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Research Article

Citrus Aurantifolia Essential Oil Composition, Bioactivity, and Antibacterial Mode of Action on *Salmonella Enterica*, a Foodborne Pathogen

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Abstract: *Salmonella enterica* is a well-known pathogenic bacterium that can cause intracellular illness and food poisoning in humans, especially salmonellosis. In the present study, 20 essential oils of aromatic plants were screened for their antibacterial activity against *S. enterica*. Among the essential oils screened, *Citrus aurantifolia* essential oil (CAEO) demonstrated the most efficient activity against *S. enterica*. CAEO had minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 8 and 16 µl/mL, respectively. A significant scavenging of radical cations was observed with CAEO using 2, 2'-diphenyl-1-picryl hydrazyl with an IC_{50} of 0.2147 mg/mL. The total phenolic content of CAEO was 10.90 mg GAE/100 µL. The treatment of bacterium with CAEO caused the leakage of cellular components, i.e., both nucleic acids and proteins. Furthermore, scanning electron microscopy (SEM) was used to measure the ultrastructure of control and treated the bacterium (due to CAEO). The results of SEM reveal a loss of cellular integrity and function of bacterial cell membrane. The GC/GC-MS analysis of CAEO revealed *E*-citral (67.97%) as a major chemical component. CAEO may be considered a safe antibacterial agent against foodborne *S. enterica*. The present study investigated a detailed analysis of the antibacterial activity of *C. aurantifolia* essential oil, which can provide insight for further studies.

***Keywords*:** foodborne pathogen, *E*-citral, antioxidant, GC and MS, phenolic content, SEM, mode of action

1. Introduction

In food system, both Gram-positive and Gram-negative bacteria cause spoilage of various food commodities worldwide [1-3]. These bacteria comprise the species of *Pseudomonas*, *Salmonella*, *Proteus*, *Shigella*, *Klebsiella*, *Vibrio cholerae*, *E. coli*, and *Staphylococcus aureus.* Among children and adults, these bacteria are the major cause of sporadic and epidemic diarrhoea [4]. The *Salmonella* groups of bacteria are recognized as leading causes of foodborne diseases. The most common cause of salmonellosis is consumption of food contaminated with *S. enterica*, which usually affects cattle and poultry, as well as domestic cats [5]. In recent years, the prevalence of human salmonellosis and typhoid fever has increased worldwide due to the consumption of contaminated foods [6].

Since synthetic antimicrobial additives cause cancer in various tissues, the food industry has been looking for

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natural products. Parabens, for example, cause breast cancer [7-8] and nitrites cause cancer in the lungs, intestines, liver, and stomach [9]. Increasing resistance to antimicrobials and higher mortality rates, make the development of new compounds increasingly important in the medical field. Currently, antibiotic resistance is a global problem [10-11]. The emergence of antibiotic resistance among bacterial strains, i.e., failure of antibiotics against foodborne pathogens has necessitated the search for new antibacterial agents. Over the years, antimicrobial plant products including extracts, latex, phenolics, alkaloids, tannins, flavones, flavonoids, and essential oils have been evaluated for their biological activity with the objective of discovering new compounds with antibacterial actions for industrial intended use [12-13]. Throughout history, essential oils and other plant extracts have attracted interest as sources of natural products. These alternative remedies have been evaluated for their prospective use in treating various types of infectious diseases [14].

Many plants produce antimicrobial compounds as secondary metabolites as part of their normal growth and development. Essential oils are good sources of biologically active compounds. There are usually 20-60 different compounds found in essential oils, over a wide range of concentrations [14]. In general, essential oils are composed of terpenes and terpenoids followed by aromatic and aliphatic compounds. There are numerous structural variations of terpenes, which include monoterpenes, diterpenes, sesquiterpenes, triterpenes, hemiterpenes, and tetraterpenes. Essential oils are mostly derived from terpenes, which come from the isoprenoid pathway, and are synthesized and secreted by specialized organs in plants [14]. The essential oils were extracted and purified from plant species through various methods such as solvent extraction, hydrodistillation, soxhlet extraction, and so forth, advanced methodologies such as supercritical fluid extraction method, solar hydrodistillation, ultrasonic, and microwave, improved essential oil processes, which improved the quality of essential oils in terms of biological efficiency, energy dissipation, and yield [15].

The use of essential oils reduces the proliferation of microorganisms. Many foods can benefit from the use of essential oils, which are natural volatiles extracted from aromatic and medicinal plants. Essential oils are natural additives capable of treating various health conditions as they possess antibacterial, antifungal, antioxidant, and anticarcinogenic mode of action [16-17]. Certain essential oils are known to have antiviral properties against coronavirus strains [18]. In earlier studies, essential oils from *Cinnamomum zeylanicum* [19], Coriander seeds [20], *Thymbra capitata* [21] and many more have shown promising antibacterial activity against *S. enterica* derived from various foodstuffs. However, the majority of these essential oils can alter the taste of food or exceed acceptable flavor thresholds [22] due to strong balsamic notes of thymol and other constituents [23].

Natural compounds with antimicrobial mode of action, low toxicity, and low cost are needed in order to protect human health from food contamination and to combat foodborne illness. The objective of the present study was to evaluate the antibacterial potential of 20 essential oils derived from aromatic plants against *S. enterica* and find out which essential oil have more antibacterial properties. In this context, the present study was intended to examine antibacterial, bactericidal effects and mode of action of *Citrus aurantifolia* (Christm.) Swingle essential oil, as well as the time of death of *S. enterica* induced by this essential oil. Further, the ultrastructure of the cell was examined for signs of possible cellular changes. We also investigated the chemical composition, antioxidant properties, and phenolic content of the potent essential oil, i.e., *C. aurantifolia* to understand the mode of action.

2. Materials and methods

2.1 *Bacterial culture*

This study used culture of *Salmonella enterica* (MTCC 9844) obtained from Institute of Microbial Technology in Chandigarh, India. A Nutrient Broth (NB, HiMedia, India) culture was maintained for 15 days and then re-cultured on nutrient agar (NA, HiMedia, India) plates. A loopful of test bacterium was inoculated into 5ml nutrient broth from above NA plates to prepare working cultures. The broths were incubated for 24 h at 37 °C. To adjust bacterial inoculum, 0.5 Mcfarland standards (10^8 CFU/mL) were used.

2.2 *Plant collection and essential oil extraction*

In 2015, leaves or shoots of twenty aromatic and medicinal plants (Table 1) were collected from different forests located in Gorakhpur division (26°46'N and 83°2'E, at an elevation 84 m), transported to the laboratory, and identified with the help of taxonomic keys and the Departmental Herbarium available with Department of Botany, DDU

Gorakhpur University. The volatile oil used in the study was obtained by hydro distillation of plant parts (300 g) in a Clevenger's apparatus [24]. Each essential oil was dried over anhydrous sodium sulphate and then stored in glass vials at 4 °C, separately until needed. The extraction of essential oil from each plant was carried out in three replicates.

Table 1. *In vitro* screening of essential oils against *S. enterica*

Plant species	Part used	Family	ZOI (mm)#* at 5 µL
Aegle marmelos (L.) Correa	Leaf	Rutaceae	19.5 ± 1.20[c]
Cannabis sativa (L.)	Leaf	Cannabaceae	11 ± 0.48[c]
Citrus aurantifolia (Christm.) Swingle	Leaf	Rutaceae	43 ± 0.91[a]
Citrus sinensis (L.) Osbeck	Peel	Rutaceae	20 ± 0.42[c]
Cymbopogon citratus (DC.) Stapf.	Leaf	Poaceae	32 ± 0.20[b]
Dysphania ambrosioides (L.) Mosyakin & Clemants	Twig	Amaranthaceae	14 ± 0.86[d]
Eucalyptus globulus Smith.	Leaf	Myrtaceae	14.3 ± 0.44[d]
Mesosphaerum suaveolens (L.) Kuntze	Leaf	Lamiaceae	11 ± 0.79[d]
Lippia alba (Mill.)	Leaf	Verbenaceae	11 ± 0.22[d]
Mentha arvensis (L.)	Leaf	Lamiaceae	31.6 ± 0.68[b]
Mentha piperita (L.)	Leaf	Lamiaceae	16.9 ± 0.39[c]
Piper betle (L.)	Leaf	Piperaceae	12 ± 0.11[d]
Piper methysticum G. Forst	Leaf	Piperaceae	NZ
Monoon longifolium (Sonn.) B.Xue & R.M.K.Saunders (Sonn.)	Leaf	Annonaceae	12 ± 0.52[d]
Psidium guajava (L.)	Leaf	Myrtaceae	NZ
Drypetes roxburghii (Wall),	Leaf	Putranjivaceae	10 ± 0.19[d]
Ocimum basilicum L.	Leaf	Lamiaceae	16 ± 0.65[c]
O. canum L.	Leaf	Lamiaceae	24 ± 0.23[b]
O. gratissimum L.	Leaf	Lamiaceae	15 ± 0.70[c]
O. sanctum L.	Leaf	Lamiaceae	17 ± 0.26[c]
SEm			1.15
CV%			48.99

#ZOI: Zone of Inhibition, NZ: No ZOI, values are mean of three replicates ± SE (standard error), *ZOI of each essential oil followed by different letters within a column are significantly different accordingly to Tukey's test ($P < 0.001$).

2.3 *Screening of essential oils through disc diffusion method against S. enterica*

Bioassays of antibacterial activity of 20 essential oils extracted from aromatic plants were conducted according to the Disc diffusion method described by Andrews [25]. The target bacterial suspension (0.1 mL) containing 10^8 CFU/mL was spread on Nutrient agar medium with a sterile spreader. Whatman filter paper discs (6 mm in diam., HiMedia, India) were individually applied with 5 µL (v/v) of each essential oil and aseptically placed in the centre of inoculated NA plates to determine the antibacterial activity, separately. In the positive control plates, the test bacterium was inoculated without essential oils. Un-inoculated plates were used as negative controls. A zone of inhibition (ZOI) was measured in mm around the paper discs, if present, by using the HiMedia ZOI scale after 24 to 36 h of incubation at 37 °C in a complete randomized design (CRD) in three replicates.

2.4 *Determination of MIC and MBC*

An agar dilution susceptibility test based on NCCLS (National Committee for Clinical Laboratory standards) [26] and CLSI (Clinical and Laboratory Standards Institute) [27] modified methods was conducted to assess the minimum inhibitory concentration (MIC) of most efficient essential oil. A series of dilutions of efficient essential oil, i.e., *C. aurantifolia* up to 16 µL/mL was prepared in a NA plate (10 mL), followed by the addition of Tween 80 (0.5% v/v) as an emulsifier. Following the solidification of plates, 5 µL of overnight-grown bacterial culture containing 10^8 CFU/mL was aseptically spotted on the plates. A plate containing media and only inoculum was kept as a negative control, whereas a plate containing only media was kept as a positive control. The plates were incubated for 24-72 h at 37 °C in a CRD in three replicates. A comparison was made between the inhibition of bacterial growth and the growth in control plates. A MIC value was determined by finding the *C. aurantifolia* essential oil (CAEO) concentration necessary to inhibit visible growth on agar plates. Further, the MIC plates that failed to grow were sub-cultured on fresh medium (NA plates without essential oil) for determining Minimum Bactericidal Concentration (MBC) [24]. The minimum concentration at which the bacteria did not grow on fresh medium was identified as the MBC.

2.5 *Antibiotic susceptibility test*

Discs of Amikacin (30 µg/disc), Aztreonam (30 µg/disc), Cefixime (5 µg/disc), Cefuroxime (30 µg/disc), Cefotaxime (30 µg/disc), Gentamycin (10 µg/disc), Moxifloxacin (5 µg/disc), Nalidixic acid (30 µg/disc), and Tobramycin (10 µg/disc) were used as standard. All antibiotics were purchased from HiMedia, India. The antibacterial activity was assessed based on the diameter of the inhibition zone (mm) generated by each antibiotic after 24 to 36 h of incubation of inoculated plates at 37 °C in a CRD in three replicates.

2.6 *Time-kill curve studies*

Based on methodology of Carson et al. [28], the killing activity of CAEO on *S. enterica* was determined by measuring the numbers of CFU/mL over 2 h. Tween 80 (0.5%) was added to 10 mL nutrient broth (NB) and the culture tubes were sterilised. In marked tubes, a sufficient amount of CAEO (at MIC value) was aseptically mixed. An aqueous suspension of bacterium (0.1 mL) was dispensed into tubes containing essential oil so that the final inoculum was 10^5 CFU/mL. The suspension was thoroughly mixed for one minute. The control was bacterium inoculated broth without essential oil and contained Tween 80 (0.5%). The tubes were incubated at 37 °C under aerobic conditions. A small aliquot of samples (1 mL) was removed at specific intervals (0, 10, 20, 30, 60, 80, 90, 100, 120 min) and serially diluted 10^{-6} times before being plated on NA medium for colony count determination. The experiments were conducted in three replicates. Over time, the mean number of survivors was determined and a plot of the number of viable bacteria was made to determine the rate of killing.

2.7 *Loss of 260 and 280 nm absorbing material*

In this study, the release of metabolites was studied using the method of Carson et al. [28] with a few modifications. The bacterial culture (10 mL in nutrient broth) at the exponential stage of growth was transferred into sterile centrifuge tubes (5 mL) and centrifuged at 5,000 rpm for 10 minutes. The supernatant was discarded, and the pellet was re-

suspended in 10 mL of nutrient broth (NB) and centrifuged again. A 10 mL aliquot of each sample was divided into three tubes. The CAEO was then added in three different concentrations (10, 20 and 30 µL) to each tube. The tubes were then incubated for 30 minutes at 37 °C on a shaker at 200 rpm. The suspension was filtered through sterile filters with a pore size of 0.2 mm, and the filtrate was used to determine nucleic acid at 260 nm and protein at 280 nm [28]. In UV-Vis spectrophotometer (Hitachi, Germany) measurements, the differences of absorption values at 260 nm and 280 nm between test and control groups were used to evaluate the release of metabolites from *S. enterica*. Each experiment was repeated three times.

2.8 *Scanning Electron Microscopy (SEM)*

In the present study, SEM analysis was performed according to methodology described by Guimarães et al. [29] with slight modifications. The bacterium, *S. enterica* was cultured on Mueller-Hinton agar. During mid-log-phase, *S. enterica* culture was transferred to Mueller-Hinton broth and treated with CAEO within 4 h at a MIC concentration. Fixations were performed by incubating cells in a 2.5% glutaraldehyde solution (HiMedia, India) and 0.1 M cacodylate buffer for at least 12 h. After post-fixation with 1% osmium tetroxide, the samples were left at room temperature for 40 minutes. A series of alcohol solutions (HiMedia, India) ranging from 20% to 100% (v/v) was used to dehydrate the samples following washing with 0.1 M cacodylate buffer. The samples were then placed in a sample basket and dried at a critical point. A spray machine was then used to coat the slides with gold before the samples were analysed with a scanning electron microscope (Hitachi, Germany).

2.9 *Determination of total phenols*

The total phenolic content of CAEO was determined by adopting methods of Taga et al. [30]. An amount of 100 µL of CAEO was dissolved in 10 mL of methanol (Sigma/Aldrich). By adding 0.3% HCl solution in water, 2 mL of above sample was made up to 5 mL. A 100 µL aliquot of resulting solution was added to 2 mL of 2% Na_2CO_3 (Sigma/Aldrich) and after 2 min, 100 µL of Folin-Ciocalteau reagent (Sigma/Aldrich) (10-fold diluted with distilled H_2O) was supplemented and mixed well. The absorbance of mixtures was measured spectrophotometrically at 750 nm after 30 minutes of incubation. The total phenolic content was calculated as gallic acid equivalent (GAE) from a calibration curve of Gallic acid (Sigma/Aldrich) standard solutions and expressed as mg of gallic acid (HiMedia, India)/100 µL of essential oil sample. Equation used for this calculation is as follows: $y = 0.021 \times +0.213$ ($R^2 = 0.973$)

2.10 *Free-radical scavenging activity*

The antioxidant activity of CAEO was evaluated using the method of Gulcin et al. [31] which involves scavenging 2,2'-diphenyl-1-picrylhydroxyl (DPPH) free radicals. A DPPH solution (Himedia, India) containing 0.004% methanol was added to essential oil samples of various concentrations. The mixture was incubated at 37 °C in dark for 30 min on shaker. Absorbance was recorded at 517 nm using a UV-Vis spectrophotometer. The percent (%) inhibition was calculated by: % inhibition = $(A_{control} - A_{test})/(A_{control})$ where $A_{control}$ = absorbance of the control reaction, A_{test} = absorbance of tested essential oil, IC_{50} values, which represented the concentration of CAEO or BHT that caused 50% scavenging, were determined from plot of inhibition percentage against concentration.

2.11 *Gas Chromatography (GC) and Gas chromatography-Mass spectrometry (GC-MS) of CAEO*

GC and GC-MS analyses of 0.1 µL of crude CAEO sample were carried out. The GC analysis was performed on an Agilent Technology 6890 N gas chromatograph data handling system equipped with a split-split less injector and fitted with a flame ionization detector (FID). During the analysis, nitrogen was used as a carrier gas. The column was an HP-5 capillary column (Film thickness: 0.25 µm; length: 30 m × Diameter: 0.32 mm). The temperature program included: initial temperature of 60 °C (hold: 2 min) programmed at a rate of 3 °C/min to a final temperature 220 °C (hold: 5 min). Injector and FID temperatures were both maintained at 210 °C and 250 °C, respectively. For the GC-MS analysis, Perkin Elmer Clarus 500 GC/MS was used, coupled to the RTX-5 capillary column (ID × film thickness 0.25 µm; 60 m × 0.32 mm). This test used Helium (1 mL/min) as a carrier gas, 210 °C as the injector temperature, and 60 to

210 °C as the oven temperature, which was then held for 15 minutes at an isothermal rate. The sample components were ionized in the EI mode at 70 eV. The chemical components were identified by determining their retention index relative to a homologous series of n-alkanes and by matching their recorded mass spectra with those stored in the spectrometer database/library (NIST/Pfleger/Wiley). GC-FID analysis provided the relative percentage area of each component.

2.12 *Statistical analysis*

All experiments were carried out in triplicate, repeated, and the results are expressed as mean ± standard error (SE). Normality distribution of all variables was verified using the Shapiro-Wilk Test. The analyses were performed using one-way analysis of variance (ANOVA). Additionally, the data obtained from antibacterial screening was subjected for Tukey's test and it was performed as post-hoc analysis to know which essential oils were significantly different in terms of efficacy, if they were found significant from ANOVA. All the analysis was performed using R software.

3. Results

3.1 *Screening of essential oils through disc diffusion method against S. enterica*

The antibacterial activity of each essential oil shown for test bacterium in terms of ZOI is summarized in Table 1. Among the essential oils screened, *Piper methysticum* and *Psidium guajava* essential oils did not induce inhibition zones against *S. enterica*, while all essential oils displayed more or less antagonist activity and significantly ($P < 0.0001$) differed from both essential oils. The test bacterium was more susceptible to *Aegle marmelos*, *Citrus aurantifolia*, *C. sinensis*, *Cymbopogon citratus,* and *Mentha arvensis* EOs at 5 µL concentration. Among these five essential oils, antibacterial activity of *C. aurantifolia* essential oil significantly differed ($P < 0.0001$) from others essential oils and showed potent results, with a zone of inhibition measuring 43.0 mm. Therefore, CAEO was chosen for detailed analysis including MIC and MBC determination, minimum time killing, SEM analysis of the test bacterium at MIC value, antioxidant property and chemical analysis.

3.2 *Determination of MIC and MBC*

The MIC and MBC of CAEO were determined. Based on the MIC and MBC results, it was found that this essential oil was highly effective against the test bacterium. The oil was able to inhibit the growth of *S. enterica* with the lowest MIC (8.0 µL/mL) and MBC (16.0 µL/mL) values as shown in Table 2.

Table 2. Bacteriosatic and bactericidal values of *C. aurantifolia* essential oil (CAEO**)** against *S. enterica* along with its antioxidant activity and total phenolics

Parameters*	CAEO
MIC	8 µL/mL
MBC	16 µL/mL
Total phenolics (mg GAE 100 µL/ml)	10.90 mg GAE 100 µL/mL
DPPH IC_{50} (mg/mL)	0.2147 mg/mL

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration, *Significantly different from controls at $P < 0.05$

3.3 *Antibiotic susceptibility test*

The test antibiotics showed more or less ZOI against *S. enterica*, except Aztreonam, Cefuroxime, Cefixime, and Nalidixic acid which did not show zone of inhibition. Besides, the antibiotics Amikacin (20 mm), Cefotaxime (9 mm), Gentamycin (24 mm), Moxifloxacin (21 mm), and Tobramycin (24 mm) had ZOI lesser than *C. aurantifolia* essential oil ($P < 0.001$).

3.4 *Time-kill curve studies*

The viability of *S. enterica* was significantly ($P < 0.05$) reduced by CAEO treatment at its MIC value. The treatment with CAEO resulted in four colony counts after 60 min of exposure, while complete inhibition of colonies was recorded after 120 minutes (Figure 1).

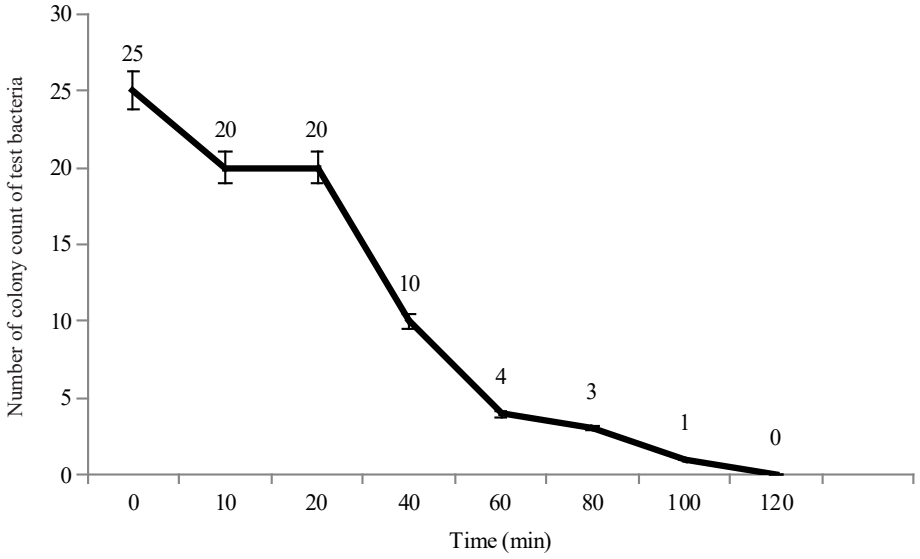

Figure 1. Graph showing time killing curve of bacterium, *S. enterica* in control and treatment (*C. aurantifolia* EO) sets at different time intervals.

3.5 *Loss of 260 and 280 nm absorbing materials*

The absorption of material cells at 260 nm and 280 nm was significantly different ($P < 0.01$) between CAEO treatment sample and control sample suspensions examined in UV spectrophotometer. The absorption value indicated that material was leaking from the cell. The absorption wavelengths of nucleic acids and proteins were 260 nm and 280 nm, respectively. A higher absorption value leads to the loss of more materials from the *S. enterica* cell. The absorption at MIC values, 10 µL/mL, 20 µL/mL, and 30 µL/mL were $0.868 < 0.885 < 0.892$ and with 0.865 control value (at 260 nm) and $0.866 < 0.873 < 0.875$ and with 0.842 control value (at 280 nm), respectively (Figure 2).

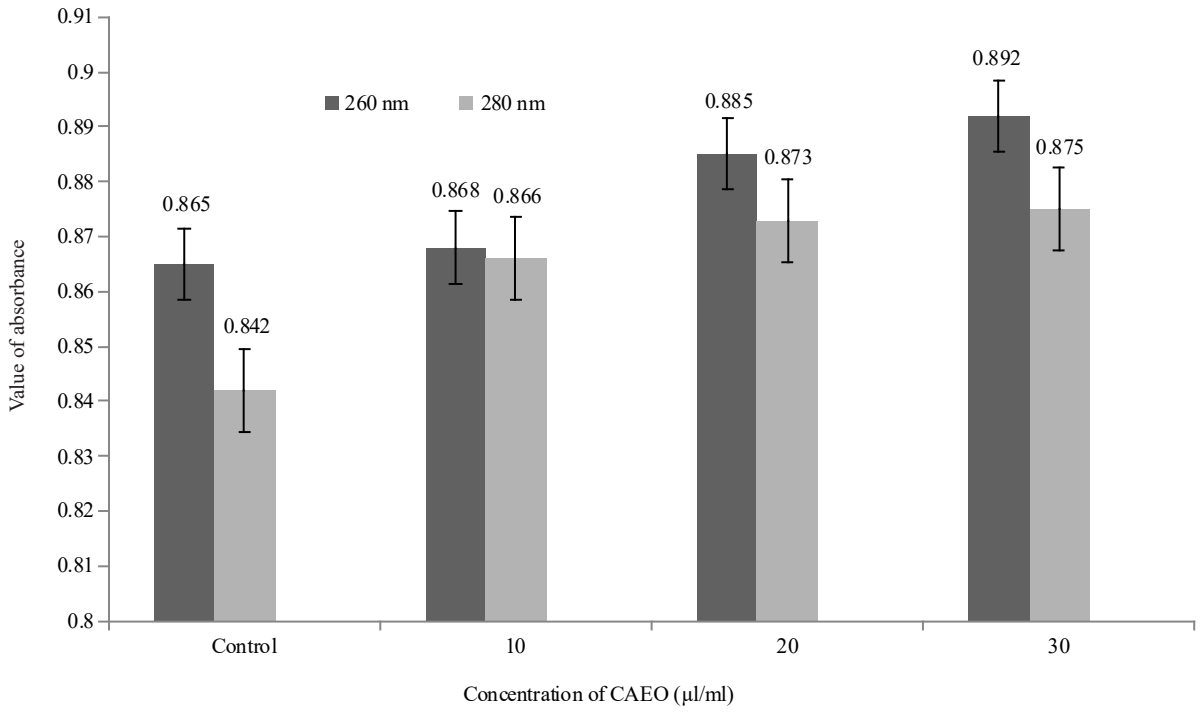

Figure 2. Leakage of cellular metabolite of *S. enterica* after treatment with *C. aurantifolia* essential oil (CAEO) at different concentrations

3.6 *SEM analysis*

The SEM analysis revealed morphological changes occurring in *S. enterica* after treatment with CAEO at its MIC value. The bacillary cells of *S. enterica* controls (Figure 3) had smooth surfaces, whereas the microbial cells treated with essential oil displayed irregular sizing with debris, possibly caused by disruptions in cell division or malfunctions of the nuclear membrane.

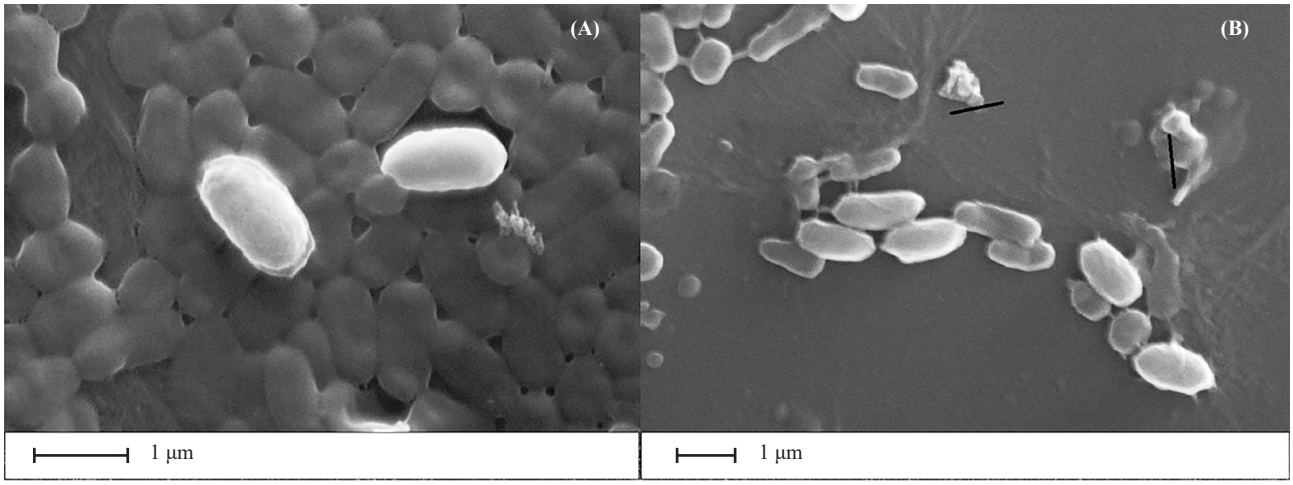

Figure 3. Scanning Electron Microscopy of untreated (A) and *C. aurantifolia* essential oil treated at its MIC value (B) *S. enterica* showing changes in morphology

3.7 *Determination of total phenols*

CAEO was subjected to determination of total phenols. The total phenolic content present in essential oil was 10.90 mg/100 µL essential oil which significantly depicts an appreciable amount of oil phenolic content (Table 2).

3.8 *Free-radical scavenging activity*

The radical scavenging ability of CAEO and the positive control was measured using spectrophotometry (Table 2). The CAEO showed 58.9%, 75.7%, 80.4%, and 81.9% inhibition of DPPH radical cations at 10, 20, 30, and 40 µL/mL concentrations, respectively. The oil was able to reduce the stable radical DPPH to the yellow-coloured DPPH-H with an IC_{50} value of 0.2147 mg/mL. BHT was used as a positive control with antioxidant activity with IC_{50} value of 0.025 mg/mL (Table 2).

3.9 *GC and GC-MS analysis of CAEO*

Table 3. Chemical composition of *C. aurantifolia* essential oil

S.N.	Components	Retention time	Percent composition
1	Caryophyllene oxide	34.5	2.17
2	D-limonene 1,2-epoxide	21.3	0.24
3	E-citral	24.2	67.97
4	Epoxy-linalool oxide	25.1	4.27
5	Farnesol	44.9	0.45
6	Geranic acid	28.9	4.43
7	Isobutanoic acid	49.0	5.15
8	Limonene-1-hydroperoxide	47.7	1.84
9	Myrcene	12.7	4.32
10	Nerol acetate	27.9	0.81
11	Pinene oxide	20.7	0.82
12	5,9-Dimethyl-4,8-decadiena	49.5	2.05
13	Terpinyl formate	47.5	0.81
14	1-Oxaspiro[4.5]decane, 2,3,6,6-tetramethyl-10-methylene	49.7	1.05
15	6,10-Dimethylundecan-2-one	31.8	0.29
	Total		96.67

GC and GC-MS analysis of the CAEO revealed presence of fifteen major and minor compounds comprising 96.67% of the total oil sample. In particular, *E*-citral (67.97%), Isobutanoic acid (5.15%), Geranic acid (4.43%),

Myrcene (4.32%), Caryophyllene oxide (2.17%), 5,9-Dimethyl-4,8-decadiena (2.05%), and Limonene-1-hydroperoxide (1.84%) were recognised as major components in essential oil. The total components identified along with their retention time is presented in Table 3.

4. Discussion

Typhoid fever remains one of the major public health problems in most resource-poor countries as a result of factors such as poor sanitation and health care infrastructure. The incidence of enteric fever is highly fluctuating in these areas based on public and private hospital records [31]. Secondary metabolites such as essential oils produced by plants can be used to treat several diseases [33-34]. In the present study, we evaluated twenty essential oils against the food-borne bacterium, *S. enterica* and found that, of all the essential oils, CAEO was the most potent. According to Penalver et al. [35], oregano essential oil had a slightly higher MIC value against *Salmonella* ranging from 2.5 to 10 µL/mL for *S. enteritidis* and *S. essen* than that of CAEO evaluated in this study. Among the natural organic compounds tested by Penvalver et al. [34], thymol had the lowest MICs of 1.0 and 1.2 mmol/L against both *S. typhimurium* and *Escherichia coli*, respectively. Friedmann et al. [36] reported bactericidal activity at 50% of oregano, thyme, clove bud, cinnamon, allspice, palmarosa, bayleaf, and marjoram essential oils (with BA_{50} values ranging from 0.045-0.14%) against *S. enterica*. In the present study, an 8 µL/mL MIC value was reported for CAEO against *S. enterica*. These differences in antibacterial activities and MIC values may be the result of differences in essential oil compositions, origin of plant species from which EOs were extracted, and variation in microbial strains used from different sources.

In the developing world, antibiotics are a key part of treating diseases of bacterial origin, which are a major cause of morbidity and mortality. This study contributes toward the availability of information on antibiotic susceptibility among users in rural areas. Madhulika et al. [37] report the emergence of multidrug-resistant (MDR) strains of *S. enterica* (resistant to chloramphenicol, ampicillin, and co-trimoxazole) throughout the world in the last two decades. The results of the antibiotic susceptibility assay among nine commercially used antibiotics were quite interesting in the present study. The results of this study showed Amikacin, Gentamycin, and Tobramycin have 20% to 25% resistance towards the test bacterium, whereas Nalidixic acid, Cefuroxime, and Cefixime showed no resistance towards *S. enterica*. A study by Muthu et al. [38] on *S. enterica* also demonstrated resistance to Nalidixic acid and Gentamycin of 95.5% and 2.5%, respectively. The above studies concluded that most *S. enterica* strains are resistant to Nalidixic acid. In the present investigation also, we found that the test bacterium (*S. enterica*) showed no zone of inhibition towards Nalidixic acid, thus confirming its resistance. However, CAEO exhibited greater toxicity than antibiotics. Earlier research also evidenced that *Citrus* EO has potential antibacterial activity against a wide range of foodborne bacteria both alone and as a synergistic application [39-40].

Fruit juice from *C. aurantifolia* (Family: Rutaceae) contains higher amounts of water and vitamin C. Leaf, fruit, and flower oils contain limonene and linalool and exhibit strong antioxidant properties [41]. Our study showed that its essential oil had good free radical scavenging activity against DPPH. A study by Reddy et al. [40] found CAEO to be able to inhibit maximum 93.3% of radical cations at 1.0 mg/mL, respectively. The high DPPH scavenging activity of this essential oil could be attributed to the high phenolic content of the sample (10.90 mg GAE/100 µL). There was a linear correlation between DPPH radical scavenging activity and total phenolic content of $R^2 = 0.812$ and 0.973 in the present study. Li et al. [42-43] reported similar linear relationships between free radical scavenging activity and total phenolic content. Besides, the total phenolic content and DPPH inhibition reported with CAEO in our study was lesser than that of *Citrus lemon* EO reported by Moosavy et al. [44] with 81.82 mg gallic acid equivalent/g of the EO total phenolic content and 55.05% DPPH inhibition. The variation in phenolic content and DPPH activity might be due to variation in species and origin of plant from different geographical regions.

The GC and GC-MS analysis of CAEO revealed that *E*-citral was the major component, while limonene was found as a minor component. On the contrary, researchers investigated that essential oil of *C. aurantifolia* was rich in monoterpenes and limonene was the most dominant terpene (< 82.84%) [45-46]. However, some studies found lower amounts of limonene in *Citrus* essential oil [46-47]. Similar results were reported in the present investigation. Investigators also reported that the antimicrobial activity of CAEO is attributed to presence of *E*-citral, Geranic acid, Myrcene, and Caryophyllene oxide in essential oil [47]. Thus, the high antibacterial activity of this essential oil found in the present study may be due to presence of these terpenic compounds [49-50].

It has been shown that the antimicrobial compounds of CAEO can inhibit and kill the bacterium at low concentrations. The results of our studies also revealed that this essential oil altered the structure of the cell membrane at minimum inhibitory levels. In Carson et al.'s [28] study, numerous antimicrobial compounds that act on the bacterial cytoplasmic membrane precipitate the loss of 260 nm-absorbing material (nucleic acid) and 280 nm-absorbing material (protein). Besides, the effect of various essential oils on the permeability of Gram-negative bacteria's membranes has been described by Helander et al. [50]. It appears that CAEO exerts its antimicrobial activity by disrupting the permeability barriers of microbial membrane structures. *S. enterica* exhibits maximum cellular leakage after being treated with CAEO. In the same way, *Kaempferia pandurata* essential oil at two MICs caused ghost cells (empty cells) in *E. coli* K1.1 cells, which means the loss of cellular metabolites [51], supporting our results. Furthermore, Figure 3 shows that CAEO affected the morphology of *S. enterica*, indicative of a mechanism of action that would involve rupture or dysfunction of the cell membrane. Guimarães et al. [52] observed similar results with terpineol and eugenol, which suggested that the mechanism of action would be related to rupture or dysfunction of the cell membrane.

5. Conclusions

Among the 20 essential oils evaluated, the majority of oil demonstrated antibacterial activity, with CAEO being the most effective against *S. enterica*. CAEO had a strong antibacterial activity with lower MIC and MBC values for the test bacterium. In addition to promising antimicrobial effects, the essential oil also showed potential antioxidant properties. SEM indicates that loss of surface integrity is the mechanism responsible for cell death in *S. enterica*. Thus, essential oil from *C. aurantifolia* can be used as potential antibacterial agent against foodborne pathogen *S. enterica*. A detailed study of the antibacterial activity of 20 essential oils including CAEO has been conducted in the current study, providing insight into mechanisms of action, synergistic effects with other adjuvants, and bioavailability of the various essential oil components for future research.

Conflict of interest

The authors declare no competing financial interest.

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