PLANT SCIENCE TODAY
Vol x(x): xx–xx
https://doi.org/10.14719/pst.13082

eISSN 2348-1900

RESEARCH ARTICLE

Phytochemical characterisation and biosafety profiling of *Elsholtzia communis* (Collett & Hemsl.) Diels essential oil using zebrafish embryos

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Received: 05 December 2025; Accepted: 08 January 2026; Available online: Version 1.0: 22 March 2026

Cite this article: Birjit SW, Sangeeta Y, Keisham NS, Lokendrajit N, Thangal Y, Maibam DD. Phytochemical characterisation and biosafety profiling of *Elsholtzia communis* (Collett & Hemsl.) Diels essential oil using zebrafish embryos. Plant Science Today (Early Access). https://doi.org/10.14719/pst.13082

Abstract

Elsholtzia communis (Collett & Hemsl.) Diels is traditionally used in herbal medicine, yet the phytochemical composition and developmental safety of its flower-derived essential oil remain insufficiently explored. This study aimed to characterise the essential oil of *E. communis* flowers (EOEC), evaluate its antioxidant and antibacterial activities and assess its developmental toxicity using zebrafish (*Danio rerio*) embryos. The EOEC was extracted by hydro-distillation and characterised using gas chromatography-mass spectrometry (GC-MS) and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. Antioxidant potential was evaluated through DPPH and ABTS radical scavenging assays, while antibacterial activity against selected Gram-positive and Gram-negative bacteria was determined using agar well diffusion and minimum inhibitory concentration (MIC) assays. Developmental toxicity was assessed in zebrafish embryos by monitoring mortality and morphological abnormalities to calculate LC_{50} values. The GC-MS analysis revealed citral-A (geranial, 45.5 %) and citral-B (neral, 37.8 %) as the major constituents of EOEC, while FTIR spectra confirmed the presence of characteristic OH, C=O and CH functional groups. EOEC exhibited moderate antioxidant activity with EC_{50} values of 45.12 µg/mL (DPPH) and 43.15 µg/mL (ABTS) and demonstrated significant antibacterial effects against both Gram-positive and Gram-negative strains. Zebrafish embryo assays showed clear dose-dependent lethality and developmental abnormalities, with LC_{50} values of 123 µg/mL for EOEC and 145 µg/mL for citral. Notably, adverse developmental effects occurred at concentrations close to those producing biological activity. In conclusion, EOEC possesses promising antioxidant and antibacterial properties but exhibits concentration-dependent embryotoxicity, emphasising the need for careful dose optimisation and biosafety evaluation prior to therapeutic or nutraceutical applications.

Keywords: antioxidant activity; *Elsholtzia communis*; essential oil; GC-MS; zebrafish embryo toxicity

Abbreviations: EOEC: Essential oil of *Elsholtzia communis*; GC-MS: Gas chromatography-mass spectrometry; FTIR: Fourier transform infrared spectroscopy; ATR-FTIR: Attenuated total reflectance-fourier transform infrared spectroscopy; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); EC_{50}: Half effective concentration; LC_{50}: Median lethal concentration; MHB: Mueller-Hinton broth; MHA: Mueller-Hinton agar; MIC: Minimum inhibitory concentration; INT: Iodonitrotetrazolium Chloride; ZFET: Zebrafish embryo toxicity test; OECD: Organisation for Economic Co-operation and Development; BSI: Botanical Survey of India; CFU: Colony forming units; UV-Vis: Ultraviolet-visible spectrophotometer; DMSO: Dimethyl sulfoxide; hpf: Hours post-fertilization; ANOVA: Analysis of variance; RSA: Radical scavenging activity; VOCs: Volatile organic compounds

Introduction

Elsholtzia communis (Collett & Hemsl.) Diels (Lamiaceae), locally known as Lomba in Manipur, is a widely consumed aromatic herb and condiment in several states of Northeast India (1, 2). Indigenous practitioners traditionally use it to manage oral ulcers, pharyngitis and inflammatory conditions. The plant's considerable ethnomedicinal importance stems largely from the essential oils derived from its aerial parts.

Past research has primarily focused on essential oil from *E. communis* leaves, identifying geranial (24.1 %) and neral (28.85 %) as key bioactive constituents. However, floral essential oils often exhibit distinct chemical compositions and biological activities compared to those from vegetative parts, a difference attributed to variations in volatile metabolite biosynthesis. Despite its traditional use, a systematic characterisation of the essential oil specifically from the flowers (inflorescences) of this species has not yet been reported. Citral, an acyclic monoterpene aldehyde, comprises 2 geometric isomers: geranial (α-citral) and neral (β-citral). It imparts the characteristic lemon-like aroma found in many aromatic plants and is a major component of numerous essential oils. Its pleasant fragrance and flavor make citral widely used as a flavoring agent in

food products like confectionery, beverages and lemon tea, as well as in cosmetic and household formulations. Regulatory bodies, such as the U S Food and Drug Administration, have classified citral as Generally Recognized as Safe (GRAS) for food use, supporting its extensive industrial application (3).

Zebrafish embryos offer an efficient *in vivo* platform for developmental toxicity testing due to their transparent chorion, rapid organogenesis and genetic similarity to higher vertebrates (4). This integrated approach, combining phytochemical analysis, bioactivity evaluation and biosafety profiling, thus offers the first comprehensive insight into the therapeutic potential and safety of EOEC. Despite citral's widespread use, its toxicological safety remains a concern, particularly at high concentrations or during sensitive developmental stages. Long-term studies in rodent models have shown dose-dependent adverse effects from dietary exposure, emphasising the need for established safe concentration limits (5). Zebrafish (*Danio rerio*) embryos have emerged as a reliable vertebrate model for assessing developmental toxicity and biosafety of food-derived and natural compounds. Their rapid embryonic development, optical transparency and high genetic similarity to humans make them particularly suitable for such evaluations (6).

Considering citral's widespread application in food and beverage products, particularly in lemon-flavored formulations, together with its strong biological activity, it is essential to conduct integrated antibacterial evaluations and *in vivo* toxicological assessments. Therefore, evaluating citral alongside. Although *E. communis* has a long history of traditional use, particularly of its aerial parts, the chemical composition and biological effects of the flower-derived essential oil remain largely unexplored. Notably, no prior studies have systematically linked tissue-specific phytochemical variation with both *in vitro* bioactivity and *in vivo* developmental safety assessment. Therefore, the present study provides the first comprehensive chemical characterisation of *EOEC* and integrates antioxidant, antibacterial and zebrafish embryo toxicity assays to evaluate both efficacy and biosafety. By combining traditional ethnobotanical insights especially cautions regarding use during pregnancy with modern toxicological evidence, this work offers a novel and translational perspective on the therapeutic potential and safety of *EOEC*. The EOEC provides meaningful insight into balancing antimicrobial efficacy with biosafety, especially for food, nutraceutical and health-related applications.

This study aims to analyse the flower-derived EOEC collected from the Indo-Myanmar biodiversity hotspot region (Manipur, Northeast India). Our research focuses on 3 key areas: (i) characterising its phytochemical composition using gas chromatography-mass spectrometry (GC-MS) and Fourier transform infrared (FTIR) spectroscopy, (ii) evaluating its antioxidant and antibacterial activities and (iii) assessing its developmental toxicity using zebrafish (*Danio rerio*) embryos as a vertebrate model system.

Materials and Methods

Collection of plant material

Flowers of *E. communis* were collected during the flowering season (November–December, 2023) from the Imphal West district of Manipur, India (Latitude 24.75515°, Longitude 93.92826°, Altitude 7.57 m). A botanist at the Botanical Survey of India (BSI) confirmed taxonomic identification, assigning voucher specimen number BSI/ERC/Tech/2018/68. The herbarium specimen is deposited in the Department of Life Sciences (Botany), Manipur University, under accession number 001537.

Chemicals

For DPPH and ABTS antioxidant assays, we used methanol (EMPLURA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich), ascorbic acid (HiMedia), ethanol (HiMedia) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich). Mueller-Hinton Broth (MHB) (HiMedia) and Iodonitrotetrazolium chloride (HiMedia) served for antibacterial activity testing and minimum inhibitory concentration (MIC) determination. 3,4-dichloroaniline (Sigma-Aldrich) was the positive control, as recommended for zebrafish embryo toxicity assays.

Essential oil isolation

Inflorescences of *E. communis* were thoroughly washed with distilled water and air-dried at room temperature (25 ± 2 °C) under shade for 15 hr. The dried plant material then underwent hydro-distillation using a Clevenger-type apparatus for 3 hr. The essential oil yield averaged 0.5 % (v/w, based on fresh weight). The isolated oil was collected in centrifuge tubes and stored at 4 °C until further analysis.

Gas chromatography and gas chromatography-mass spectrometry analysis

GC-MS analysis of the essential oil was performed using a Shimadzu GCMS-QP2010 Plus system equipped with an Rtx-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.21 mL min^{-1}. The injector temperature was maintained at 250 °C, with a solvent cut time of 2.50 min. The oven temperature program began at 50 °C (held for 2 min), increased to 250 °C at a rate of 5 °C min^{-1} and was then held for 10 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV, with the ion source temperature set at 220 °C and the interface temperature maintained at 270 °C. Chemical constituents were identified by comparing their mass spectra with those in the Wiley 8, NIST 14 (National Institute of Standards and Technology) and FFNSC 2 (flavor and fragrance natural and synthetic compounds) spectral libraries.

Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR)

The FTIR spectra of EOEC were recorded using a Shimadzu IRAffinity-1S spectrophotometer (Japan) equipped with an ATR (single-bounce diamond crystal). Spectra were acquired from 4000 –500 cm^{-1} at 4 cm^{-1} resolution with 40 scans, using air as background and processed with LabSolutions software.

Antioxidant activity

DPPH radical scavenging assay

The DPPH radical scavenging activity of EOEC was determined following, with minor modifications (7). Briefly, 50 µg of EOEC at different concentrations (expressed as µg/mL in methanol) was mixed with 2.5 mL of 0.1 mM methanolic DPPH solution. The mixture was shaken and incubated in the dark at room temperature (25 ± 2 °C) for 30 min. Absorbance was measured at 517 nm using a UV-visible spectrophotometer, with methanol as the blank. Radical scavenging activity (RSA) was calculated as

percentage inhibition and EC_{50} values (µg/mL) were determined from plots of percentage inhibition versus concentration.

ABTS radical cation scavenging assay

The ABTS radical cation scavenging activity was evaluated according to, with slight modifications (8). The $ABTS^{+}$• radical was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature for 16 hr. The resulting solution was diluted with ethanol (1:8, v/v) to achieve an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 200 µg of EOEC at various concentrations (µg/mL) was added to 2 mL of $ABTS^{+}$• solution and incubated at 25 °C for 6 min. Absorbance was recorded at 734 nm and RSA was calculated as percentage inhibition. EC_{50} values were obtained from concentration-response curves.

The percentage radical scavenging activity (% RSA) was calculated using the equation:

% RSA = (Abs of control - Abs of sample) / Abs of control × 100

Where, Abs control = Absorbance of radical solution Abs sample = Absorbance of test sample

The EC_{50} value (half efficient concentration) was determined to assess the sample's antioxidant capacity.

Antibacterial activity

Standardization of bacterial inoculum

Selected bacterial strains were cultured in MHB and incubated for 24 hr at 37 °C. The bacterial suspension's turbidity was adjusted to approximately 1.5×10^8 CFU/mL by measuring absorbance at 625 nm using a UV-Vis spectrophotometer (Thermo Scientific Multiskan SkyHigh Microplate Reader). A turbidity range of 0.08–0.10 absorbance units correspond to the 0.5 McFarland standard, equivalent to roughly 1.5×10^8 CFU/mL, prepared following standard procedure (9).

Agar well diffusion method

EOEC antibacterial activity was tested against 4 bacterial strains: *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Salmonella typhi* (MTCC 531) and *Staphylococcus aureus* (MTCC 121), using the agar well diffusion method (10). Each bacterial strain was cultured overnight at 37 °C. The inoculum density was adjusted to approximately 1.5×10^8 CFU/mL and 100 µL of each suspension was evenly spread onto Mueller-Hinton Agar (MHA) plates. Four wells (6 mm in diameter) were created on each plate with a sterile Cork borer. Pure EOEC and Citral were added to the wells at volumes of 25 µL (0.059 µg/mL of EOEC & 0.036 µg/mL of Citral) and 50 µL (0.118 µg/mL of EOEC & 0.072 µg/mL of Citral). 2 % DMSO served as the negative control, while tetracycline (1 mg/mL) was used as the positive control. Plates were left at room temperature for 30 min to allow sample diffusion into the agar, then incubated at 37 °C for 24 hr. Zones of inhibition were measured in millimeters to assess antibacterial activity.

Minimum inhibitory concentration (MIC)

The MICs of the essential oil were determined using a modified 96-well microtiter plate method, following protocol (11). In each row, the first well was left blank, while wells in columns 2 to 8 contained 100 µL of sterile Mueller-Hinton broth (MHB). Varying volumes of EOEC: 100, 80, 60, 40, 20, 10, 5 and 2.5 µL were added to wells in columns 1 to 8, respectively, establishing a concentration range of 100 to 2.5 µL/mL. Tetracycline (10 µL of 1 mg/mL solution) was added to the wells in column 9 as the positive control. Subsequently, 100 µL of standardised bacterial inocula were added to each row as follows: Row 1: *E. coli* (EC), Row 2: *S. typhi* (ST), Row 3: *B. subtilis* (BS), Row 4: *S. aureus* (ST). Microplates were incubated at 37 ± 1 °C for 24 hr. After incubation, 20 µL of Iodonitrotetrazolium chloride (INT) solution (1 mg/mL) was added to each well, followed by another 60 min incubation at 37 ± 1 °C. The MIC was recorded as the lowest concentration of EOEC inhibiting visible bacterial growth, indicated by the absence of pink coloration.

Zebrafish embryotoxicity assay

Zebrafish maintenance

Wild-type zebrafish (*Danio rerio*) were purchased from a local fish supplier. All experiments involving zebrafish were approved by the Institutional Animal Ethics Committee (IAEC), Manipur University, under project proposal number IAEC/MU/8 before study initiation. Adult fish were kept in a 50 L glass aquarium under a controlled 12:12-hr light-dark photoperiod. They received commercial flake food (Tetra, Melle, Germany) 3 times daily. Water quality parameters, including temperature (26 ± 1 °C), pH (6.5-8.5) and O_2 saturation (≥ 80 %) under a 14 hr light: 10 h dark photoperiod as per OECD guidelines (12), were regularly monitored and maintained. For spawning, 2 pairs of healthy adult zebrafish (2 males:2 females) were placed in a covered breeding chamber containing 3 L of dechlorinated tap water. Following natural mating, fertilised embryos were collected from the breeding tank and transferred to clean Petri dishes. Embryos were examined under a stereomicroscope and only viable, healthy embryos were selected for developmental toxicity studies.

Treatment of essential oil of *E. communis* to zebrafish embryos

Six concentrations each of EOEC (10, 20, 40, 80, 160 and 220 µg/mL) and citral (10, 20, 40, 80, 160 and 320 µg/mL) were administered to zebrafish embryos at 6 hr post-fertilisation (hpf) to assess developmental toxicity, following protocol (12). Each concentration was tested in triplicate, with 10 embryos per replicate. Embryos were placed in 24-well plates and toxicological endpoints such as mortality, hatchability and morphological abnormalities were observed under a stereomicroscope at 24-hr intervals up to 96 hpf. Embryos treated with 1 % dimethyl sulfoxide (DMSO) served as the negative control. The median lethal concentration (LC_{50}) for EOEC and citral was calculated using probit analysis in SPSS software (version 21.0) based on the linear regression-derived dose-response relationship.

Statistical analysis

Microsoft Excel and SPSS (Version 21.0) were used to calculate EC_{50} and LC_{50} values for antioxidant and toxicity assays, respectively. Zebrafish embryo mortality and teratogenic effects were analysed using GraphPad Prism (Version 8.0). FTIR spectra of both EOEC and citral were generated using OriginPro 2026. One-way analysis of variance (ANOVA) was performed, followed by Tukey's post hoc multiple comparison test. Results were considered statistically significant at $p < 0.05$.

Results

Detection and identification of essential oil of *Elsholtzia communis* (EOEC) chemical composition

The GC-MS analysis identified 25 compounds in the EOEC, accounting for 100 % of the total mass. The dominant constituents were geranial (41.50 %), neral (37.80 %), isogeranial (5.49 %), trans-β-caryophyllene (2.75 %), sulcatone (1.65 %) and humulene (1.49 %). These compounds were identified by comparing their retention times and mass spectral data with the Wiley 8, NIST 14 and FFNSC 2 databases (Table 1).

Confirmation of essential oil of *Elsholtzia communis* (EOEC) functional groups and chemical bonds

The FTIR analysis of both EOEC and Citral is presented in Fig. 1A. The EOEC's FTIR spectra showed fingerprint peaks between 4000 and 400 cm^{-1}, indicative of absorption bands from its complex mixture of volatile components (13–17). The peak at 1674.21 cm^{-1} is attributed to C=O stretching of ketonic groups, suggesting a significant presence of ketones (Fig. 1B). A peak at 3614.60 cm^{-1} corresponds to OH stretching of alcohol groups (18). Another peak at 1442.83 cm^{-1} corresponds to C-H bending of alkanes (19). Additional shared peaks at 2915, 1192, 1118 and 841 cm^{-1} in both EOEC and Citral spectra confirm common functional groups (Fig. 1B & C).

Antioxidant activity

Both DPPH and ABTS assays evaluated EOEC's antioxidant potential. The EC_{50} value in the ABTS assay (43.15 µg/mL) was slightly lower than that in the DPPH assay (45.12 µg/mL), indicating a marginally higher free radical scavenging efficiency in the ABTS system (Table 2 and 3). Lower EC_{50} values signify higher antioxidant potency. However, these values were higher than those of the reference antioxidant ascorbic acid (EC_{50} = 3.941 µg/mL for DPPH assay and EC_{50} = 3.513 µg/mL for ABTS), suggesting EOEC possesses moderate antioxidant activity relative to this established standard. This quantitative comparison provides clearer context for assessing EOEC's antioxidant efficacy.

Antibacterial activity

Both EOEC and citral effectively inhibited all four tested bacterial strains. EOEC exhibited the largest zone of inhibition against *S. aureus* (28.3 mm) and the smallest against *E. coli* (19.6 mm) (Table 4 & Fig. 2). The MIC of EOEC was consistently 6230 ± 0.0 µg/mL across all tested strains (Fig. 3). For citral, the largest zone of inhibition was observed against *S. typhi* (26.6 mm) and the smallest against *S. aureus* (18.6 mm) (Table 5 & Fig. 4); its lowest MIC (3556 ± 0.0 µg/mL) was recorded against *S. aureus* (Fig. 5). Comparative and statistical analysis of EOEC and citral-mediated growth inhibition for *E. coli*, *S. typhi*, *B. subtilis* and *S. aureus* using the zone of inhibition method is presented in Fig. 6 & 7.

Table 2. Antioxidant activity of the essential oil of *Elsholtzia communis* (EOEC) measured by DPPH assay (mean ± SD, n = 3)

Concentration (µg/mL)	Radical scavenging activity of ascorbic acid[a] (%)	Radical scavenging activity of EOEC[b] (%)
20	9.256 ± 0.31	8.76 ± 0.27
40	19.173 ± 0.45	12.727 ± 0.33
60	32.066 ± 0.62	15.206 ± 0.38
80	45.454 ± 0.74	23.801 ± 0.52
100	55.371 ± 0.88	26.766 ± 0.63

EC_{50} values: [a]ascorbic acid = 3.941 µg/mL; [b]EOEC = 45.12 µg/mL.

Table 3. Antioxidant activity of the essential oil of *Elsholtzia communis* (EOEC) measured by ABTS Assay (mean ± SD, n = 3)

Concentration (µg/mL)	Radical scavenging activity of ascorbic acid[a] (%)	Radical scavenging activity of EOEC[b] (%)
10	4.08 ± 0.21	2.332 ± 0.11
20	24.49 ± 0.37	15.743 ± 0.31
30	35.87 ± 0.42	28.862 ± 0.47
40	42.57 ± 0.46	40.816 ± 0.59
50	58.89 ± 0.52	47.23 ± 0.66

EC_{50} values: [a]ascorbic acid = 3.513 µg/mL; [b]EOEC = 43.15 µg/mL.

Table 4. Antibacterial activity of *Elsholtzia communis* (EOEC) against four pathogenic bacterial strains

Bacterial pathogens	Clear zone diameter (mm)			MICs (µg/mL)
	EOEC		Tetracycline[b] (0.017 µg/mL) (10 µL/mL)	
	0.059 µg/mL (25 µL/mL)	0.118 µg/mL (50 µL/mL)		
Escherichia coli	16.3 ± 1.1	19.6 ± 0.5	16.6 ± 1.1	119 ± 0.0
Salmonella typhi	27 ± 1.0	25.6 ± 1.1	18.6 ± 0.5	119 ± 0.0
Bacillus subtilis	20.3 ± 0.5	23.6 ± 0.5	18.6 ± 0.5	119 ± 0.0
Staphylococcus aureus	26.6 ± 1.5	28.3 ± 1.1	23.6 ± 1.5	119 ± 0.0

[a]Inhibition zones are expressed as mean ± SD (n = 3). MIC values are reported in µg/mL after conversion using oil density. The negative control (2 % DMSO) showed no inhibition. [b]Tetracycline was used as the positive control.

Table 5. Antibacterial activity of citral against four pathogenic bacterial strains

Bacterial pathogens	Clear zone diameter (mm)			MICs (µg/mL)
	Citral		Tetracycline[b] (0.017 µg/mL) (10 µL/mL)	
	0.036 µg/mL (25 µL/mL)	0.072 µg/mL (50 µL/mL)		
Escherichia coli	15.3 ± 0.5	19.6 ± 0.5	16.6 ± 0.5	102 ± 0.0
Salmonella typhi	20.6 ± 0.5	26.6 ± 0.5	22.6 ± 0.5	119 ± 0.0
Bacillus subtilis	16.6 ± 0.5	19.6 ± 0.5	19.6 ± 0.5	85 ± 0.0
Staphylococcus aureus	15.3 ± 0.5	18.6 ± 0.5	20.6 ± 0.5	68 ± 0.0

[a]Inhibition zones are expressed as mean ± SD (n = 3). MIC values are reported in µg/mL after conversion using oil density. The negative control (2 % DMSO) showed no inhibition. [b]Tetracycline was used as the positive control.

Table 1. Chemical composition of the essential oil identified by gas chromatography - mass spectroscopy (GC-MS) analysis

Sl. No.	Compound (standard nomenclature)	RT[a] (min)	RI[b] (Exp.)	RI[c] (Lit.)	Peak area (%)	Identification[d]
1	Geranial (Citral A) (2)	21.700	1270	~1244	41.56	MS
2	Neral (Citral B) (2)	20.127	1247	~1215	37.80	MS
3	Isocitral (13)	16.846	1166	~1175	5.49	MS
4	trans-β-Caryophyllene (14)	26.991	1417	~1418	2.75	MS
5	6-Methyl-5-hepten-2-one (Sulcatone) (15)	8.413	965	~986	1.65	MS
6	α-Humulene (16)	28.410	1457	~1454	1.49	MS

Only compounds with relative area percentages > 1 % are listed. [a]RT, retention time (min); [b]RI (Exp.), experimental retention index; [c]RI (Lit.), literature retention index on non-polar capillary columns; [d]MS, tentative identification based on mass spectral library comparison (NIST/Wiley).

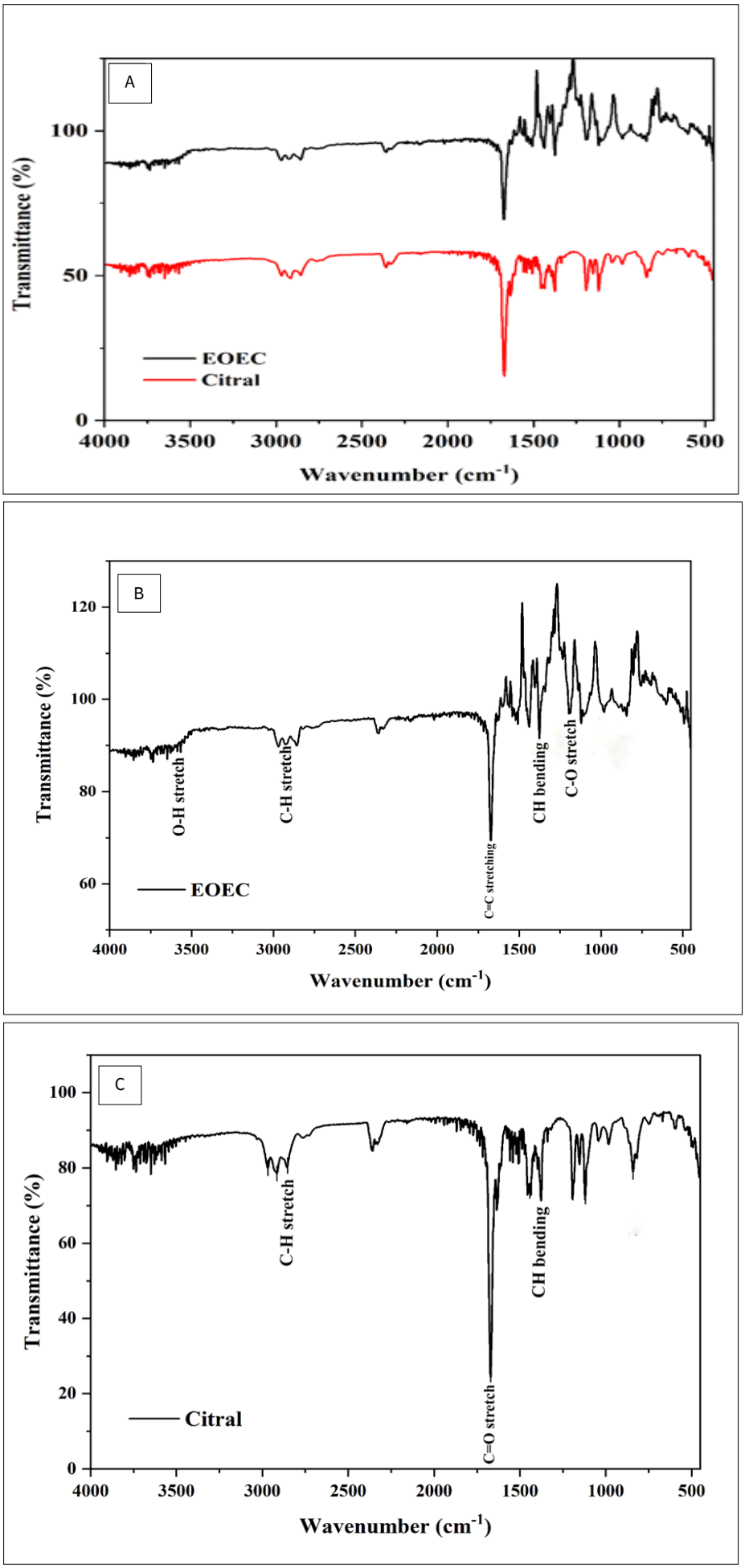


Fig. 1. FTIR spectra showing peaks of both the essential oil of *Elsholtzia communis* (EOEC) and Citral [A]. FTIR spectrum of only EOEC [B] and Citral [C].

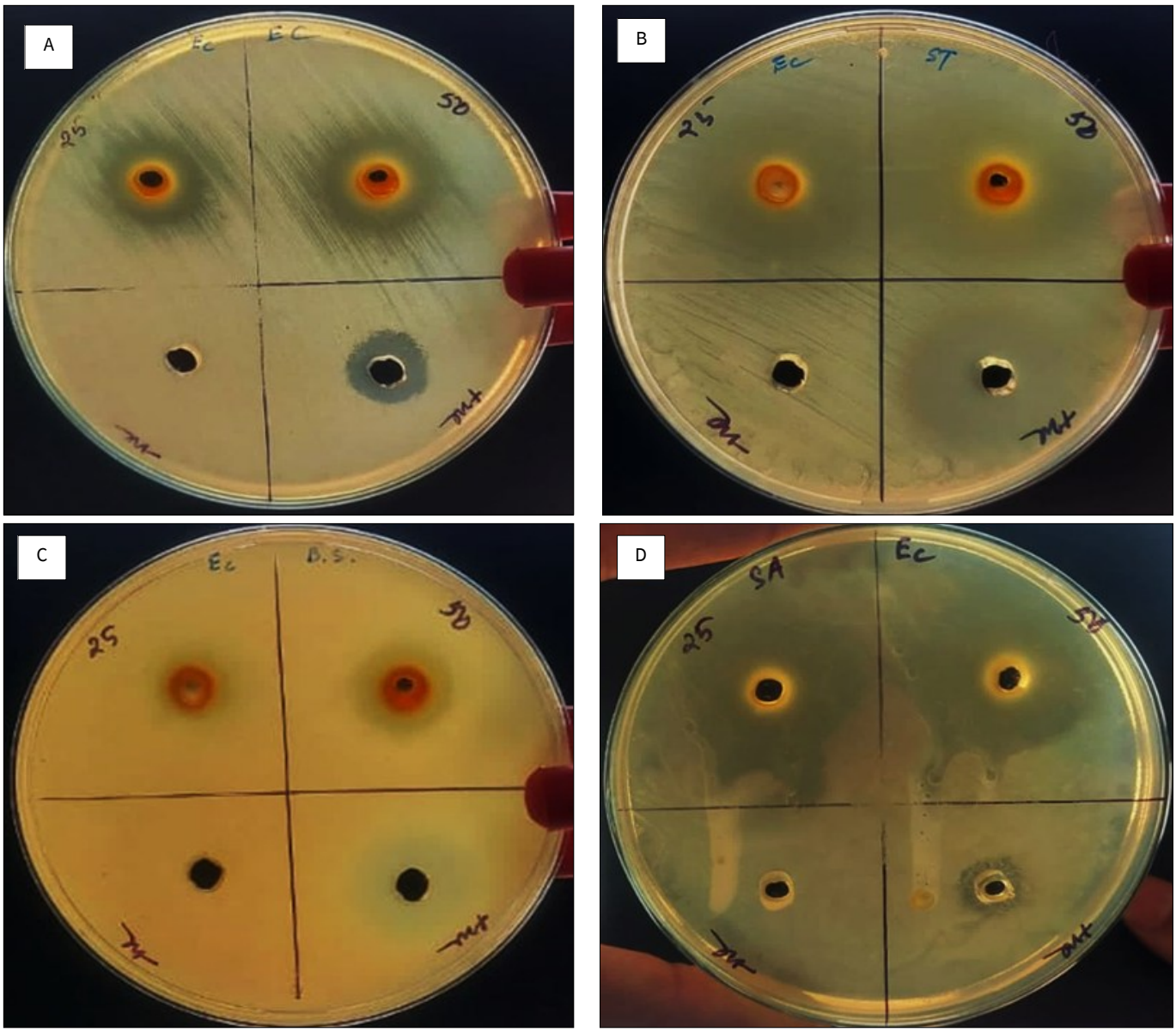


Fig. 2. Antibacterial activity of essential oil of *Elsholtzia communis* (EOEC) by agar well diffusion method against pathogenic bacteria (A) *Escherichia coli* (EC), (B) *Salmonella typhi* (ST), (C) *Bacillus subtilis* (BS) and (D) *Staphylococcus aureus* (SA).

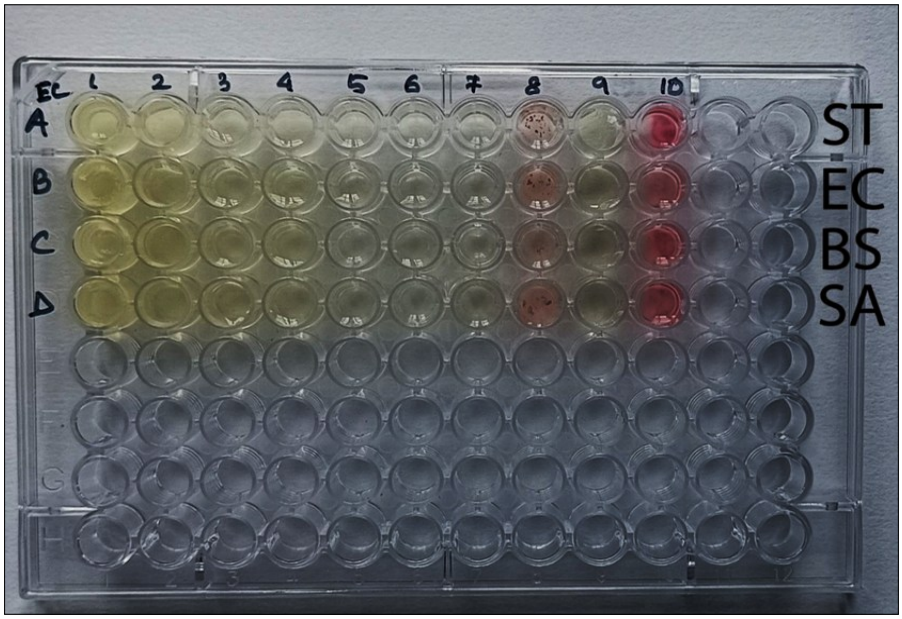


Fig. 3. Calculation of minimum inhibitory concentration (MICs) of essential oil of *Elsholtzia communis* (EOEC) by using 96-well microtiter plate method against pathogenic bacteria, where 1st row is inoculated with *Salmonella typhi* (ST), 2nd row with *Escherichia coli* (EC), 3rd row *Bacillus subtilis* (BS) and 4th row with *Staphylococcus aureus* (SA). Well 1: Blank; Wells 2–8: EOOB (100–2.5 µL + sterilised Mueller-Hinton (MH broth); Well 9: 10 µg/mL tetracycline (positive control). Rows 1–4: *E. coli*, *S. typhi*, *B. subtilis*, *S. aureus*.

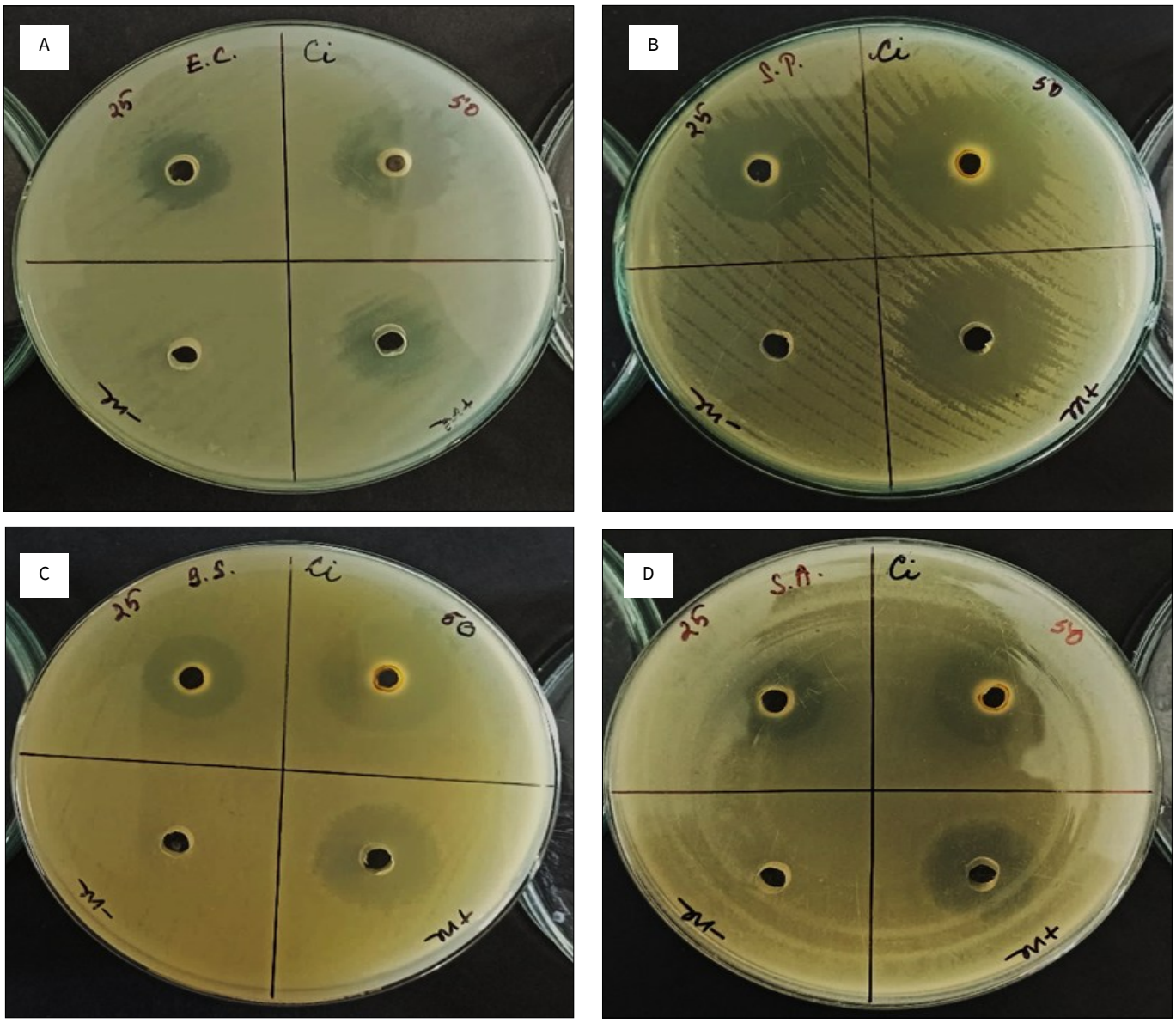


Fig. 4. Antibacterial activity of citral by agar well diffusion method against pathogenic bacteria (A) *Escherichia coli* (EC), (B) *Salmonella* typhi (ST), (C) *Bacillus subtilis* (BS) and (D) *Staphylococcus aureus* (SA).

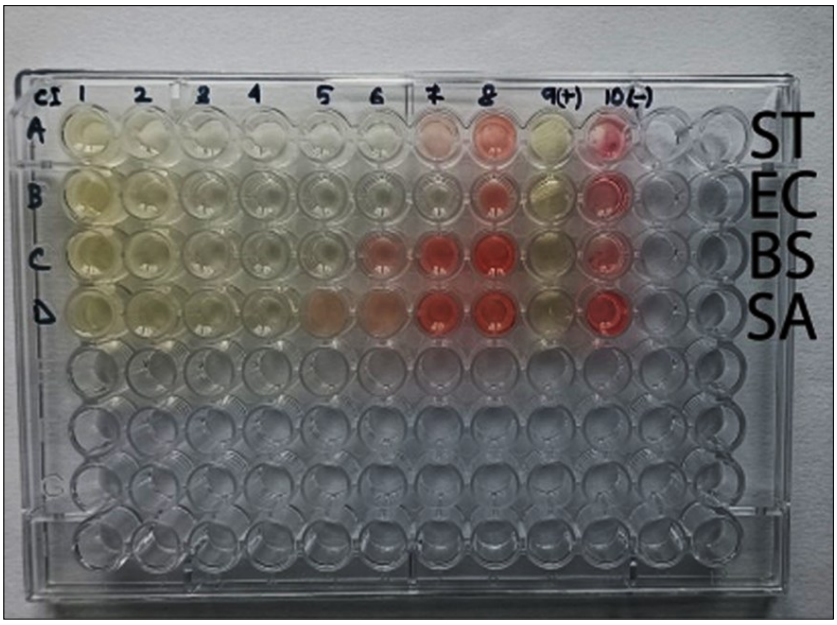


Fig. 5. Calculation of minimum inhibitory concentration (MICs) of citral by using 96-well microtiter plate method against pathogenic bacteria, where 1st row is inoculated with *Salmonella typhi* (ST), 2nd row with *Escherichia coli* (EC), 3rd row *Bacillus subtilis* (BS) and 4th row with *Staphylococcus aureus* (SA). Well 1: Blank; Wells 2–8: EOOB (100–2.5 µL + sterilized Mueller-Hinton (MH broth); Well 9: 10 µg/mL tetracycline (positive control). Rows 1–4: *E. coli*, *S. typhi*, *B. subtilis*, *Staphylococcus aureus*

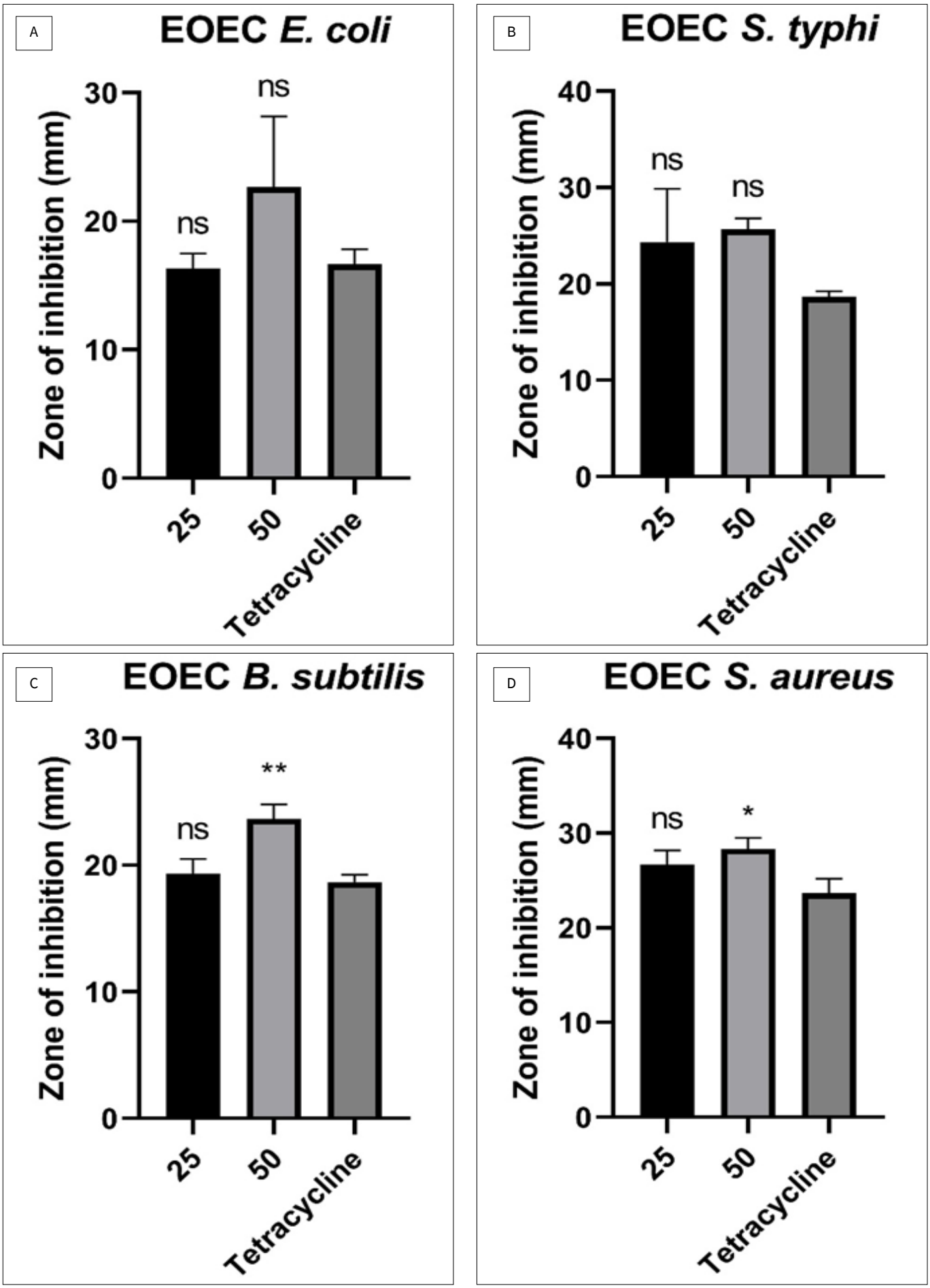


Fig. 6. Antibacterial activity of essential oil of *Elsholtzia communis* (EOEC) against *E. coli* [A], *S. typhi* [B], *B. subtilis* [C], *S. aureus* [D] by zone of inhibition assay. Bars represent ±SD (n=3). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. "ns" indicates no statistical difference, whereas * and ** denote the statistical significance difference at $p < 0.05$ and $p < 0.01$. 10 µg/mL of Tetracycline in 20 % DMSO was used as the positive control.

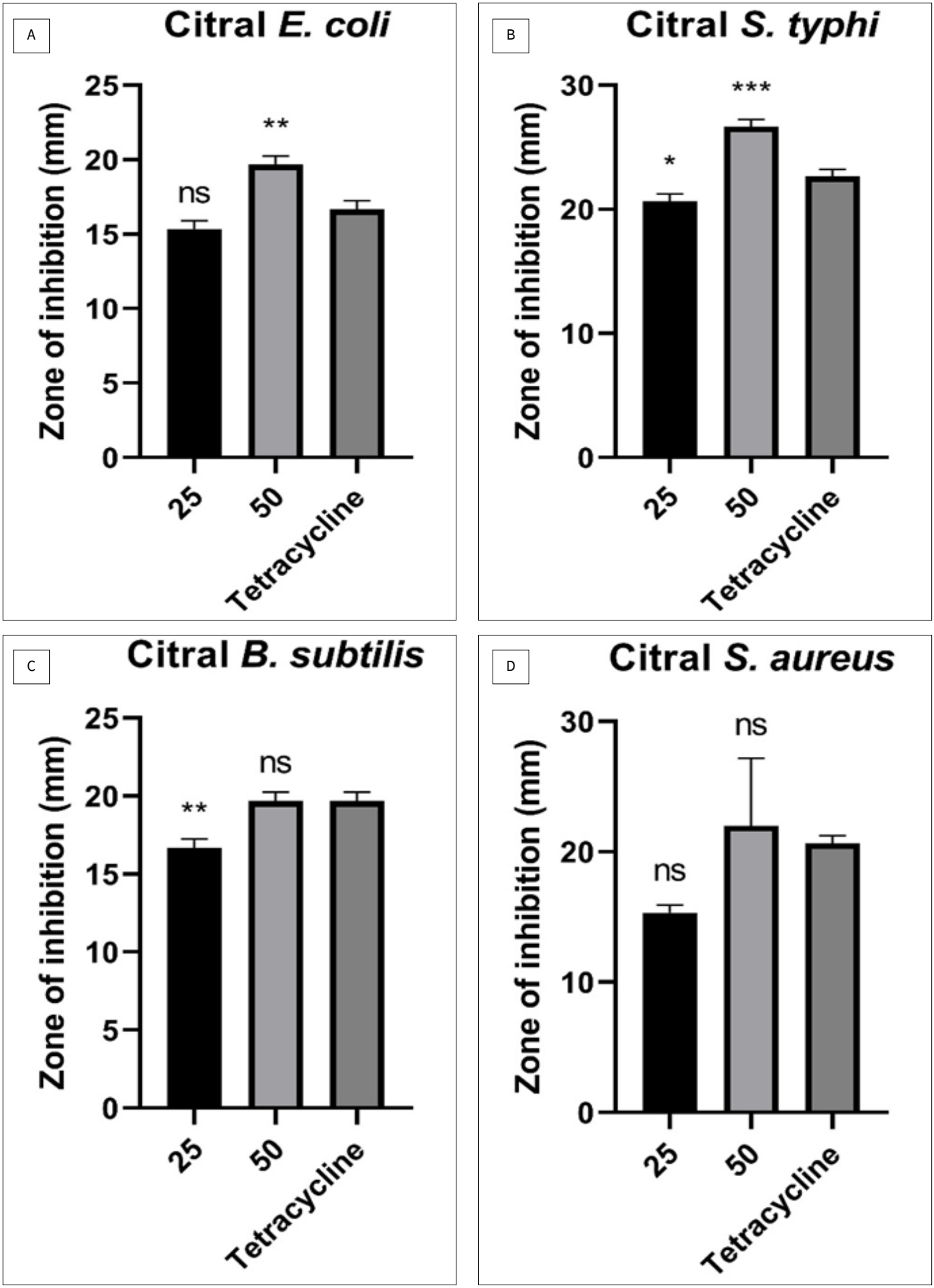


Fig. 7. Antibacterial activity of citral against *E. coli* [A], *S. typhi* [B], *B. subtilis* [C], *S. aureus* [D] by zone of inhibition assay. Bars represent ± SD (n=3). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. "ns" indicates no statistical difference, whereas * and ** denote the statistical significance difference at $p < 0.05$ and $p < 0.01$. 10 µg/mL of tetracycline in 2 % DMSO was used as the positive control.

Developmental and embryotoxic potential of essential oil of *Elsholtzia communis* (EOEC)

Zebrafish embryo lethality

Mortality rates of zebrafish embryos exposed to EOEC and citral were recorded up to 96 hpf (Fig. 8A & B). A significant decline in embryo survivability occurred at higher concentrations (220 µg/mL for EOEC and 320 µg/mL for citral). Embryos treated with EOEC and citral showed higher mortality than the negative control (1 % DMSO) but lower than the positive control (4 µg/mL 3,4-dichloroaniline).

Heart rate analysis showed normal embryonic heartbeats ranged between 120 and 180 bpm (20, 21) (Fig. 9A & B). At lower concentrations, EOEC and citral elevated heart rates, which then declined at higher concentrations, likely indicating embryonic distress or lethality. Complete absence of a heartbeat at the highest concentrations confirmed embryo death.

Morphological deformities

Zebrafish embryos treated with various concentrations of EOEC and citral developed several teratogenic deformities. Common abnormalities included pericardial and yolk sac edema, spinal curvature, ocular malformations, scoliosis, lordosis, dented tail and delayed hatching (Fig. 10 & 11). No deformities were observed in embryos exposed to 1 % DMSO (negative control) or egg water. However, the frequency and severity of deformities increased with treatment concentration.

Discussion

Investigating the phytochemical constituents of *E. communis* is crucial for understanding its biological activity and safety profile. This study provides a detailed chemical and biological evaluation of the flower-derived EOEC. The GC-MS analysis revealed EOEC is predominantly composed of monoterpenes and sesquiterpenes, with geranial and neral as the major components. These concentrations are considerably higher than those previously reported from leaf-derived oils, suggesting significant tissue-specific variation in volatile biosynthesis within the species.

This shift in composition towards citral-enriched monoterpenes is significant, as citral (a blend of geranial and neral) is well-known for its antioxidant potency. Our current investigation into EOEC from flowers reveals a moderate, yet consistent, capacity for scavenging free radicals, with the ABTS assay showing slightly higher efficiency than the DPPH assay. In stark contrast, an earlier study reported significantly more potent antioxidant activity from *E. communis* leaf essential oil, with considerably lower IC_{50} values across DPPH, H_2O_2 and nitric oxide scavenging assays (2). This marked difference clearly highlights a tissue-specific variation in antioxidant potential between the flower and leaf oils. We attribute such variation to differing phytochemical compositions, particularly in the relative abundance of oxygenated monoterpenes or phenolic constituents and distinct metabolic pathways active in each plant part. These findings underscore that EOEC antioxidant efficacy heavily depends on the plant part used, making tissue selection a critical factor for its functional and therapeutic assessment.

Previous research on *E. communis* leaf essential oil indicated moderate antimicrobial effects, reporting inhibition zones against *Mycobacterium smegmatis*, *S. aureus* and *Candida albicans* (2). Our current investigation, however, found that the essential oil extracted from EOEC exhibited considerably more potent antibacterial action. Both EOEC and its primary component, citral, successfully inhibited all 4 bacterial strains tested: *E. coli*, *B. subtilis*, *S. typhi* and *S. aureus*. The EOEC achieved its strongest inhibition against *S. aureus* and its weakest against *E. coli*, with a consistent MIC across all strains. Citral, meanwhile, showed maximum inhibition against *S. typhi* and minimum against *S. aureus*; notably, its lowest MIC was recorded for *S. aureus*.

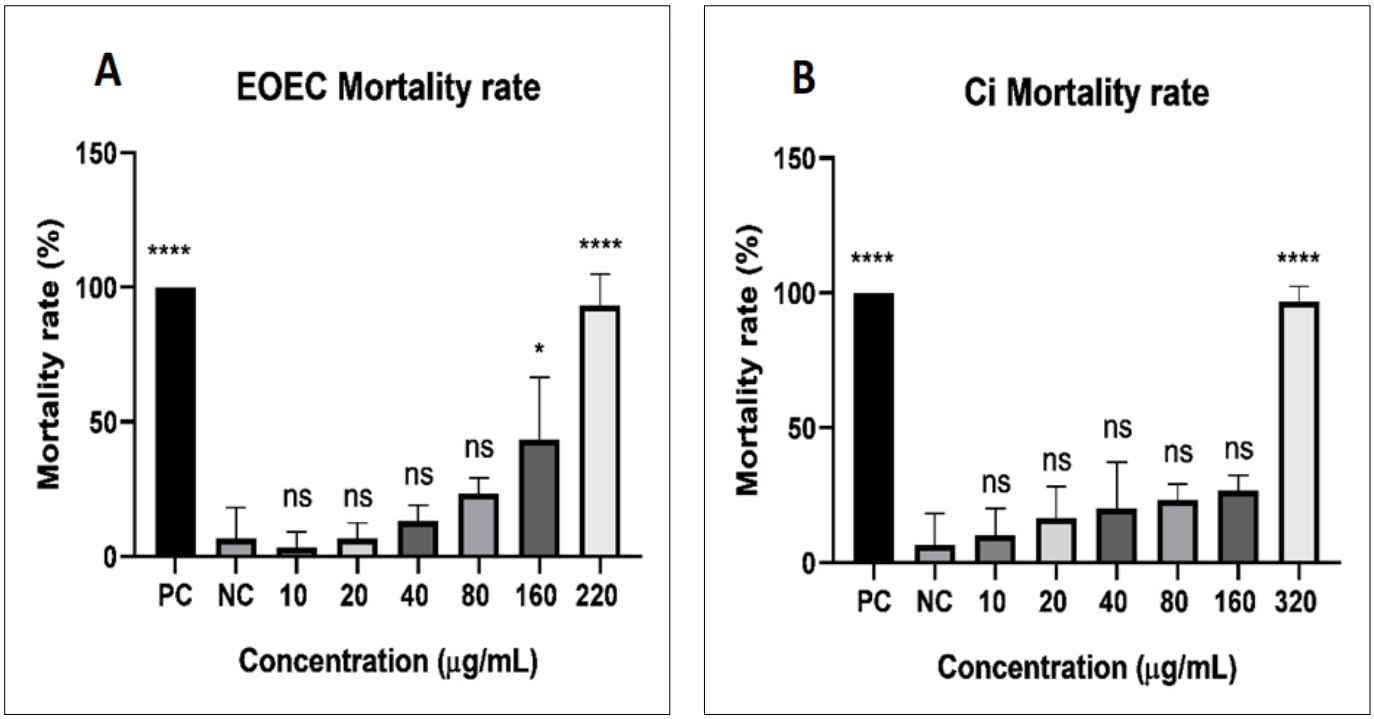


Fig. 8. Embryotoxicity of different concentrations of essential oil of *Elsholtzia communis* (EOEC) (A) and citral (B) in zebrafish embryo at 96 hpf. 3,4-Dichloroaniline and 1 % Dimethylsulfoxide were used as positive control (PC) and negative control (NC). All experiments were performed using at least 3 independent biological replicates. Data are expressed as mean ± SD. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$.

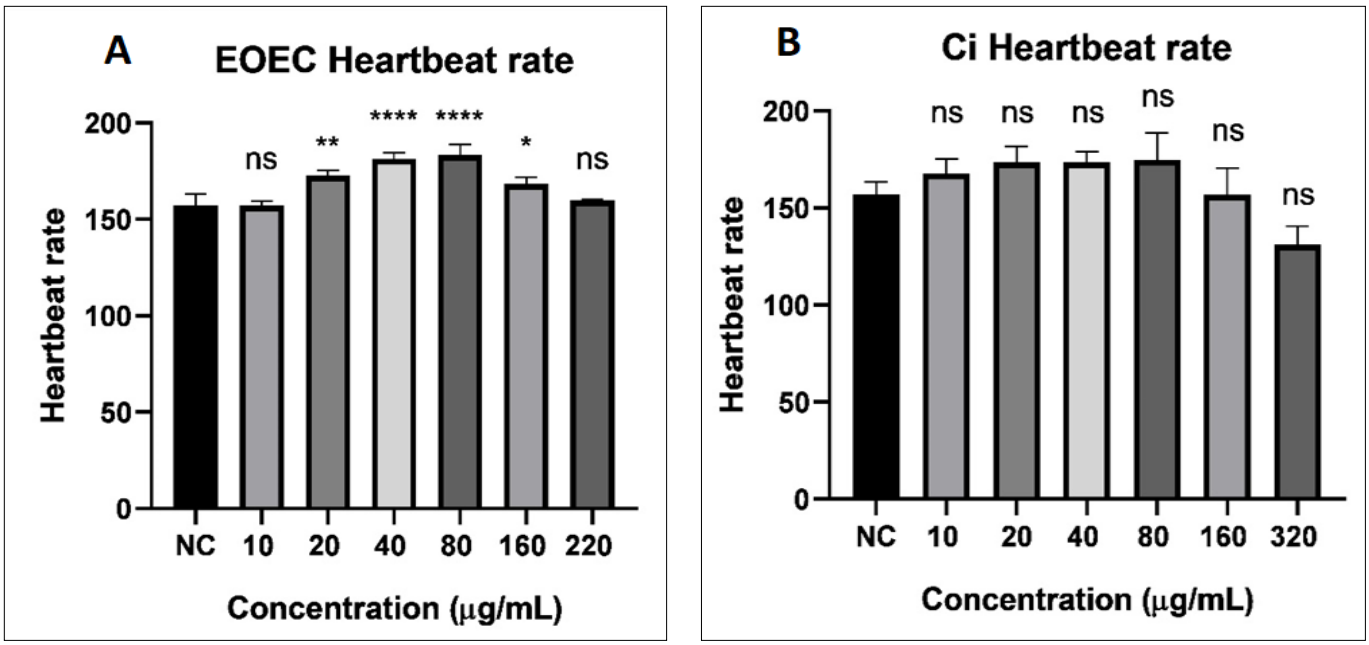
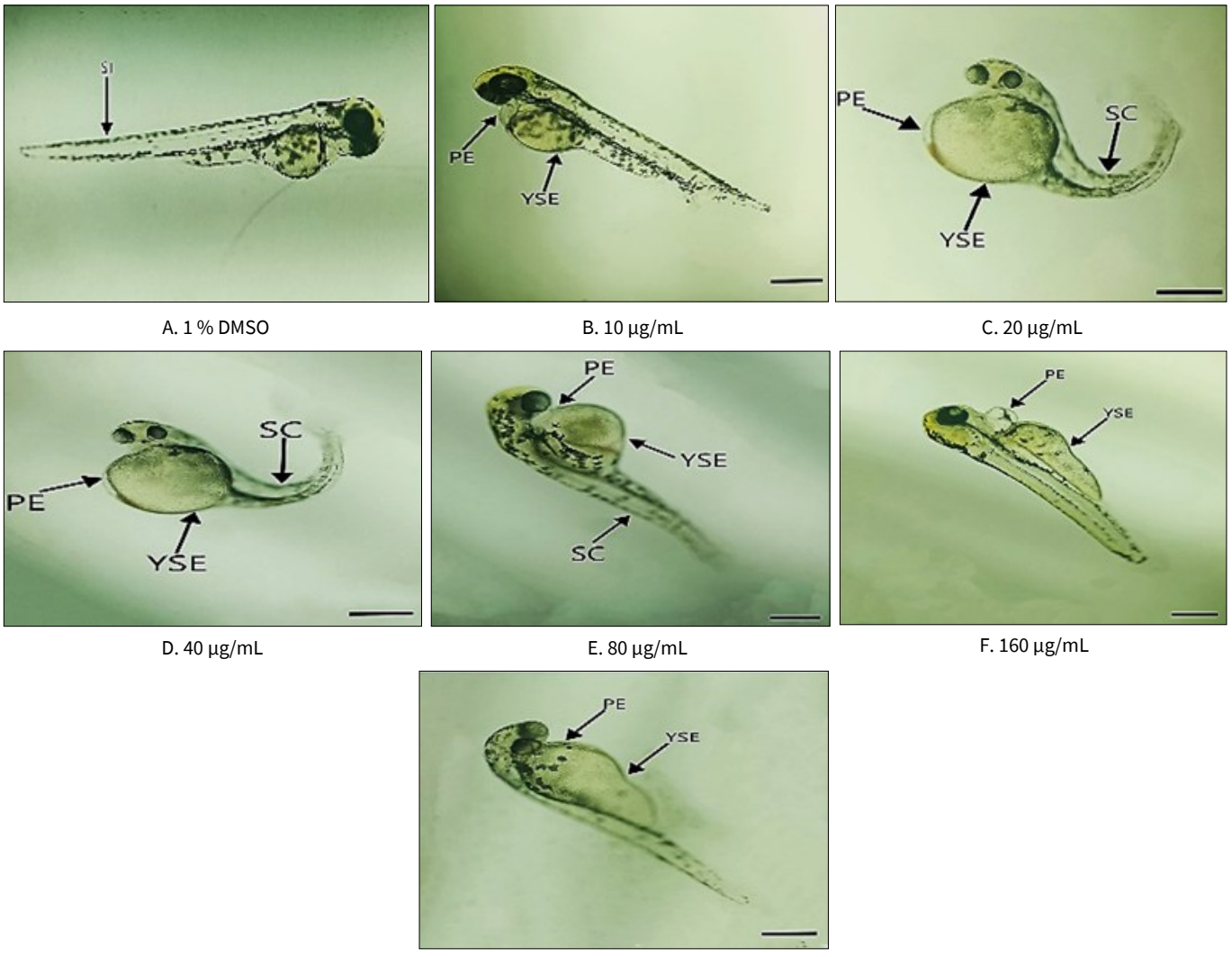


Fig. 9. Heart beat rate of different concentrations of A: EOEC and citral (Ci) in zebrafish embryo at 72 hpf. 1 % Dimethylsulfoxide was used as negative control (NC).

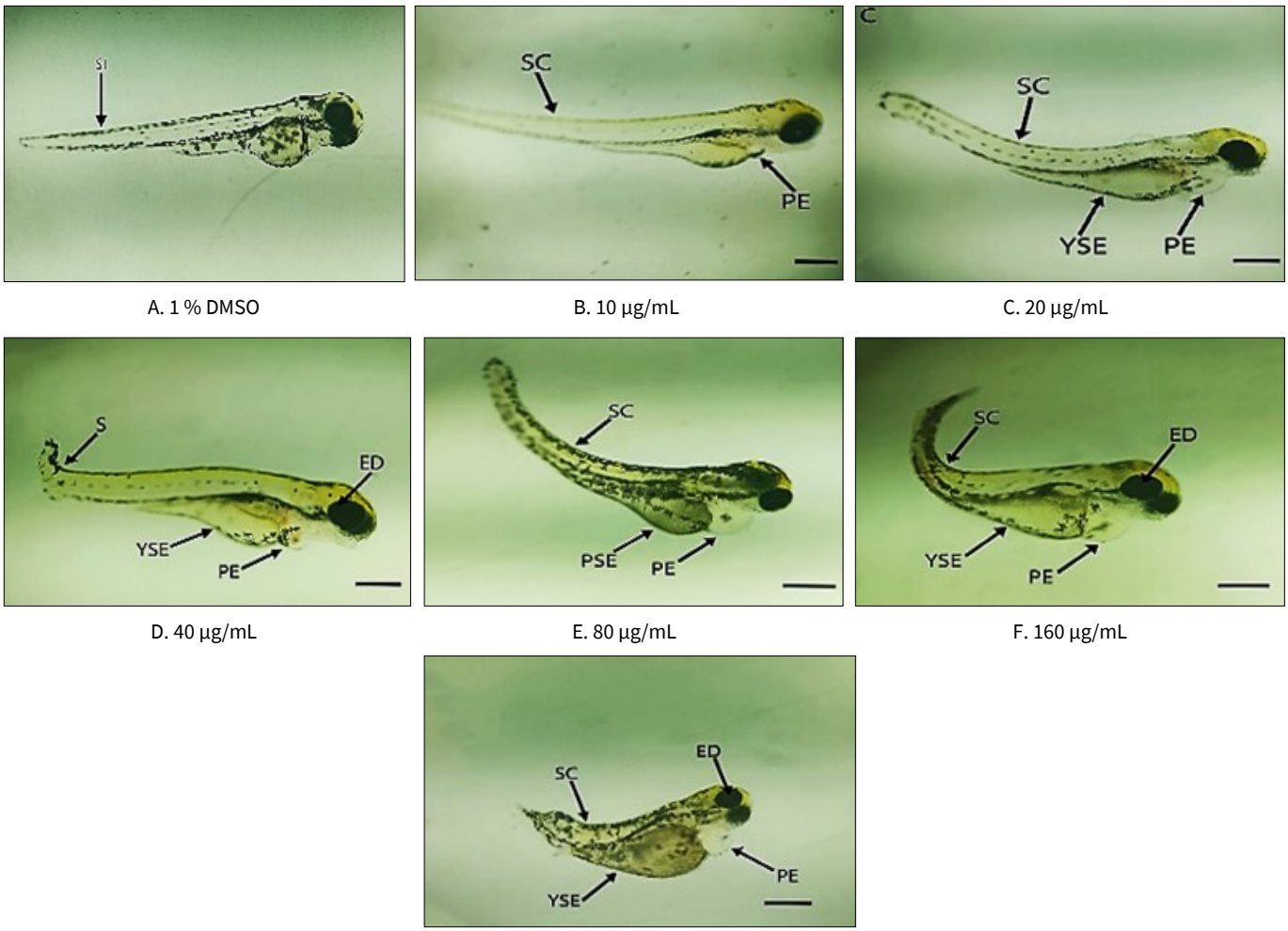


A. 1 % DMSO B. 10 µg/mL C. 20 µg/mL

D. 40 µg/mL E. 80 µg/mL F. 160 µg/mL

G. 220 µg/mL

Fig. 10. Microscopic image of zebrafish embryos showing the abnormalities after exposure to different concentrations of EOEC. (A) Control embryo at 96 hpf showing normal development with straight tail (ST); (B) 96 hpf embryo showing YSE and PE after treatment with 10 µg/mL ECEO; (C) 96 hpf embryo showing SC, PE and YSE after treatment with 20 µg/mL EOEC; (D) 96 hpf embryo showing SC, YSE and PE after treatment with 40 µg/mL EOEC; (E) 96 hpf embryo showing SC, YSE and PE after treatment with 80 µg/mL EOEC; (F) 96 hpf embryo showing SC, YSE, PE after treatment with 160 µg/mL EOEC; (G) 96 hpf embryo showing YSE and PE after treatment with 220 µg/mL EOEC. Scale (—) is equal to 100 µm.



A. 1 % DMSO B. 10 µg/mL C. 20 µg/mL

D. 40 µg/mL E. 80 µg/mL F. 160 µg/mL

G. 320 µg/mL

Fig. 11. Microscopic image of zebrafish embryos showing abnormalities after exposure to different concentrations of citral (A) Control embryo at 96 hpf showing normal development with straight tail (ST); (B) 96 hpf embryo showing SC, YSE and PE after treatment with 10 µg/mL citral, (C) 96 hpf embryo showing SC, PE, YSE and ED after treatment with 20 µg/mL citral; (D) 96 hpf embryo showing SC, YSE and PE after treatment with 40 µg/mL citral (E) 96 hpf embryo showing SC, YSE, PE and ED after treatment ED after treatment with 80 µg/mL Citral (F) 96 hpf embryo showing SC, YSE, PE and ED after the treatment with 160 µg/mL citral (G) 96 hpf embryo showing SC, YSE, PE and ED after treatment with 320 µg/mL citral. ST: Straight tail, SC: Spine curvature, YSE: Yolk-sac edema, PE: Pericardial edema, S: Scoliosis, ED: Eye defect, ST: Straight tail, SC: Spine curvature, YSE: Yolk-sac edema, PE: Pericardial edema. Scale (—) is equal to 100 µm.

These findings collectively underscore that *E. communis*'s antimicrobial activity is profoundly influenced by the specific plant part used. The strong antibacterial efficacy and notably low MIC values observed in this study further validate the biological significance of the flower oil's chemical profile. This research marks the first report directly establishing a link between the floral essential oil composition of *E. communis* and its antibacterial, as well as antioxidant, properties. The EOEC's potent activity against both Gram-positive and Gram-negative bacteria suggests broad-spectrum potential. Its low MIC values and substantial inhibition zones, particularly against *S. aureus* and *S. typhi*, highlight its promise as a natural antimicrobial agent. Discovering such pronounced activity from the flower oil, rather than the leaf oil, adds a new pharmacognostic dimension to the plant, significantly broadening its potential applications in natural product research and pharmaceutical development.

In the present study, citral served as a reference compound to help interpret the antibacterial and embryotoxic effects observed for EOEC. Citral is well-documented for its strong antibacterial activity, primarily linked to its interaction with bacterial cell membranes. This interaction increases membrane permeability, dissipates the proton motive force and consequently reduces intracellular ATP levels (22, 23). These mechanisms are characteristic of essential oils rich in oxygenated monoterpenes, reinforcing citral's role as a major bioactive constituent. Numerous studies have reported citral's inhibitory effects against a broad range of Gram-positive and Gram-negative bacteria, including foodborne and opportunistic pathogens. These effects are mainly attributed to its ability to disrupt bacterial membrane integrity, cause leakage of intracellular components and interfere with cellular energy metabolism (22–24). Owing to these properties, citral and citral-rich essential oils have been explored as natural antimicrobial agents for food preservation and as alternative strategies to control microbial contamination. Previous investigations have demonstrated citral's antibacterial efficacy against a wide range of clinically and food-relevant pathogens, including resistant bacterial strains, highlighting its potential in food safety and antimicrobial applications (24). However, citral's antimicrobial benefits must be weighed against its toxicological profile. Rodent studies have shown that high-dose citral exposure can lead to adverse systemic effects, indicating its safety is strongly dose-dependent (5).

Despite these bioactivities, safety remains a critical concern for plant-derived oils. No prior study has examined EOEC's *in vivo* developmental toxicity. *Danio rerio* (zebrafish) is an established vertebrate model for toxicological screening (25) and this work uniquely employs the OECD-approved Zebrafish embryo toxicity test (ZFET). This allowed us to evaluate various developmental parameters such as coagulation, heartbeat, somite formation, pigmentation and tail detachment at 24, 48, 72 and 96 hpf to assess EOEC's embryotoxic potential (26–29).

The results showed a concentration-dependent increase in mortality and morphological deformities, including pericardial and yolk sac edema, scoliosis and delayed hatching (30, 31). These teratogenic endpoints align with citral-associated developmental toxicity, likely stemming from the disruption of early organogenesis. Notably, the high citral content (≈ 79 %) in the flower oil may explain its pronounced embryotoxicity, as aldehyde-rich monoterpenes can readily penetrate biological membranes and interfere with embryonic enzyme systems (32, 33). These findings provide a scientific basis for the traditional caution against using EOEC preparations during pregnancy, as reported among indigenous Meitei communities of Northeast India. Evidence from zebrafish embryo-based assays further supports these findings, as exposure to citral or citral-rich essential oils has been linked to concentration-dependent embryotoxic effects. Reported abnormalities include reduced survival rates, delayed hatching, pericardial edema, spinal deformities and alterations in heart rate (34). These developmental effects are consistent with the embryotoxic endpoints observed in the present study and emphasise the heightened sensitivity of early developmental stages to biologically active monoterpenes.

Overall, the results indicate that while citral exhibits notable antibacterial efficacy, careful optimization of exposure levels is necessary to ensure biosafety, particularly for applications involving repeated exposure or developmental stages. The zebrafish embryo model employed in this study provides a valuable *in vivo* platform for defining safe concentration ranges and for balancing antimicrobial effectiveness with potential toxicological risks. Such integrated assessments are essential for the responsible application of citral-rich essential oils in food, beverage and nutraceutical formulations.

Therefore, this study not only identifies the biochemical and pharmacological uniqueness of the flower-derived EOEC but also provides the first empirical evidence of its developmental toxicity, thereby connecting ethnomedicinal knowledge with modern toxicological validation.

Conclusion

In conclusion, the flower-derived EOEC, characterised by a high content of citral, demonstrates strong antioxidant and antibacterial activities, supporting its potential bioactivity. Concurrently, zebrafish embryo assays conducted according to OECD TG 236 revealed dose-dependent embryotoxic effects, such as delayed hatching, edema and reduced heart rate. This indicates that the citral-rich composition may pose developmental risks at higher concentrations. These findings provide a scientific foundation for dose standardisation and risk assessment prior to therapeutic or commercial application. This study represents the first integrated evaluation of the flower-derived EOEC, demonstrating clear chemotypic differences from previously reported leaf oils and linking these differences to distinct antibacterial and antioxidant activities. Importantly, the zebrafish embryo model provides the first *in vivo* evidence supporting traditional cautions associated with the plant, revealing concentration-dependent developmental effects consistent with citral-rich EOEC. By bridging traditional knowledge with modern phytochemical and toxicological approaches, this work makes a meaningful contribution to the understanding of tissue-specific bioactivity and safety and establishes a foundation for future pharmacological and risk-assessment studies of EOEC.

Acknowledgements

The authors gratefully acknowledge the Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, for GC-MS analysis; and the Department of Chemistry Instrumentation Facility, Manipur University, for ATR-FTIR measurements. This research received funding from the UGC under the Non-NET Fellowship Scheme from the DBT Ramalingaswami Re-entry Fellowship Project (BT/RLF/Re-entry/56/2021) at the Department of Life Sciences (Botany), Manipur University.

Authors' contributions

BSW conceptualised, designed and investigated the study as well as wrote the original draft. SY helped in formal analysis and manuscript preparation. KNS helped in antibacterial studies. LN helped in FTIR studies and TY helped in designing the experiment and data interpretation. MDD helped in designing, validation and supervision of the study. All authors read and approved the final manuscript.

Compliance with ethical standards

IAEC approval/reference number All zebrafish (*Danio rerio*) experiments were authorised and approved by the Institutional Animal Ethics Committee (IAEC) of Manipur University (Proposal No. 8) and were conducted in accordance with the approved IAEC guidelines. Zebrafish embryo toxicity assay (OECD TG 236).

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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