In vitro and *in situ* Antibacterial Potential of *Citrus aurantifolia*

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Abstract

Research on plant sources and screening of plant materials for new compounds has fostered increased interest in replacing synthetic antibacterial agents with natural ones. Spices and their essential oils have had varying degrees of antibacterial activity since antiquity. The need to find new antimicrobials has arisen from the emergence of bacterial resistance to currently marketed antimicrobial compounds. Thus, the aim of this study was to characterize the antibacterial properties of *Citrus aurantifolia* essential oil against these plant pathogenic bacteria: *Bacillus subtilis* CCM 2217, *Pseudomonas putida* CCM 7156, *Xanthomonas arboricola* CCM 1441, *Pectobacterium carotovorum* CCM 1008 and *Priestia* (*Bacillus*) *megaterium* CCM 2007. In this experiment, we measured antibacterial activity using two different methods. The antibacterial activity of the investigated bacterial strains was compared and their antibiotic resistance was evaluated using the disc diffusion method under *in vitro* conditions. The species strategy used on the surface of carrots was the antimicrobial activity under *in situ* conditions. The essential oil of *C. aurantifolia* was found to have the strongest antibacterial effect against *B. subtilis in vitro*. In addition, *in situ* monitoring of antibacterial activity was carried out and a concentration of 6.25-500 µg/L gave the best results against *X. arboricola*.

Keywords: *Citrus aurantifolia*, *in situ*, disk diffusion, antimicrobial activity

1. Introduction

Citrus is a fruit that is often consumed due to its many health benefits [1]. *Citrus aurantifolia* is one of the citrus species that is utilized extensively. The family Rutaceae, which has 900 species and 150 genera, includes the *species C. aurantifolia* [2]. Cultivation of this plant is widespread worldwide [3]. The small herb *C. aurantifolia* has a unique scent. The fruits have a tapering end and a slightly rounded shape. The fruits also have a distinctive aroma and a very sour, juicy taste. The *C. aurantifolia* plant is widely used as a culinary flavouring, a flavour enhancer in beverages, a cosmetic raw material and a traditional medicinal ingredient [4].

Numerous biological activity are present in *C. aurantifolia* [5]. According to several studies, *C. aurantifolia* has the following biological activities: antiseptic, antiviral, antifungal, astringent, anticholesterol, diuretic, appetite stimulant, anticonstipating, anti-inflammatory and analgesic [6], antioxidant, anticancer, and antimicrobial [7], and more. These different biological functions are caused by secondary metabolites that *C. aurantifolia* contains. Alkaloids, coumarins, flavonoids [8], carotenoids, phenolics, terpenes, limonoids [9]. and essential oils [10] are some of the secondary metabolites identified in *C. aurantifolia*. The concentration of these secondary metabolites can be influenced by many variables, including physicochemical properties, soil composition, solar radiation, geographical

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coordinates, and the part of the plant being exploited [11].
Bacterial growth is now a major cause of food quality deterioration and shelf-life reduction [12,13]. Therefore, it is necessary to use chemical additives as preservatives to prevent lipid oxidation and microorganisms that cause food decomposition. However, consumers are becoming increasingly aware of the shortcomings of artificial antimicrobials and their relationship to risks to human health when used to extend the shelf life of food [14]. The current trend is therefore to use naturally occurring antimicrobial chemicals to extend the shelf life of foods [15]. These properties include antibacterial properties that can be applied to food products derived from citrus, including peel, seeds, pulp and essential oils (EOs). Since EOs are known to contain significant concentrations of biophysical substances that protect against microorganisms, they can also be added to foods to improve quality and extend shelf-life [14].
The aim of our research was to assess the antibacterial properties of *Citrus aurantifolia* against a range of plant pathogens in both an *in vitro* study and an *in situ* study in a carrot model.

2. Materials and methods

2.1 Essential oil

This study's essential oil (EO) came from fresh, cold-pressed *Citrus aurantifolia* (CAEO) pericarp, which was purchased from Hanus s.r.o. in Nitra, Slovakia. *C. aurantifolia* pericarps were sourced from Italy and the EO was carefully stored at 4 °C in the dark for future research. The main components were citral, D-limonene, α pinene, fenchone.

2.2. Microorganisms tested

Different bacterial strains were used to assess the antibacterial activity of the investigated CAEO. Gram-positive (G^+) bacteria were among these strains; they were *Bacillus subtilis* CCM 2217, *Priestia (Bacillus) megaterium* CCM 2007, and Gram-negative (G^-) bacteria *Xanthomonas arboricola* CCM 1441, *Pectobacterium carotovorum* CCM 1008, and *Pseudomonas putida* CCM 7156. All bacterial strains were obtained from the Czech Collection of Microorganisms, located in Brno, Czech Republic. The bacterial inoculum was grown for 24 hours at 37 °C in Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK) before investigation. The optical density of the bacterial inoculum was calibrated to 0.5 McFarland on the day of the experiment.

2.3 Disc diffusion method

The above microbial strains were used in the disk diffusion susceptibility experiment. Using Mueller Hinton agar (MHA; Merck, Germany), bacterial strains were inoculated into Mueller Hinton broth (MHB; 0.1 mL). After moistening with 10 µL of the studied CAEO, six-millimetre sterile discs were placed on the agar medium. The bacterial cultures were incubated at 37 °C for twenty-four hours. After the 24-hour incubation period, the inhibitory activity was quantified and the results were expressed in millimeters. The two antibiotic treatments (ATBs) were gentamicin and chloramphenicol (30 µg/disc, Oxoid, Basingstoke, UK), which also served as positive controls for the bacteria. Each measurement experiment was performed three times.

2.4 Antimicrobial activity in vapor phase

The antibacterial efficacy of CAEO was assessed *in situ* against a variety of bacterial species, encompassing both G^+ and G^- bacteria. One model vegetable that was utilized as a substrate to promote bacterial growth was carrots. The experimental approach used in the evaluation is in line with the techniques given by Kačániová et al. [16]. After drying and slicing the carrot into 0.5 mm pieces, it was washed with distilled water. Then, 60 mm Petri plates containing prepared substrates supported by agar were filled with bacteria. After dissolving the tested CAEO sample in ethyl acetate at concentrations of 500, 250, 125, and 62.5 µg/L, it was placed on sterile filter paper. As a control, filter sheets that were only exposed to ethyl acetate were employed.
The prepared Petri dishes were to be incubated for seven days at 37 °C. The assessment of the *in situ* bacterial growth was conducted using standard operating procedures. The ImageJ tool from the National Institutes of Health, Bethesda, Maryland, USA, was used to calculate the volume density of

bacterial colonies (vv). The volume density of bacterial colonies was calculated using the formula below: vv (%) = P/p
where P represents the stereological grid points that hit the colonies and p stands for the points that are inside the growth substrate's reference space.
The following is an expression of the percentage (%) of bacterial growth inhibition (BGI) brought about by the EO vapor phase treatment:
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

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

3. Results and discussion

Research into the antibacterial properties of lime juice, both alone and in combination with other herbs, has shown that it has strong antibacterial properties [17,18].
The antibacterial activity of CAEO ranged from 4.33 to 9.33 mm (Table 1). The best antimicrobial activity of CAEO was found against *B. subtilis* from the group of G^+ bacteria and against *P. carotovorum* from group of G^- bacteria.
The antibacterial efficacy of CAEO against *Salmonella* spp., *Bacillus* spp., *Staphylococcus aureus*, and *Escherichia coli* was assessed by Onyeagba et al. [18]. The antibacterial activity of CAEO against *S. aureus, S. epidermidis, E. coli*, and *K. pneumoniae* was examined by Julaeha et al. [19]. In the study of Al-Aamriet al. [20] were determined inhibitory zones for *S. aureus* and *E. coli*. There was stronger efficacy on G^+ bacteria, which is consistent with our findings. The CAEO inhibitory zones for *S. aureus, B. cereus, S. typhi*, and *P. aeruginosa* were identified by Chi et al. [21]. Eight harmful bacteria (*S. epidermidis, P. aeruginosa, S. aureus, M. luteus, E. coli, S. typhimurium, L. monocytogenes*, and *E. faecium*) were tested by Ben Bnina et al. [22] to determine the antibacterial activity of CAEO.
The most sensitive bacteria against antibiotic Gentamycin was *P. carotovorum* and against chloramphenicol was *X. arboricola*.

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

Microorganism	Inhibition zone	Gentamycin	Chloramphenicol
Gram positive bacteria			
Bacillus subtilis	9.33±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	4.33±0.58^c	30.33±0.58^a	30.67±0.58^a
Pectobacterium carotovorum	4.67±0.58^c	32.33±0.58^c	30.33±0.58^a
Pseudomonas putida	4.33±0.58^c	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 aurantifolia* in the vapor phase on carrot

Food model	Microorganisms	Concentration of EO in µg/L			
		62.5	125	250	500
Carrot					
	Gram-positive				
	Bacillus subtilis	44.58±1.21[a]	33.10±1.36[a]	54.98±2.81[a]	64.00±3.22[a]
	Priestia megaterium	65.22±2.78[b]	66.47±2.27[c]	24.99±1.69[c]	14.71±2.04[c]
	Gram-negative				
	Pectobacterium carotovorum	64.43±3.07[b]	53.94±2.59[b]	15.35±2.76[b]	7.06±2.11[b]
	Pseudomonas putida	7.37±1.49[c]	23.14±1.31[d]	35.27±0.85[d]	66.85±1.77[a]
	Xanthomonas arboricola	96.40±2.29[d]	89.26±0.62[e]	78.57±1.43[e]	7.77±0.94[b]

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

In view of its interesting antibacterial properties, another aim of this work was to investigate the antibacterial effects of the tested CAEO in the vapour phase. The efficacy of CAEO was evaluated with respect to G^- and G^+ bacteria that proliferate on carrots (Table 2).

Examining the inhibitory effects on G^+ bacterial strains in the carrot model, it was discovered that CAEO was most effective against *P. megaterium* at concentrations of 125 µg/L (66.47 %), while *B. subtilis* showed the highest levels of suppression at concentrations of 500 µg/L (64.00 %). Significantly, the vapor phase of CAEO demonstrated the highest effectiveness against G^- bacteria at the lower dosage (62.5 µg/L), with reported inhibitory effects of 96.40 % against *X. arboricola* in the carrot model.

Various pathogenic bacteria, including *Staphylococcus aureus, Escherichia coli* O157:H7, *Bacillus cereus, Campylobacter jejuni*, and *Listeria monocytogenes*, have been demonstrated to be susceptible to the antimicrobial effects of EOs derived from bergamot (*Citrus bergamia*), lemon (*Citrus lemon*), and sweet orange (*Citrus sinensis*). When compared to their susceptibility *in vitro*, all of the evaluated bacteria in the food system (cabbage leaf and chicken skin) were less vulnerable [23]. Kasaai and Moosavi [24] carried out a second study to look into citrus wastes, including mandarin peel and leaf, and how to treat food-grade kraft paper with them.

The result showed that the air permeability, peroxide value (PV) and water vapour permeability (WVP) of the modified films were lower than that of the original materials. They concluded that there might be a way to modify the paper used to wrap and package food using citrus fruit waste to prevent and prolong food spoilage caused by air and moisture. Simas et al. [25] examined the protective properties of citrus essential oils against fruit fungal infections. The extracts from *C. limon* and *C. limonia* presented 312 µg/mL, while the extracts from *C. latifolia* and *C. aurantifolia* showed 625 µg/mL. The results demonstrated that the minimal inhibitory concentration was effective against *B. cinerea*. Tyagi et al. [26] looked into the effectiveness of lemon grass oil against a number of yeasts that cause food to deteriorate. In mixed fruit juices, both lemon grass oil by itself and a combination of all oil and heat treatment were used. The outcome shown that combining lemon grass oil with heat produced a higher-quality preservative than either method alone.

4. Conclusions

The packaging industry is interested in a unique form of shelf-life treatment that controls microbial contamination of food surfaces to improve the microbiological safety of products and extend their shelf life. This treatment mixes food packaging materials with antimicrobial compounds. In line with the current movement to recover natural and renewable resources, natural antibacterial compounds are increasingly used, especially in the food and healthcare industries. The most popular method of application is the direct addition of natural compounds to foods, but many attempts have been made to develop other ways to minimize undesirable inactivation. Food products are currently treated with active solutions

prior to packaging by dipping, spraying and coating, which are viable methods. Further research using different food models and storage settings is recommended to maximize the use of EO from *Citrus aurantifolia* as a natural replacement for artificial preservatives.

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