The Antimicrobial Potential of *Citrus sinensis* Essential Oil *in vitro* and *in situ*

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Abstract
Essential oils (EOs) are naturally occurring complex secondary metabolites of plants that play a role in the body's defence against pathogens, environmental factors and physiological stresses. They have a range of biological properties such as antibacterial, anticancer, anti-inflammatory and antioxidant properties. The mechanisms of their antibacterial activity have been thoroughly investigated. It is known that EOs can inactivate bacteria by targeting their cytoplasm, cell wall or cell membrane. The aim of our research was to observe the antibacterial activity of red orange (*Citrus sinensis*) EO *in situ* via vapor phase and *in vitro* using the disc diffusion method. In addition to the antimicrobial activity, the antibiotic activity against five plant diseased bacteria was also monitored. The results of our analyses showed that the disk diffusion approach and vapor phase were the most effective antibacterial strategies against *Pectobacterium carotovarum*. Plants have an innate ability to produce a diverse range of compounds, especially secondary metabolites, which, due to their biological properties, have been attributed a protective role against diseases. For integrated crop pest management, biological control is not a new idea and has recently attracted much attention.

Keywords: *in situ, in vitro,* antimicrobial activity, red orange essential oil.

1. Introduction

With recent declines in the effectiveness of medications to treat bacterial illnesses, antibiotic resistance represents a hazard to the entire world [1]. Natural antibiotic resistance has increased as a result of the overuse of antibiotics in both humans and animals [2]. Consequently, this has had detrimental effects on mortality rates, length of hospital stays, and healthcare costs [3]. It is essential to promote the appropriate use of antibiotics and develop novel, potent antibiotics to maintain global health security [4]. A promising solution to this worldwide issue can be found in natural resources, particularly in essential oils (EOs) [5–7].

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There are many different types of cultivated trees worldwide that belong to the Rutaceae family, which includes the *Citrus genus* [8]. Citrus fruits are commonly consumed raw, pureed into drinks, or processed into jams and juices [9]. Their unique flavors and high vitamin content, especially vitamin C, make them well-known [10].
The impacts of different food components on health are increasingly concerning for consumers and the food industry, making food safety a primary concern. The use of natural and organic molecules in food has become increasingly important because they are less expensive, more ecologically friendly, and have fewer negative health consequences than non-organic synthetic compounds. Consequently, for enhancing food quality and safety, naturally occurring antimycotics derived from plants have emerged as perfect substitutes for commercially available synthetic chemical preservatives [11–13].

Due to their broad-spectrum insecticidal, antibacterial, and antifungal qualities, as well as their strong aromas and flavors, citrus essential oils (CEOs) have garnered the most attention among plant essential oils in this regard [14–17]. To ensure food quality and safety, CEOs are widely used in food formulation, packaging, and preservation [18].

Therefore, the focus of this research was on various techniques for extracting, purifying, and detecting EOs from different citrus species, as well as their composition and potential uses in food safety, packaging, and preservation.

The goal of this study was to evaluate the antimicrobial potential of *Citrus sinensis* against five plant bacteria using the disc diffusion method, minimal inhibition concentration, and vapor phase on a carrot model.

2. Materials and methods

2.1 Essential oil

The essential oil (EO) utilized in this study was produced by cold-pressing fresh pericarp of *Citrus sinensis* red oranges from Hanus s.r.o. in Nitra, Slovakia (CSEO). The EO contains the main ingredients: limonene (95.5%), myrcene (1.9%), and aldehyde C-10 (0.2%). The fruits originated from Italy, and the EO was carefully preserved at 4°C in the dark for future research.

2.2. Microorganisms tested

To assess the antibacterial activity of the investigated CSEO, a variety of bacterial strains were utilized. These included Gram-positive (G^+) bacteria such as *Bacillus subtilis* CCM 2217, *Priestia* (*Bacillus*) *megaterium* CCM 2007, and Gram-negative (G^-) bacteria including *Xanthomonas arboricola* CCM 1441, *Pectobacterium carotovarum* CCM 1008, and *Pseudomonas putida* CCM 7156. All bacterial strains were obtained from the Czech Collection of Microorganisms, based in Brno, Czech Republic. The bacterial inoculum was cultured for 24 hours at 37°C in Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK) prior to investigation. The optical density of the bacterial and yeast inoculum was adjusted to 0.5 McFarland on the day of the experiment.

2.3 Disc diffusion method

The disk diffusion susceptibility experiment utilized the microbial strains previously reported. Mueller Hinton Agar (MHA; Merck, Germany) was used to inoculate Mueller Hinton Broth (MHB) with the generated bacterial strains (0.1 mL each). Six-millimeter sterile discs were moistened with 10 µL of the analyzed CSEO and placed on the agar medium. The bacterial cultures were then incubated at 37 °C for twenty-four hours. The inhibitory activity was assessed, and the results were reported in millimeters following the 24-hour incubation period. Gentamicin and chloramphenicol (30 µg/disc, Oxoid, Basingstoke, UK) served as two well-known antibiotics (ATB) and positive controls for bacteria, respectively. The experiment was conducted in duplicate for each iteration.

2.4 Antimicrobial activity in vapor phase

In situ testing was conducted to evaluate the antimicrobial efficacy of CSEO against a variety of bacterial species, including both Gram-positive G^+ and G^- bacteria. Carrots were chosen as a popular vegetable substrate to promote bacterial growth. The experimental approach used in the evaluation aligns with the techniques outlined by Kačániová et al. [19]. After drying and slicing the carrot into 0.5 mm pieces, it was washed with distilled water. Subsequently, 60 mm Petri plates containing prepared substrates supported by agar were inoculated with bacteria. The CSEO sample under examination was dissolved in ethyl acetate at concentrations of 500, 250, 125, and 62.5 mg/L before being applied to sterile filter paper. Filter sheets exposed only to ethyl acetate were used as a control.

The prepared Petri dishes were incubated for seven days at 37°C. The assessment of in situ bacterial growth was conducted following standard operating procedures. The volume density of bacterial colonies (vv) was calculated using the ImageJ tool from the National Institutes of Health, Bethesda, Maryland, USA. The volume density of bacterial colonies was determined using the following formula: vv (%) = P/p, where p indicates the points that are inside the reference space (the growth substrate that was employed), and P indicates the stereological grid points that hit the colonies.

Following the EOs vapor phase treatment, the percentage (%) of bacterial growth inhibition (BGI) was expressed as follows: BGI = (C-T)/C x 100, where T stands for the treatment group and C for the control group. Both groupings indicate bacterial growth expressed as v/v. results attained since negative values signify growth stimulation.

2.5 Statistical analyses

At a significance level of $p < 0.05$, the One-way Analysis of Variance (ANOVA) test was used to evaluate statistically significant variances, followed by the Tukey's Significant Difference (HSD) test. Astatsa Anova One Way, an internet tool, was used for this investigation.

3. Results and discussion

The inhibitory zones in the study ranged in length from 3.33 to 9.33 mm (Table 1). The largest inhibitory zone (7.33 mm) against *P. megaterium* was found in G^+ bacteria, with *B. subtilis* following closely behind (3.33 mm). Conversely, when it came to *P. carotovorum* (9.33 mm), CSEO was most effective against G^- bacteria.

Our findings support those of Burt et al. [20], who demonstrated that G^- bacteria typically exhibit greater resistance to citrus EOs than G^+. Burt et al. [20] state that the structural complexity of G^- bacteria, in contrast to G^+ bacteria, partly accounts for their resistance to EOs. Similarly, Djenane [21] found lemon essential oil to have the strongest effect when testing EOs of orange, bergamot, and lemon against *S. aureus*. Cuca et al. [22] previously reported on the activity of citrus EO against pathogenic bacteria, finding EO from the peel of Bingtang sweet oranges (*Citrus sinensis* Osbeck), high in limonene, to be effective in inhibiting *E. coli* ATCC 25922. Fisher and Phillips [17] also demonstrated that citrus essential oil from sweet oranges (*Citrus sinensis*) had potent antibacterial activity against B. cereus, *S. aureus*, and *E. coli* O157. Fernández-López et al. [23] found orange EO to be ineffective against *L. mesenteroides*, corroborating their findings. Similar outcomes were noted by Ambrosio et al. [24] when using orange EO against *L. plantarum.*

The most sensitive bacteria to gentamicin was *P. carotovorum* (32.33 mm), while the most sensitive bacteria to chloramphenicol was *X. arboricola* (30.67 mm).

Worldwide crop losses due to plant pathogenic bacteria (PPB) are predicted to be as high as five billion euros [25,26] or one billion dollars [27,28] with PPBs most common in vineyards, pear, and apple orchards.

Table 1. Disc diffusion method antimicrobial activity of *Citrus sinensis* in mm

Microorganism	Inhibition zone	Gentamycin	Chloramphenicol
Gram positive bacteria			
Bacillus subtilis	3.33±0.58^a	30.33±0.58^a	29.67±0.58^a
Priestia megaterium	7.33±0.58^b	26.67±0.58^b	27.67±0.58^b
Gram negative bacteria			
Xanthomonas arboricola	3.67±0.58^a	30.33±0.58^a	30.67±0.58^a
Pectobacterium carotovorum	9.33±0.58^c	32.33±0.58^c	30.33±0.58^a
Pseudomonas putida	7.33±0.58^b	29.67±0.58^a	29.67±0.58^a

Data are the mean (± SD) of 3 samples. Different letters in each column refer to significant differences (Tukey, $p \leq 0.05$).

Table 2. *In situ* analysis of the antimicrobial activity (in %) of *Citrus sinensis* in the vapor phase on carrot

Food model	Microorganisms	Concentration of EO in µg/L			
		62.5	125	250	500
Carrot					
	Bacillus subtilis	54.15±2.61[a]	45.22±1.83[a]	36.08±1.65[a]	22.71±0.85[a]
	Pectobacterium carotovorum	94.63±1.12[b]	86.00±3.31[b]	77.14±2.30[b]	25.51±2.16[a]
	Priestia megaterium	5.00±1.70[c]	75.98±2.69[c]	15.00±2.29[c]	46.08±2.25[b]
	Pseudomonas putida	6.44±2.25[c]	13.74±0.90[d]	25.80±2.76[d]	33.28±1.05[c]
	Xanthomonas arboricola	32.51±0.47[d]	25.29±2.30[e]	14.86±1.70[c]	7.76±0.94[d]

Data are the mean (± SD) of 3 samples. Different letters in each column refer to significant differences (Tukey, $p \leq 0.05$).

Commercial orange oils produced through cold-pressing (EOP) and cold-pressing followed by a hot distillation system (EOPD) demonstrated moderate to high levels of antibacterial activity against *S. aureus* but not *P. aeruginosa*. Our findings indicate that *Citrus sinensis* essential oils exhibited greater antibacterial activity against G^- bacteria responsible for food deterioration and pathogenicity compared to G^+ bacteria [29]. It is widely recognized that phenolic and terpenoid chemicals possess various biological properties, including antimicrobial and antibacterial activities. Specifically, our sample's pure primary components, limonene and myrcene, have been shown to be effective antibacterial agents against G^+ pathogens [30,31].

Food packaging is intended to shield food from various environmental elements, such as light, temperature, humidity, and microbes, as well as shocks, vibrations, and dust [32,33]. This helps to prolong the shelf life and quality of food [34]. Maintaining the meals' original organoleptic qualities is one of the most important components of food packing. To increase the shelf life of food, active packaging has been developed in this area. In order to improve the organoleptic qualities and shelf life of the food as well as to guarantee food safety, active packaging may include components that are meant to be released into the food [35–38].

The G^+ and G^- bacteria that proliferate on carrots were used to assess the efficacy of CSEO (Table 2). Through analysis of the inhibitory effects on G^+ bacterial strains in the carrot model, it was observed that CSEO was most effective against *P. megaterium* at 125 µg/mL (75.98%), whereas *B. subtilis* showed the highest levels of suppression at 62.5 µg/mL (54.15%). Interestingly, the vapor phase of CSEO had the most effectiveness against G^- bacteria at the lower dosage (62.5 µg/L), with reported inhibitory effects of 94.63% against *P. carotovorum* in the carrot model.

Greater quantities of EOs are typically required for food applications, even if *in vitro* investigations have yielded some useful information on EO interactions. The differences in fruit and vegetable surface tissues, the hydrophobicity of plant surfaces, the internalization of pathogens within cut plant tissue, the capacity of bacteria to form protective biofilms, interactions with suspended organic matter, fat, and protein, the availability of nutrients, and water content are just a few of the many variables that can impact how EOs interact with food [39–41]. These justifications call for the necessity of *in situ* validation studies on fresh-cut vegetables.

4. Conclusions

This study suggests that *Citrus sinensis* fresh pericarp can be extracted with EO and used as a natural antibacterial agent in the food industry. It was also interesting to assess the antibacterial efficiency of *Citrus sinensis* against relevant illnesses. To maximize the potential of *Citrus sinensis* EO as a natural alternative to artificial preservatives, more research using a variety of food types and storage conditions is recommended.

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