.

Other fatty acid derivatives

Seven compounds were categorized as other fatty acid derivatives 9,12: 9,12-octadecadienal (Compound 5), trans-geranic acid (Compound 8, Compound 22), monolinolenin (Compound 9), isopropylmalic acid (Compound 10), itaconic acid (Compound 11), furan fatty acid F6 (Compound 13), and 11-phenylundecanoic acid (Compound 14). These compounds had varied structures with a variation in mass error of between -0.69 and 2.18 ppm. Itaconic acid had the lowest mass error (2.18 ppm), possibly due to its small molecular size; however, its presence was confirmed by the MS/MS fragmentation patterns.

Table 4 Fatty acids identified in soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS

Compound name	Molecular formula	RT (min)	Ion mode	Theoretical *m/z*	Observed *m/z*	Mass error (ppm)
Linolenelaidic acid	C18H30O2	11.483	$[M+H]^+$	278.22460	279.23180	-0.10
Eicosapentaenoic acid	C20H30O2	11.163	$[M+H]^+$	302.22440	303.23150	-0.71
13(S)-HOTrE	C18H30O3	10.259	$[M–H]^–$	294.21960	293.21230	0.21
Corchorifatty acid F	C18H32O5	9.368	$[M–H]^–$	328.22530	327.21800	0.91
9,12-Octadecadienal	C18H32O	11.817	$[M+H]^+$	264.24513	265.25241	-0.69
(±)13-HODE	C18H32O3	10.449	$[M–H]^–$	296.23529	295.22801	0.49
(E)-Parinaric acid	C18H28O2	9.476	$[M+H]^+$	276.20874	277.21601	-0.70
trans-Geranic acid	C10H16O2	8.823	$[M+H]^+$	168.11494	169.12219	-0.56
Monolinolenin	C21H36O4	11.077	$[M+H]^+$	352.26117	353.26848	-0.55
Isopropylmalic acid	C7H12O5	6.591	$[M–H]^–$	176.06868	175.06140	1.16
Itaconic acid	C5H6O4	1.207	$[M–H]^–$	130.02689	129.01962	2.18
Palmitic acid	C16H32O2	11.635	$[M–H]^–$	256.24028	255.23300	0.19
Furan fatty acid F6	C22H38O3	11.821	$[M+H]^+$	350.28197	351.28917	-0.36
11-Phenylundecanoic acid	C17H26O2	10.059	$[M+H]^+$	262.19312	263.20044	-0.62
Oleic acid	C18H34O2	12.382	$[M–H]^–$	282.25595	281.24875	0.25
Aleuritic acid	C16H32O5	9.308	$[M–H]^–$	304.22528	303.21800	1.01
9,10-Dihydroxystearic acid	C18H36O4	10.294	$[M–H]^–$	316.26141	315.25414	0.17
Ricinoleic acid	C18H34O3	11.555	$[M–H]^–$	298.25087	297.24359	0.25
2-Hydroxy lignoceric acid	C24H48O3	10.729	$[M–H]^–$	384.36067	383.35339	0.85
Juniperic acid	C16H32O3	10.471	$[M–H]^–$	272.23527	271.22800	0.48
Myristic acid	C14H28O2	11.459	$[M–H]^–$	228.20913	227.20185	0.86

Strong intensities were apparent in the TIC, and strong intensities were apparent on palmitic acid, oleic acid, and linolenelaidic acid, suggesting that these are very concentrated in the extract. The combination of ESI + and ESI - modes allowed for the detection of all polar and non-polar fatty acids. There was high mass accuracy (<= 2.18 ppm) and confident annotation of compounds. Ethanol Soxhlet extraction supported the extraction of various classes of fatty acids.

Terpenoids

The UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 11 terpenoids, encompassing monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetraterpenoids. These compounds were detected in both positive ($[M+H]^+$) and negative ($[M–H]^-$) ionization modes, with retention times (RT) ranging from 7.503 to 13.616. Table 5 summarizes the molecular formulas, ionization modes, theoretical and observed *m/z* values, mass errors (ppm), and retention times for the identified terpenoids.

Sesquiterpenes

The UHPLC-ORBITRAP-MS/MS analysis of Soxhlet-extracted CLEE (*M. koenigii*) identified 11 terpenoids, encompassing monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetraterpenoids. These compounds were detected in both positive ($[M+H]^+$) and negative ($[M–H]^-$) ionization modes, with retention times (RT) ranging from 7.503 to 13.616. Supplementary Table 5 summarizes the molecular formulas, ionization modes, theoretical and observed *m/z* values, mass errors (ppm), and retention times for the identified terpenoids.

Sesquiterpenes

Four compounds were recognized as sesquiterpenes: curcumene (Compound 1), (+)-nootkatone (Compound 3), (E,E)-alpha farnesene (Compound 4), and phaseic acid (Compound 9). Curcumene showed a strong peak in the total ion chromatogram (TIC), showing that it was very abundant. Its mass error (-0.75 ppm) in ESI + mode confirms correct identification, as did the mass errors of phaseic acid (0.41 ppm) observed in ESI + mode, whereas + -nootkatone and (E,E)-alpha farnesene had mass errors of -0.90 and -1.11 ppm, respectively.

Triterpenes

Three compounds were triterpenes: cucurbitacin S (Compound 2), (+)-absinthin (Compound 6), and maslinic acid (Compound 11). Cucurbitacin S and (+)-absinthin were observed in ESI+ mode with mass error of 1.23 and 4.49 ppm, respectively. The low mass error of 0.40 ppm of maslinic acid was detected in ESI- mode. The error in mass of the higher mass of (+)-absinthin is possibly because of the complex

Table 5 Terpenoids identified in soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS

Compound name	Molecular formula	RT (min)	Ion mode	Theoretical *m/z*	Observed *m/z*	Mass error (ppm)
Curcumene	C15H22	9.469	$[M+H]^+$	202.17200	203.17927	-0.75
Cucurbitacin S	C30H42O6	12.202	$[M+H]^+$	498.29875	499.30603	1.23
(+)-Nootkatone	C15H22O	10.619	$[M+H]^+$	218.16687	219.17414	-0.90
(E,E)-alpha Farnesene	C15H24	12.728	$[M+H]^+$	204.18757	205.19485	-1.11
Genipin	C11H14O5	7.503	$[M–H]^-$	226.08426	225.07699	0.62
(+)-Absinthin	C30H40O6	12.170	$[M+H]^+$	496.28472	497.29199	4.49
1,4-Cyclohexadiene	C6H8	9.865	$[M+H]^+$	80.06269	81.06997	1.11
Calicoferol E	C27H42O2	11.838	$[M+H]^+$	398.31861	399.32584	0.34
Phaseic acid	C15H20O5	8.444	$[M–H]^-$	280.13119	279.12391	0.41
Dehydroeburicoic acid	C31H48O3	13.492	$[M–H]^-$	468.36060	467.35332	0.54
Maslinic acid	C30H48O4	13.616	$[M–H]^-$	472.35545	471.34817	0.40

dimeric structure which can be problematic when trying to determine the accurate mass.

Other terpenoid derivatives

Four compounds were categorized as terpenoid derivatives as genipin (Compound 5, iridoid), 1, 4 cyclohexadiene (Compound 7, a monoterpene precursor), calicoferol E (Compound 8, a diterpene), and dehydroeburicoic acid (Compound 10, a triterpenoid). They had varied structures in which the mass error ranged between 0.34 and 1.11 ppm. The nearest error in mass was also the lowest (0.34 ppm) with calicoferol E, which supports the correct identification. The presence of 1,4-Cyclohexadiene could be a biosynthetic intermediate or artifact at some point of the extraction. The TIC showed some definite peak intensities with high signals of curcumene, cucurbitacin S, and maslinic acid, indicating high concentrations in the extract. Using ESI- and ESI+ made possible the detection of polar and non-polar terpenoids comprehensively. High mass accuracy (errors 4.49 ppm) and a high confidence of compound annotation were achieved.

Saponins

Bruceoside A (Compound 1) was identified as a saponin. It was identified in ESI - mode with low error of mass -0.42 ppm with a confirmed identification. Bruceoside A had an intermediate value in the total ion chromatogram (TIC), which indicates a relatively lower concentration when compared to other compounds.

Carotenoids

A single compound, namely echinenone (Compound 2) was found to be a derivative of beta-carotene (carotenoid). It was identified in ESI+ mode, with a very low mass error value of -0.09 ppm, which justifies a high confidence of annotation. It had a good TIC signal, which means that it was abundant in the extract.

Vitamins

There were five vitamins identified: choline (Vitamin B4, Compound 3), niacin (Vitamin B3, Compound 4), vitamin A (Compound 5), tocopherol (Vitamin E, Compound 6), and dehydroascorbic acid (one of the Vitamin C derivatives, Compound 7). These were found in both ESI + and ESI - modes with mass errors of between -2.25 and 0.75 ppm. The smallest mass error was found with Niacin (0.06 ppm) and tocopherol (the highest -2.25 ppm), which is likely attributed to its size (larger molecular structure). The choline and niacin quality of TIC showed strong signals of high concentration, and dehydroascorbic acid and vitamin A had moderate signals (Table 6).

Phytochemical analysis and antioxidant estimation of the CLEE

Antioxidant activity of Soxhlet-extracted CLEE (*M. koenigii*) was assessed using various tests, the findings of which are presented in Table 1. The total phenolic content was calculated as 163.112±1.182mg/g gallic acid equivalent (GAE), total flavonoid content mg/g quercetin equivalent of 56.167±0.541, showing high concentration of phenolic and flavonoid compounds. Tannin was determined at 3.974 ± 0.169 mg/g tannic acid equivalents (TAE). The antioxidant was found to have a high antioxidant capacity of DPPH at 73.391 ±

Table 6 Saponins, Beta-Carotene Pigments, and Vitamins identified in soxhlet-extracted CLEE via UHPLC-ORBITRAP-MS/MS

Compound name	Molecular formula	RT (min)	Ion mode	Theoretical *m/z*	Observed *m/z*	Mass error (ppm)
Bruceoside A (Saponin)	C32H42O16	7.509	$[M–H]^-$	682.24728	681.82198	-0.42
Echinenone (Beta-Carotene)	C40H54O	14.605	$[M+H]^+$	550.41742	551.42469	-0.09
Choline (Vitamin B4)	C5H13NO	0.970	$[M+H]^+$	103.10000	104.11000	-0.19
Niacin (Vitamin B3)	C6H5NO2	1.190	$[M+H]^+$	123.03000	124.04000	0.06
Vitamin A	C20H30O	9.785	$[M+H]^+$	286.22942	287.23672	-0.85
Tocopherol (Vitamin E)	C29H50O2	13.401	$[M+H]^+$	430.38011	431.38739	-2.25
Dehydroascorbic acid	C6H6O6	1.240	$[M–H]^-$	174.02000	173.01000	0.75

0.84%. Likewise, DMSO free radical scavenging activity was found to be 69.176 +1.40, which once again confirmed the strong antioxidant properties of CLEE.

Disc diffusion assay

The 100 mg/ml (4 mg/disc) and 150 mg/ml (6 mg/disc) concentrations did not show any inhibition zones. Whereas the 200 mg/ml (8 mg/disc) concentration had an inhibition zone of 5.5 mm and the 250 mg/ml (10 mg/disc) concentration had an inhibition zone of 7.5 mm. The positive control, gentamicin (10 mg/disc) produced an inhibition zone of 11 mm. These findings suggest that M. koenigii extract possesses antibacterial activity against A. hydrophila at higher concentrations (200 and 250 mg/ml) and bigger inhibition zones are noted in 250 mg/ml.

Challenge study

After being challenged with *A. hydrophila*, infected rohu fingerlings (B) show hemorrhages on the skin, Whereas the non-infected rohu fingerlings (A) show no hemorrhages on the skin, and internal organs are normal.

Histopathological findings

Liver

The histopathological observation showed that the negative control group presented circles of hepatocytes (CH) with no pathological variations, which defines the control state. On the other hand, the positive control group showed various histopathological abnormalities, such as vacuolation (V), which is characterized by cytoplasmic vacuoles, erythrocyte infiltration (EI) in the tissue, multinucleated cells (MNC), karyorrhexis (KR), which indicates nuclear breakdown, and vacuolated melano-macrophage centers (VMMC). The frequency and intensity of vacuolation (V), erythrocyte infiltration (EI), multinucleated cells (MNC), and karyorrhexis (KR) decreased significantly in the CLEE-treated group compared to the positive control, and vacuolated melano-macrophage centers (VMMC) were completely absent. These results suggest that CLEE treatment reduces the pathological alteration observed in the positive control, which could be attributed to an antibacterial and possible protective effect (Figure 7).

Kidney

Histopathological analysis of renal tissue showed that the control negative group had intact glomeruli (G),

Table 7 Antioxidant properties of soxhlet- extracted CLEE

Antioxidant property	Curry leaf Ethanolic extract (CLEE)
Total phenolic content (mg/g GAE)	163.112 ± 1.182
Total flavonoid content (mg/g QE)	56.167±0.541
Total tannin content (mg/g TAE)	3.974 ± 0.169
DPPH (Free Radical Scavenging Activity %)	73.391 ± 0.84
DMSO (Free Radical Scavenging Activity %)	69.176 ± 1.40

Values are expressed as mean ± standard error.

Table 8 MIC of *M. koenigii* against *A. hydrophila*

Plant species	Standard concentration mg/mL	Zone of inhibition (mm)
Murraya koenigii	Negative control(ethanol)	0
	4 mg (100 mg/mL),	0
	6 mg (150 mg/mL),	0
	8 mg (200 mg/mL),	5.5± 0.29
	10 mg (250 mg/mL).	7.5 ± 0.29
	Positive control (10 mg Gentamycin)	11± 0.58

Values are expressed as mean ± standard error.

normal nephrons (N), and healthy proximal tubules (PT), which can be taken as the baseline condition. Conversely, the positive control group exhibited intense pathological alterations including severe shrinkage of glomeruli (SG), melanomacrophage centers (MMCs), vacuolization and degeneration of tubules (VDT),

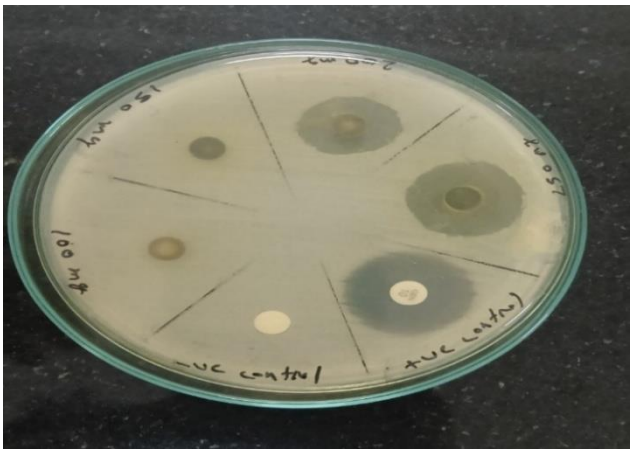


Fig. 5 Agar plate of CLEE, 100 mg/mL – 4 mg, 150 mg/mL – 6 mg, 200 mg/mL – 8 mg, 250 mg/mL - 10 mg, negative control -ethanol, positive control – 10 mg Gentamycin.



Fig. 6 A: non-infected rohu fingerlings with no haemorrhages on the skin and normal internal organs and B: Ruhu fingerling infected with the presence of haemorrhages on the skin.

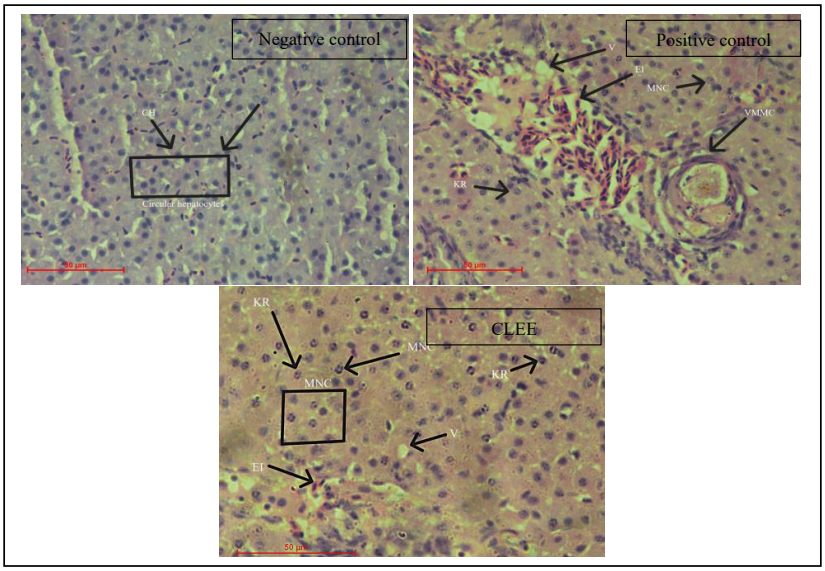


Fig. 7 Representative histopathology of the Liver of *L. rohita* fed with CLEE and challenged with *A. hydrophila*. Exp. Control: no bacterial infection; Bac. Control: bacterial infection, CLEE : 1 g CLEE/kg feed, and bacterial infection. H & E staining. Scale bar=50 µm

and occlusion (O) in the renal structure. There was less glomerular shrinkage (SG) and proximal tubule degeneration (DT) in the CLEE-treated group than in the positive control, and sustained occlusion (O) and significantly compact nephrons (N) with no melanomacrophage centers (MMCs). The findings imply that CLEE treatment has a mitigative effect than the positive control, meaning it partially reduced the renal histopathological changes (Figure 8).

Discussion

UHPLC-ORBITRAP-MS/MS analysis of curry leaf extract

Phenolic compounds

UHPLC-ORBITRAP-MS/MS of Soxhlet-extracted CLEE (*M. koenigii*) provided a diverse phenolic profile, which comprised 14 compounds belonging to phenolic acid, non-phenolic acid phenolics, and complex phenolics. This overall description is consistent with the literature on curry leaves, which emphasizes a high phenolic content and related bioactivities such as antioxidant, anti-inflammatory, and antimicrobial (Verma et al. 2024). Both ESI+ and ESI- errors (0.434 ppm) and detection errors demonstrate the method's capability to recover various phenolic structures. It has also been demonstrated that Soxhlet extraction using ethanol provides a better yield of phenolic acids in plant matrices than other techniques.

Phenolic acids

The presence of eight phenolic acids, most of which are hydroxycinnamic acids (e.g., chlorogenic acid, caffeic acid 3-glucoside, cynarine) and one hydroxybenzoic acid (gallic acid), highlights the preponderance of these compounds in CLEE (*M. koenigii*). The high abundance of chlorogenic acid is seen in the total ion chromatogram (TIC), which confirms a high level of chlorogenic acid in the CLEE (*M. koenigii*) as previously reported (El-Shiekh et al. 2024; Verma et al. 2024). The error of mass of 0.09 ppm and the absence of the compound in the ESI+ mode prove that it is reliably identified as the compound is generally well-ionized in the negative mode because of its acidic nature. Likewise, caffeic acid 3-glucoside and cy-

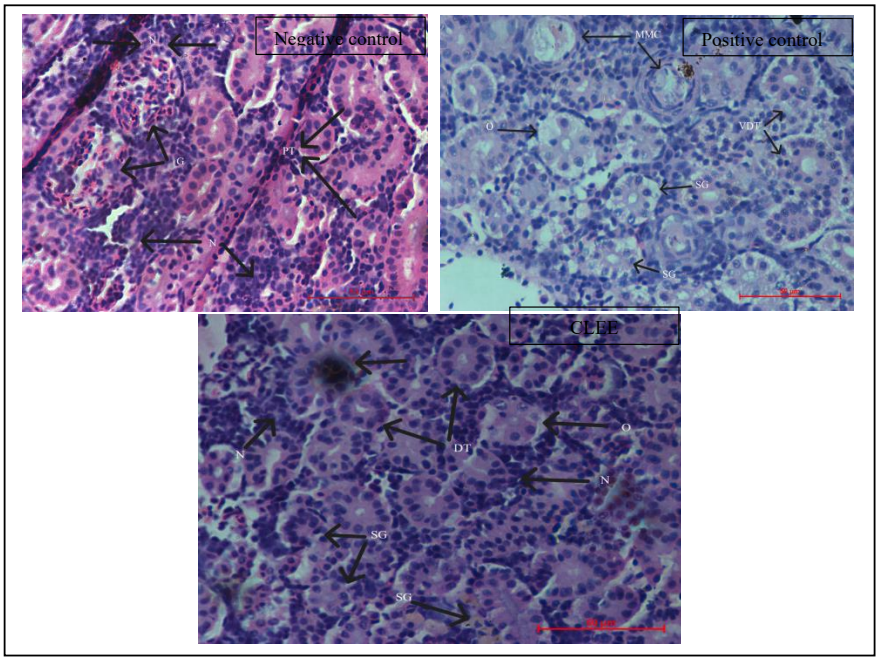


Fig. 8 Representative histopathology of the kidney of *L. rohita* fed with CLEE and challenged with *A. hydrophila*. Exp. Control: no bacterial infection; Bac. Control: bacterial infection, CLEE : 1 g CLEE/kg feed, and bacterial infection. H & E staining. Scale bar=50 µm

narine had low errors in mass (0.58 and 0.13 ppm, respectively), which allows for an accurate annotation. These compounds are described as antioxidants, which can also play a role in the therapeutic utility of curry leaves (Rajendran et al. 2014). Nonetheless, the provisional formation of a cinnamic acid derivative as a silylated structure indicates the possibility of derivatization artifacts in the process of Soxhlet extraction, which may also be caused by high temperatures (Santos et al. 2017). This highlights the need for careful extraction of such compounds and potential optimization of different extraction protocols to minimize artifacts (Abeysinghe et al. 2021a, b).

Non-phenolic acid phenolics

Five non-phenolic acid phenolics (phloroglucinol, vanillin, guaiacol, and 10-gingerol) contribute to the chemical variety of the curry leaf extracts. Phloroglucinol and 10-gingerol, which are detected in ESI+ mode, are consistent with their chemical characteristics, because polyphenols and the derivatives of gingerols tend to protonate easily (Zhang et al. 2021). On the other hand, vanillin and guaiacol were identified in ESI 3.6 mode as expected of their single-phenolic structures. Their mass data (-1.03 to 2.32 ppm) is of good quality in terms of analytical accuracy, and that 10-Gingerol is present in the CLEE is important in its own right since the gingerols are traditionally attributed to the herb *Zingiber officinale*, and their occurrence could be used to define the flavor and bioactivity of curry leaves (Zhang et al. 2021).

Complex phenolics

The complex phenolics represented by copalliferol B and lusitanicoside are likely glycosides or esters due to their higher molecular weight. Their appearance in ESI+ and ESI- modes, respectively, demonstrates their structural complexity, which can contain polar glycosidic. The error in the mass determination of Copalliferol B (2.43 ppm) may be attributed to its complex structure, which is not easily measured using the UHPLC-ORBITRAP-MS/MS system of analysis (Brenton and Godfrey 2010). These molecules may be associated with the pharmacological effects of curry leaves, as glycosylated phenolics are frequently linked to increased bioavailability. The use of ESI and ESI-ionization methods enabled the extensive identification of polar and semi-polar phenolic compounds, which is also in line with other LC-MS metabolomics protocols, such as UHPLC-ORBITRAP-MS/MS. Chlorogenic, gallic, and sinapinic acids were identified as having the highest levels in the TIC, which is consistent with previous studies on the curry leaf (Ningappa et al. 2008; Singh et al. 2023). The mass accuracy (±4.34 ppm) of the UHPLC-ORBITRAP-MS/MS system ensures its reliability in identifying the compound, as errors of less than 5 ppm are typically acceptable in high-resolution MS/MS (Zhou et al. 2022; Verma et al. 2024).

Alkaloids

UHPLC-ORBITRAP-MS/MS analysis of the Soxhlet-extracted CLEE (*M. koenigii*) showed a complex profile of alkaloids with 14 compounds classified as carbazole alkaloids, piperidine and pyridine alkaloids, isoquinoline alkaloids, and other derivative forms of alkaloids. This detailed description is in line with the other prior investigations of curry leaves that note its richness in alkaloids and the existence of other bioactivity potentials, such as anti-microbial property, anti-cancer property, and neuroprotective property (Ganesan et al. 2013). Moderately polar ethanol, combined with the repeated extraction cycles of Soxhlet, probably improved the solubility of polar (e.g., guvacine) and semi-polar (e.g., murrayanine) alkaloids. Low mass errors (1.30 ppm) indicate good detection accuracy, which is justified by the fact that ethanol can be analyzed by UHPLC-Orbitrap-MS/MS. It has been shown in the literature that Soxhlet extraction is more effective than ultrasound-assisted extraction to extract alkaloids in medicinal plants because of its vigorous extraction process. However, thermolabile alkaloids are destroyed under the thermal conditions of Soxhlet extraction and can target other compounds such as (−)-strychnine (Santos et al. 2017).

Carbazole alkaloids

Three alkaloids of carbazole are known, namely evocarpine, murrayanine, and mahanimbin, indicating

their dominance in the curry leaf. The high abundance of Mahanimbin was shown by a high TIC peak, which is in line with reports of its abundance in CLEE (*M. koenigii*) (Chatterjee et al. 2024). The fact that it was identified in ESI + mode and the fact that its mass error is only -0.25 ppm is evidence of consistent identification because carbazole alkaloids are usually easy to ionize in positive mode because of the presence of basic nitrogen-containing structures. The validity of the UHPLC-ORBITRAP-MS/MS technique is further confirmed by Murrayanine and evocarpine with mass errors of -1.30 and -1.00 ppm, respectively. All these compounds add up to the medicinal value of the curry leaves, as these are anticancer and antimicrobial compounds.

Piperidine and pyridine alkaloids

Four alkaloids of the piperidine and pyridine group have been found to add to the chemical diversity of curry leaf extract: 1-piperidine, guvacine, and arecoline. These are compounds that have been identified in ESI 3 mode and hence are consistent with their chemical characteristics, since derivatives of piperidines and pyridines typically protonate easily. High analytical precision is denoted by the low mass errors (-0.24 to 1.06 ppm). The high concentrations of guvacine and arecoline are indicated by their high TIC values, which are in agreement with their pharmacochemical findings in curry leaves (El-Shiekh et al. 2024). Arecoline is a cholinergic pyridine alkaloid that can be an additional contributor to the pharmacological effects of curry leaves (Wadanambi et al. 2023).

Isoquinoline alkaloids

The structural diversity of alkaloids in curry leaves is indicated by the discovery of cryptopleurine and ethaverine as isoquinoline alkaloids. The high mass error of cryptopleurine (0.05 ppm) in ESI+ mode demonstrates the high quality of the UHPLC-ORBITRAP-MS/MS system, whereas the ability of ethaverine to be detected in ESI+ mode (mass error 0.29 ppm) supports its presence of phenolic hydroxyl groups, which are prone to deprotonation (Di Sotto et al. 2023). Cryptopleurine is believed to be cytotoxic which may increase the anticancer properties of the curry leaf extracts (Aniq et al. 2022). Ethaverine is an isoquinoline derivative and may also play a role in the vasodilatory processes that have been described in the pharmacological literature.

Other alkaloids

The other six alkaloid derivatives, namely: conanine, dioncopeltine A, 4'-O-methylnorbelladine, (−)-strychnine, cammaconine and codeinone represent the diversity of the alkaloid profile in curry leaves. The positive TIC value and nearly zero mass error (-0.19 to 1.06 ppm) prove the existence of (−)-Strychnine, an Amaryllidaceae-type alkaloid that may potentially contribute to the effects of neuroprotection. The codeinone finding in ESI - mode is expected due to its ketone functionality, whereas the situation with the presence of codeinone in the curry leaves is atypical and could be considered an artifact or contamination since the use of this compound is normally restricted to Papaver species. Dioncopeltine A and cammaconine might be glycosylated/hydroxylated complex alkaloids and, that is why the structure should be elucidated further. The analysis uncover the presence of all polar and semi-polar alkaloids when using both positive ionization (ESI +) and negative ionization (ESI -) modes, as per the standard protocol of LC-MS-based metabolomics (UHPLC-ORBITRAP-MS / MS) . The mahanimbin, guvacine, and (−)-strychnine peak signals were high in the total ion chromatogram (TIC), indicating that the three compounds are present in large quantities, which conforms to prior quantitative studies on the curry leaves (Ningappa et al. 2008; Singh et al. 2023; Patil et al. 2024). The high mass accuracy of the UHPLC-ORBITRAP-MS/MS system (≤1.30 ppm) indicates that it is a reliable system in annotating compounds because an error of less than 5 ppm is generally defined as acceptable in the context of high-resolution mass spectrometry (Brenton and Godfrey 2010; Zhou et al. 2022).

Flavonoid

The UHPLC-ORBITRAP-MS/MS of the Soxhlet-extracted CLEE (*M. koenigii*) demonstrated a complex

flavonoid profile that showed the presence of 12 compounds that were classified as flavonols, flavanones, and flavonoid glycosides. This holistic description is consistent with the prior research of curry leaves, which emphasize their high levels of flavonoid compounds and their bioactivity, such as anti-oxidant, anti-inflammatory, and anticancer, activity (Ganesan et al. 2013). The literature supports the assertion that Soxhlet solvent extraction provides a more significant flavonoid content than ethanol cold solvent extraction in plant leaves because heat aids in breaking down glycosylated flavonoids in cell walls. Nevertheless, the marginally bigger error in mass of rutin (1.82 ppm) could be explained by the partial decomposition of complex glycosides during the long-term heating, one of the known shortcomings of Soxhlet extraction (Santos et al. 2017).

Flavonols

The presence of seven flavonols including hyperoside, 6 acetylisoquercitrin, quercetin, kaempferol, trifolin, isorhamnetin, and isorhamnetin 3 glucoside, is an indicator of their abundance in the curry leaves. The high abundance of quercetin and kaempferol is reflected in their high TIC values, which are also in line with their reported abundances in CLEE (*M. koenigii*) (Ningappa et al. 2008; Singh et al. 2011, 2023; Aniq et al. 2022). Their detectability in the ESI+ and ESI- modes (-0.30 and 0.02 ppm) and their mass errors (low mass) confirm their reliable identification because flavonols ionize well in both ESI modes, since they are usually hydroxylated (Singh et al. 2011). The correctness of this UHPLC-ORBITRAP-MS/MS system is further proven by hyperoside and trifolin, which mass errors of -0.15 and -0.02 ppm. These are antioxidant compounds that have been associated with a therapeutic effect of curry leaves (Rajendran et al. 2014; Verma et al. 2024).

Flavanones

The presence of the flavanone rac 8-prenylnaringenin also increases the chemical diversity of *M. koenigii*. That was identified in ESI+ mode with a mass error of -0.49 ppm can be ascribed to the chemical properties of flavanones, which tend to protonate easily because of being less polarized than flavonols. It is in the moderately high TIC signal, which indicates a lower concentration, and, therefore, flavanones are lower in quantity in curry leaves (Singh et al. 2011). Rac 8-prenylnaringenin is known for its phytoestrogenic activity, which may enhance the antibacterial profile of curry leaves.

Flavonoid glycosides

The detection of three flavonoid glycosides -rutin, tiliroside, and gossypin -highlights the presence of complex flavonoids in curry leaves. These compounds are observed in ESI mode and identified by their glycosylated structures, which are prone to deprotonation because of greater polarity. The mass errors (0.39 to 1.82 ppm) show a high level of analytical accuracy, but the larger mass error of rutin (1.82 ppm) might be related to its structure as a diglycoside that makes it hard to measure its mass accurately (Brenton and Godfrey 2010). These glycosides have been reported to possess greater bioavailability and antioxidant properties that may add nutraceutical value to curry leaves (Singh et al. 2023). ESI+ and ESI− modes allowed strong identification of polar and semi-polar flavonoids, according to the criteria of LC-MS metabolomics (Sarkar et al. 2025). The highest peak of quercetin, kaempferol, and hyperoside means are high, and the results are consistent with curry leaf (Ningappa et al. 2008; Singh et al. 2023). The annotation is reliable because the UHPLC-ORBITRAP-MS/MS mass error (<1.82 ppm) is acceptable (<5 ppm) (Zhou et al. 2022).

Fatty acids

The UHPLC-ORBITRAP-MS/MS profile of Soxhlet-extracted CLEE (*M. koenigii*) showed a rich fatty acid profile, which identified 21 compounds (saturated fatty acids, unsaturated fatty acids, hydroxy fatty acids, and other fatty acid derivatives). This holistic description is consistent with earlier research on curry leaves that has placed emphasis on its bountiful lipid content and related bioactive effects, such as anti-inflammatory, antimicrobial, and cardiovascular effects (Ganesan et al. 2013; Verma et al. 2022).

Saturated fatty acids

The three fatty acids have been identified as saturated fatty acids (palmitic acid, myristic acid, and juniperic acid), which highlights their occurrence in curry leaves. The highest TIC peak of the palmitic acid also shows its high abundance, which matches its abundance in plant lipids. It is correctly identified by its low mass error (0.19 ppm) and its detection in ESI^- mode, because saturated fatty acids normally ionize well in negative mode owing to their carboxyl groups. Further proof of the UHPLC-ORBITRAP-MS/MS technique accuracy is myristic acid and juniperic acid with mass errors of 0.86 and 0.48 ppm, respectively. These compounds may preserve the integrity of plant membranes and have antimicrobial properties.

Unsaturated fatty acids

The five unsaturated fatty acids present in CLEE, including linolenelaidic acid, eicosapentaenoic acid, (E)-parinaric acid, oleic acid, and ricinoleic acid, increase the chemical diversity of *M. koenigii*. The high concentrations of Linolenelaidic acid and oleic acid are indicated by their high TIC, which confirms their high abundance in *M. koenigii*. Low mass errors (-0.10 to 0.25 ppm) are signs of high precision in the analysis. Eicosapentaenoic acid is an anti-inflammatory polyunsaturated fatty acid that is considered to increase the therapeutic potential of curry leaves (Soni et al. 2021). Ricinoleic acid contains a hydroxyl group, and this may be considered in the context of antimicrobial activity as well.

Hydroxy fatty acids

The presence of functionalized fatty acids in the curry leaves is indicated by the identification of six hydroxy fatty acids, including 13(S)-HOTrE, (±)-13-HODE, corchorifatty acid F, aleuritic acid, 9,10-dihydroxystearic acid, and 2-hydroxy lignoceric acid. These are compounds, mainly observed in the ESI - mode, which are aligned with their polar hydroxyl groups that prefer deprotonation (Aniq et al. 2022). The low mass errors (0.17 to 1.01 ppm) reflect effective identification, the very low mass error of 9,10-dihydroxystearic acid (0.17 ppm) demonstrates the good work of UHPLC-ORBITRAP-MS/MS system. Hydroxy fatty acids are regarded as participants of plant protection against microbes and potentially have the anti-inflammatory and antimicrobial effects (Patil et al. 2024).

Other fatty acid derivatives

Other fatty acid derivatives, including 9,12-octadecadienal, trans-geranic acid (duplicate entry), monolinolenin, isopropylmalic acid, itaconic acid, fatty acid F6 and 11-phenylundecanoic acid, are indicative of the complexity of the lipid profile in curry leaf. The low mass errors (-0.69 to 2.18 ppm) testify to its high identification, but the high mass error (2.18 ppm) of itaconic acid could be explained by its low molecular size, which can be a problem in measuring its mass accurately (Brenton and Godfrey 2010). The antioxidant properties of curry leaves may be contributed by monolinolenin, which is a monoglyceride, and by furan fatty acid F6, which is a cyclic fatty acid. Itaconic and isopropylmalic acids are usually associated with metabolic systems. The TIC values of the palmitic, oleic, and linolenelaidic acids gave very high values, which are comparable to the palmitic, oleic, and linolenelaidic acids of curry leaves (Singh et al. 2011, 2023). The precision of LC-MS/MS (0.218 ppm) can be used to annotate, and errors below 5 ppm in the precision are standard (Brenton and Godfrey 2010; Zhou et al. 2022).

Terpenoids

Analysis of Soxhlet-extracted CLEE (*M. koenigii*) by UHPLC-ORBITRAP-MS/MS showed a variety of terpenoid profiles, with 11 different compounds belonging to the sesquiterpenes, triterpenes, and additional terpenoid derivatives. This overall description is consistent with past research on curry leaves that has emphasized their richness in terpenoids and other bioactivity properties such as antioxidant, anti-inflammatory and antimicrobial activities (Ganesan et al. 2013; Verma et al. 2024). It is supported by the literature that Soxhlet extraction is the best method to extract terpenoids in aromatic plants because heat and solvent

polarity interfere with the cells containing the essential oil (De Castro and Priego-Capote 2010). This is not a requirement of the presence of an intermediate, but the finding of 1,4-cyclohexadiene as a potential biosynthetic intermediate indicates the possibility of thermal artifacts, which Soxhlet will need to consider, given its high working temperature (Santos et al. 2017).

Sesquiterpenes

Four sesquiterpenes, namely, curcumene, (+)-nootkatone, (E,E)-alpha farnesene and phaseic acid, are identified, highlighting their preeminence in the curry leaves. The high abundance of curcumene is evidenced by its peak TIC, which is still in line with its prevalence in *M. koenigii* essential oils (Rajendran et al. 2014). Its low mass error (-0.75 ppm) and its appearance in ESI consummative mode indicate that the UHPLC-ORBITRAP-MS/MS technique is reliable at identifying Sesquiterpenes because they are generally easy to ionize in positive mode, as they are hydrophobic. (+)-Nootkatone and (E, E)-alpha farnesene have mass errors of -0.90 and -1.11 ppm, respectively which further Phaseic acid, ESI^- mode, 0.41 ppm, is a polar derivative of abscisic acid and may be related to plant stress responses. These are some of the compounds that give curry leaves their characteristic aroma and pharmacological qualities (Verma et al. 2024).

Triterpenes

Three triterpenes are detected in the CLEE: cucurbitacin S, (+)-absinthin, and maslinic acid, which contribute to the chemical diversity of curry leaf extracts. Cucurbitacin S and (+)-absinthin, which are found in ESI in addition mode, are consistent with their complex, oxygenated backbones, which are in turn protonated. It is possible that the increased error of mass of (+)-absinthin (4.49 ppm) was due to its dimeric sesquiterpene lactone structure, which can make the mass difficult to measure in LC-MS/MS systems (Brenton and Godfrey 2010). The low mass error (0.40 ppm) of the ESI in ESI-mode confirms the high-quality identification of Maslinic acid, and it corresponds with the hydroxylated triterpenoid structure. It has anticancer and anti-inflammatory properties that have expanded the therapeutic capacity of curry leaves.

Other terpenoid derivatives

The structural complexity of terpenoids in curry leaves is exemplified by the identification of four other terpenoid derivatives, viz. genipin, 1,4-cyclohexadiene, calicoferol E, and dehydroeburicoic acid. The iridoid glycoside precursor genipin was found in the ESI-mode (mass error 0.62 ppm), which aligns with the polarity nature of this molecule. At the ESI + mode, the calicoferol E mass error (0.34 ppm) was small, and it was annotated correctly. The mass deviation of dehydroeburicoic acid was 0.54 ppm at ESI-mode, as expected of its oxygenated structure. It has a mass error (1.11 ppm) that allows it to be identified reliably, though additional research should be conducted to establish its cause. TIC crescendos that correspond with curry leaf quantifications are preceded by the abundance of curcumene, cucurbitacin S, and maslinic acid (Singh et al. 2023). The level of clarity of compounds is anchored on UHPLC-ORBITRAP-MS/MS precision (≤4.49 ppm), which is characteristic of high-resolution MS/MS.

Saponins

The presence of bruceoside A as a saponin further indicates that glycosylated products exist in the curry leaves. Its identification in ESI - mode with a low mass error (-0.42 ppm) confirms the high reliability since saponins usually ionize easily in negative mode because of polar glycosidic moieties Patil et al. (2024). The intermediate TIC pattern indicates a lower concentration of saponin than of other compounds, which is in line with the idea that saponins are secondary metabolites in *M. koenigii*. Bruceoside A is known and reported for its potential anticancer and antimicrobial activities, contributing to the therapeutic potential of curry leaves (Balakrishnan et al. 2020; Verma et al. 2024).

Carotenoids

Curry leaves (CLEE) contain carotenoids as detected by the identification of a beta-carotene derivative, echinenone. The very low mass error (-0.09 ppm) in ESI + mode and high TIC signal suggest high abundance and good identification and are in line with hydrophobic nature of carotenoids, which prefers protonation (Bijttebier et al. 2013). Carotenoids in curry leaves are also linked to antioxidant activity and potential role in eye health, so, unsurprisingly, it contains Echinenone. High concentration indicates that the curry leaves might be an important dietary source of carotenoids.

Vitamins

The nutritional quality of curry leaves has been indicated by the presence of five vitamins, including: choline, niacin, vitamin A, tocopherol, and dehydroascorbic acid. The presence of choline and niacin in ESI + mode with low mass errors (-0.19 and 0.06 ppm, respectively) and high TIC signals indicated that the concentration of choline and niacin was high. Mass errors of -0.85 ppm and -2.25 ppm were observed in ESI+ mode, followed by vitamin A and tocopherol, respectively. One of the Vitamin C derivatives, dehydroascorbic acid, was found in ESI -mode (mass error 0.75 ppm), which indicates oxidative change during extraction or analysis, since it is not as stable as ascorbic acid. These vitamins increase the nutraceutical value of the curry leaves. The range of saponins, carotenoids and vitamins present in curry leaves advocates the application of the leaves in traditional medicine and functional foods. Moreover, investigation of the effects of environmental factors on the content of these compounds in curry leaves may also shed light on their variability under different cultivation conditions.

Phytochemical analysis and antioxidant estimation of the extracts

Total phenolic content (TPC)

The total phenolic content (TPC) of 163.112 mg/g gallic acid equivalents (GAE) in CLEE is a strong indication of a phenolic profile, in line with UHPLC-ORBITRAP-MS/MS-identified phenol-like compounds, including gallic acid, caffeic acid 3-glucoside, and their derivatives, which also display phenolic-like antioxidant properties (Ningappa et al. 2008). The capability to donate hydrogen atoms and cancel out free radicals and the relationship between TPC and antioxidant capacity are highly correlated ($R^2 = 0.85$ -0.92) when utilizing DPPH and FRAP as antioxidants (Ganesan et al. 2013). In fact, Malaysian study found the value of TPC to be 63.8 to 92.3 mg/g dry weight (DW) in samples of different regions, with the highest being in samples of Selangor, which the authors attributed to favorable soil and climatic conditions (Ghasemzadeh et al. 2014). These are lower than 163.112 mg/g GAE in the current study, and hence it appears that Soxhlet extraction in ethanol increases the yield of phenolics relative to the traditional methods. By comparing the results of this study to other works that have been carried on *M. koenigii*, we observe discrepancy with TPC, extraction process, solvents and environmental conditions.

In a study conducted in Bangladesh, the TPC of *M. koenigii* methanolic extracts was 65.02 mg of GAE/g, which is significantly lower than the present results and may be explained by the use of methanol and the shortest extraction time (Dahlia et al. 2017). A second study that optimized extraction conditions determined that ethanolic extract (50%) had the highest TPC value (63.055 mg GAE/g DW) followed by water extract (32.495 mg GAE/g DW) of *M. koenigii*, which is closely similar to the current study and demonstrates the effectiveness of ethanol-based extraction (Sablania et al. 2019). The TPC determined by Folin-Ciocalteu method could be due to the presence of compounds such as saponin bruceoside A (RT 7.509 min) and maslinic acid (RT 13.616 min), which were identified by UHPLC-ORBITRAP-MS/MS, and have redox reducing capacities (Sparg et al. 2004). Also, non-phenolic antioxidants can be used to supplement the overall antioxidant activity of CLEE and stabilize phenolics, such as tocopherol (RT 13.401 min) and dehydroascorbic acid (RT 1.240 min).

The difference in the quoted TPC values reflects the effects of extraction processes and the geographical differences. In the current study and in the literature reviewed, ethanol-water solutions are always more soluble to polar phenolics resulting in a higher TPC (Sablania et al. 2019). The TPC of CLEE (*M. koenigii*)

is significantly higher than that of *Micromelum minutum* (26.44 mg GAE/g) indicating the high phenolic content and antioxidant activity of *M. koenigii* compared to that of *M. minutum* (Abeysinghe et al. 2021b, a). The results of this study confirm that CLEE (*M. koenigii*) has great potential in nutraceutical and food applications due to its high TPC.

Total flavonoid content (TFC)

A total flavonoid content (TFC) of 56.17 mg/g quercetin equivalents (QE) of CLEE indicates a pronounced flavonoid profile, in line with the UHPLC-ORBITRAP-MS/MS-identified compounds including quercetin, myricetin, and their glycosylated derivatives, that have been noted to have strong antioxidant activities Singh et al. (2023). Flavonoids also help to neutralize free radicals by donating electrons, and researchers have found that TFC has a strong correlation (R^2 = 0.88-0.94) with DPPH scavenging activity (Ganesan et al., 2013). Comparative cross-study analyses of *M. koenigii* indicate that there were significant variations in total flavonoid content (TFC), which were influenced by the methods of extraction, the nature of solvents, and the location. An example is a Sri Lankan *M. koenigii* study that reported a TFC of 9.75 ± 0.05mg quercetin equivalents (QE)/g in methanolic leaf extracts, which is substantially lower than the results reported in the present study, presumably due to differences in solvent polarity and extraction efficacy (Abeysinghe et al. 2021b). Contrastingly, another study, conducted in Malaysia, found that the TFC values were between 2.801 and 3.771 mg/g DW in various regions with Kelantan having the highest TFC (3.771 mg/g DW), which can be attributed to environmental factors such as soil fertility and climate (Ghasemzadeh et al. 2014). These values are significantly less than the 56.17 mg/g QE of the current study and there are indications that Soxhlet extraction using ethanol could be more effective than other methods in extracting flavonoid.

In a study of *M. koenigii* in Bangladesh, the TFC of 16.65 mg catechin equivalents (Cat E)/g in the methanol extracts was significantly lower than in this study, presumably because catechin was used as a reference standard rather than quercetin and differences in solvent systems (Dahlia et al. 2017). Recent optimization analysis reported that 60 % Acetonic extracts of *M. koenigii* obtained high TFC, but not quantified in QE, and 50 % ethanol extracts had better DPPH scavenging activity, which aligns with the results of the present study (Sablania et al. 2019). The high TFC noted is probably due to the presence of glycosylated flavonoids, including quercetin-3-O-glucoside (RT 6.832 min), which were detected under UHPLC-ORBITRAP-MS/MS, and which have been shown to have strong reducing abilities. TFC variability is related to the methods of extraction, as well as the aspects of the area, and in this case, ethanol-water mixtures 50 % increased flavonoid production (Sablania et al. 2019). *M. koenigii* has a significantly better TFC than *M. minutum* (9.16 mg QE/g), which indicates the excellent flavonoid composition and antioxidant ability of CLEE (*M. koenigii*) compared to *M. minutum* (Abeysinghe et al. 2021b). These results indicate that *M. koenigii* (CLEE) has a high potential as a functional ingredient in nutraceuticals and food products due to its strong TFC.

Total tannin content

Although the tannin content of 3.974 mg/g tannic acid equivalent (TAE) in the CLEE is insignificant in terms of its total phenolic content, it is corresponding to the compounds identified in UHPLC-ORBITRAP-MS/MS that bear polyphenolic characteristics. The presence of tannins as antioxidants also destroys metal ions and free radicals, which contributes to the antioxidant property of CLEE (Farha et al. 2020). . The glycosylated structure of the saponin bruceoside A (RT 7.509 min) could be partly the cause of the tannin content measured, as not all saponins lack tannin-like reducing effects in colorimetric procedures (Parithy et al. 2021). According to recent trials involving curry leaves, ethanol solvent is more favorable compared to water in extracting the tannin to 42.36 mg CE/100 g, as its solvent is more penetrating and less cohesive (Patil et al. 2024)”URL”:”https://www.cell.com/heliyon/fulltext/S2405-8440(23. It is probable that tannins also play a role in the DPPH and DMSO scavenging activities of CLEE, but since the concentration of these compounds is low, they are probably secondary to flavonoids and phenolic acids (Ningappa et al. 2008; Singh et al. 2011). The environmental conditions (sunlight and soil type) across regions vary with tannin biosynthesis in curry leaves (Abeysinghe et al. 2021a). The presence of saponins and tannins, which

enhance antioxidant activity along with terpenoids and vitamins, is shown by data obtained in UHPLC-Orbitrap-MS/MS (Parithy et al. 2021).

DPPH scavenging activity

The DPPH scavenging activity of 73.391% in CLEE is closely associated with its heterogeneous phytochemical composition as determined by UHPLC-ORBITRAP-MS/MS, in particular its high phenolic content (163.112 mg/g GAE) (Ningappa et al. 2008). Phenolic compounds are the major DPPH radical quenching sources, including those structurally similar to gallic acid and quercetin, because the correlations between them are strong ($R^2 = 0.85$) (Balakrishnan et al. 2020). More recent research on curry leaves suggests that curry leaf ethanolic extracts have a DPPH scavenging potential of 70-80% and contain flavonoid (myricetin) and phenolic acid (Parithy et al. 2021). This activity can be increased by the saponin bruceoside A (RT 7.509 min), glycosylated compounds are likely to donate hydrogen atoms to stabilize the radicals (Sparg et al. 2004). The presence of terpenoids such as curcumene (RT 9.469 min) and maslinic acid (RT 13.616 min) also helps scavenge the DPPH due to their antioxidant properties, which have been reported in our present study (Rajendran et al. 2014). Lipid-soluble antioxidants include the beta-carotene pigment echinenone (RT 14.605 min) and tocopherol (RT 13.401 min), and work with phenolics to increase the scavenging of DPPH (Abeysinghe et al. 2021b).

An optimum extraction condition study indicated that the ethanolic and acetonic extract IC50 of DPPH scavenging activity of the *M. koenigii* leaves was 1.556 µg/mL and 3.765 µg/mL, respectively, which was slightly lower than the present study, probably because acetone has a wider non-polar antioxidant extraction profile (Sablania et al. 2019). Conversely, a Sri Lankan study had recorded that in methanolic leaf extracts, DPPH scavenging activity was 55.6 percent, much lower than the current results, which may be owing to inefficient extraction methods or a geographical difference in the phenolic content (Abeysinghe et al. 2021b).

DMSO scavenging activity

The DMSO scavenging activity of 69.176% in the CLEE is slightly lower than the DPPH activity (73.391%), which can be attributed to the presence of diverse phytochemicals detected by UHPLC-ORBITRAP-MS/MS, especially those exhibiting radical-quenching activity (Parithy et al. 2021). This indicates a high level of phenolic (163.112 mg/g GAE) and flavonoid compounds (gallic acid and quercetin) are major players in the DMSO radical scavenging, as the phenolic and flavonoid compounds donate electrons, which neutralize the hydroxyl or superoxide radical (Ningappa et al. 2008).In recent reports on curry leaves, high antioxidant activity of ethanolic extracts of curry leaves is reported, with phenolic-rich fractions (Microwave-assisted curry leaf extract up to 120.60 mg GAE/g) reporting up to 78% scavenging activity in the same radical assays, which supports the DMSO activity in CLEE (Parithy et al. 2021). This activity could be increased by the saponin bruceoside A (RT 7.509 min) since glycosylated compounds can stabilize complex radicals (Sparg et al. 2004). DMSO scavenging effects are on terpenoids curcumene (RT 9.469 min) and maslinic acid (RT 13.616 min), and are antioxidants, which are found in CLEE (*M. koenigii*) of our present study (Rajendran et al. 2014).Lipid-soluble antioxidants echinenone (RT 14.605 min) and tocopherol (RT 13.401 min) are also synergists with phenolics to improve scavenging in DMSO systems (Abeysinghe et al. 2021b). Optimization of the bioactive recovery of CLEE using ethanolic Soxhlet extraction was in agreement with its high antioxidant activities in ethanolic extracts.

Disc diffusion assay

The bioactive compounds found in the Soxhlet-extracted CLEE through UHPLC-ORBITRAP-MS/MS (Tables 1-6) could be attributed to the antibacterial activity. The phenolic acids, including chlorogenic acid, gallic acid, and sinapinic acid (Table 1), have been reported to interfere with the bacterial cell membrane and inhibit the activity of enzymes. The involvement of flavonoids, such as quercetin, kaempferol, and rutin (Table 3) is likely made possible by their capacity to form complexes with bacterial cell wall proteins, which disrupt membrane activity. Alkaloids, such as mahanimbine and murrayanine (Table 2), can also be

used to enhance the effects of antibacterials by disrupting DNA replication or protein synthesis processes (Samanta et al. 2018). The combination of these anti-oxidant and antibacterial effects is probably one of the reasons of the effectiveness of CLEE (*M. koenigii*) on *A. hydrophila*.

Comparative analyses of the disc diffusion method show different levels of plant extracts which are effective against *A. hydrophila*. The 300 mg/mL ethanolic *M. koenigii* extract generated 4.7 mm of inhibition zone with *Edwardsiaceae tarda* (Syaifurrisal et al. 2024). By contrast, *M. koenigii* leaf essential oil had 16 mm and 15 mm against *Staphylococcus aureus* and *Klebsiella pneumoniae* (Rajendran et al. 2014), and methanolic *M. koenigii* leaf extract had 15.63 mm against *Staphylococcus aureus* ATCC 700699 (Abuga et al. 2020). The *M. koenigii* (CLEE) extracted by Soxhlet, as in the study mentioned, was probably concentrated with phenolic and flavonoid compounds, which is confirmed by the UH-PLC-ORBITRAP-MS/MS analysis. The moderate antibacterial effects of *M. koenigii* (CLEE) and its high level of antioxidant activity make it possible to introduce the extract as a natural antimicrobial to control *A. hydrophila* in aquaculture. The higher concentrations (200-250 mg/mL) exhibit an inhibitory effect; however, alternative methods, such as combinations with other plant extracts or antibiotics, could prove to be effective.

Ameliorative effects of CLEE against *A. hydrophila*

Recent studies on the pharmacological properties of *M. koenigii* (CLEE) support the protective effects of this extract against the damage caused by *A. hydrophila* (Debbarma et al. 2025). As an example, Mushtaq et al. (2019) showed that ethanolic curry leaf extract had antibacterial activity against *S. aureus* resistant to multiple antibiotics, attributable to its phytochemicals, including mahanine and gallic acid, which also could potentially suppress *A. hydrophila* growth or virulence. This antimicrobial effect likely contributed to the lower inflammatory reaction in the CLEE-treated group, as evidenced by the absence of melano-macrophage centers (MCs) and lower levels of erythrocyte infiltration (EI). Further, (Desai et al. 2012) found that the ethanolic extract of curry leaf lowers lipid peroxidation and increases antioxidant enzyme (e.g., superoxide dismutase, catalase) activity in CCl4-induced hepatotoxic rats, which supports the reported decrease in hepatic vacuolation and karyorrhexis in the current study. The mechanism by which CLEE partially inhibits glomerular atrophy and tubular necrosis in the kidney is consistent with other reports on the use of plant extracts with nephroprotective effects. Essentially, (Ekasari et al. 2022) We have also observed that Cassia spectabilis ethanolic leaf extract prevented degenerative changes in the renal histology of *Plasmodium berghei*-infected mice at a dose of 200 mg/kg. The fact that the process of occlusion (O) has persisted in the kidney group treated with CLEE suggests that the treatment may need to be prolonged to completely correct the renal damage caused by *A. hydrophila*.

Abkhoo and Jahani (2017) also observed that ethanolic extracts of medicinal plants, *Mentha spicata* and *Glycyrrhiza glabra*, containing phenolic and flavonoid compounds had notable antibacterial activity against pathogens such as *E. coli* and *S.aureus*, which could also explain the activity of CLEE against *A. hydrophila* through various possible mechanisms, such as disruption of bacterial cell membranes. CLEE-treated groups might be characterized by the absence of melano-macrophage centres, possibly due to reduced aggregation of immune cells, or possibly owing to the anti-inflammatory actions of CLEE that counteract the inflammatory response caused by *A. hydrophila*. The groups treated with CLEE showed a considerable reduction of pathological alterations. CLEE treatment decreased vacuolation (V), erythrocyte infiltration (EI), multinucleated cells (MNC) and karyorrhexis (KR) in the liver with no vacuolated melano-macrophage centers (VMMC). At the kidney CLEE reduced glomerular shrinkage (SG) and proximal tubule (DT) degeneration, compact nephrons (N), and no melanomacrophage centers (MMCs), but occlusion (O) was present. These results indicate that CLEE has hepatoprotective and nephroprotective effects, which is probably induced by its bioactive constituents such as phenolic compounds, flavonoids, and alkaloids that are previously known to have antioxidant, anti-inflammatory, and antibacterial effects. Finally, the histopathological alterations suggest that CLEE has a strong capacity to prevent *A. hydrophila*-induced liver and kidney injury, which is probably due to its antibacterial, antioxidant, and anti-inflammatory activity. These results highlight curry leaf ethanolic extract as a potential therapeutic agent for treating bacterial infections and the resultant organ injuries, especially in aquaculture.

Conclusion

This present study critically analyzed the phytochemical profile and bioactivity of CLEE (*M. koenigii*) Soxhlet-extracted using UHPLC-Orbitrap-MS/MS, and 79 compounds were identified as phenolics, alkaloids, flavonoids, fatty acids, terpenoids, saponins, carotenoids, and vitamins with mass errors of ≤4.49 ppm. Chlorogenic acid, mahanimbin, quercetin, palmitic acid, and curcumin were some of the important compounds that indicated the chemical diversity of *M. koenigii* (CLEE). The high total phenolic content (163.112 ± 1.182 mg/g GAE), flavonoid content (56.167 ± 0.541 mg/g QE), moderate tannin content (3.974 ± 0.169 mg/g TAE) and robust free radical scavenging (73.391 ± 0.84% DPPH, 69.176 ± 1.40% DMSO) confirmed the presence of strong antioxid Antibacterial activity against *A. hydrophila* was dose dependent with an inhibition zone of 5.5 ± 0.29 mm (200 mg/mL) and 7.5 ± 0.29 mm (250 mg/mL) compared to gentamicin (10 mg/disc) which had an inhibition zone of 11 ± 0.58 mm, probably because of the effects of phenolics, flavonoids and alkaloids on bacterial membranes and enzymes. Histopathological examination of the liver revealed normal circular hepatocytes in the negative control, and vacuolation, erythrocyte infiltration, multinucleated cell, karyorrhexis, and vacuolated melano-macrophage centers in the positive control, which were decreased in CLEE-treated groups, eliminating vacuolated melano-macrophage centers. Glomeruli, nephrons, and proximal tubules were intact in the negative control, whereas severe glomerular shrinkage, melanomacrophage centers, tubular degeneration, and occlusion were observed in the positive control, and CLEE treatment reduced glomerular and tubular damage and compact nephrons, without melanomacrophage centers. The results of the present study demonstrate the antioxidant properties, moderate antibacterial activity, and mitigative effects on histopathology of CLEE from *M. koenigii*, indicating its potential use as a prophylactic and therapeutic supplement in aquaculture diets. Notably, this study provides one of the first UHPLC-Orbitrap-MS/MS profiles of Soxhlet-extracted *M. koenigii* and demonstrates its therapeutic potential in Labeo rohita. However, to develop *M. koenigii* as an effective antimicrobial and antioxidant agent, future investigations could explore the in vivo roles of specific compounds, such as mahanimbin and quercetin, in mediating these antimicrobial and antioxidant activities.

Ethical statement The experiment was conducted in accordance with the standard guidelines laid down by the Government of India's Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) for experimentation on fish (2021). https://ccsea.gov.in/WriteReadData/userfiles/file/Guidelines%20of%20CPCSEA%20for%20Experimentation%20on%20Fishes-2021.pdf

Author credit statement SD: Investigation, laboratory analysis, investigation, software, original draft preparation; BC: Conceived and designed the experiments, methodology, software, data curation, review & editing; PS, SKP: technical supervision, data curation & editing; ND, PS: technical supervision, data curation; MM, MS, MKP: laboratory support, data curation.

Declaration of competing interest The authors declare no competing interests.

Data Availability The data supporting this study's findings can be obtained from the corresponding author upon reasonable request.

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