The Antimicrobial Activity of *Citrus aurantium* Amara

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Abstract

Citrus aurantium L. is an evergreen tree that can reach a height of up to five metres. It is also referred to as bigarade, bitter orange, sour orange, and Seville orange. A number of studies have been carried out on the bioactivity of substances extracted from *C. aurantium*. Plant disease-causing bacteria also have a significant financial impact. Plant pathogenic bacteria can occur both inside plants as pathogens and outside plants as saprophytes in a variety of environments. The main objective of this paper is to assess the antibacterial efficacy of *C. aurantium* essential oil against five plant bacterial pathogens both *in vitro* and *in situ*. *C. aurantium* essential oil (EO) has the potential to be used in various food applications due to its natural antibacterial properties. Using the disc diffusion method, we found the best antimicrobial potential against *Pectobacterium carotovorum*. Using vapor phase antimicrobial activity on carrot model, the best microbial activity was found against *Xanthomonas arboricola* bacteria. The results of the antibacterial test showed that EO exhibited antibacterial effect against each bacteria tested.

Keywords: bitter orange essential oil, plant bacteria pathogens, antibacterial activity.

1. Introduction

Vegetables and fruits provide ecological niches for a dynamic and diversified microbiota. Fresh food usually contains a complex mixture of yeasts, fungi, and bacteria unique to each fruit or vegetable, with varied types and populations [1]. Soil organisms dominate the microbiota on vegetables. Through agricultural techniques, pathogens can contaminate raw vegetables and persist through processing and distribution [2,3]. The organisms that eventually dominate the population on traded fresh produce will depend on several factors, including the composition of the initial population, post-harvest conditioning procedures, distribution time, distribution temperature, and packaging atmosphere [1].

To keep fresh produce fresher for longer, it is essential to control the growth of microbial populations. Several post-harvest procedures, such as washing and removing damaged tissues, are used to reduce initial high counts. Since storage life is shortened with large initial microbial loads, it is well established that maintaining clean sanitation is crucial for minimizing microbial populations [4]. However, the overwhelming body of research suggests that preserving local microbes is important because they inhibit the growth of infectious diseases [5].

Foods are shielded by natural products from bacteria and pathogens that cause food spoilage [6], rancidity, discoloration, and auto-oxidation-related degradation [7]. Plant oils and extracts contain many components with potent antibacterial activity against infections resistant to multiple drugs as well as biofilms [6,8]. Compared to antibiotics, which are frequently composed of only one molecular entity, it is more difficult for bacteria to become resistant to multi-component essential oils (EOs) [9–11].

Citrus aurantium L. (*C. aurantium*), commonly referred to as sour orange or bitter orange, is a member of the Rutaceae family and the order Geraniales [12]. In Cyprus, sour orange trees are

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commonly planted around fruit gardens, providing wind protection for the trees and benefiting from their blossoms. Citrus species are believed to have bioactivities including anti-inflammatory [6], anti-carcinogenic [13], and antioxidant properties. They may also play a role in preventing cancer and degenerative diseases [14]. Citrus species exhibit bioactivities due to bioactive substances such as vitamins, flavonoids, phenolics, and essential oils [15].

The purpose of this study was to evaluate the antibacterial activity of *Citrus aurantium* Amara against selected plant pathogens using the disc diffusion method, minimal inhibition concentration *in vitro*, and vapor phase on a carrot model.

2. Materials and methods

2.1 Essential oil

The fresh pericarp of bitter oranges, *Citrus aurantium* Amara (CAEO), that were purchased from Hanus s. r. o. in Nitra, Slovakia, was cold-pressed to produce the essential oil (EO). The *C. aurantia* pericarp was supplied by Italy, and the EO was stored for future use at 4 °C in the dark. The main constituents were limonene (92.9%), myrcene (1.8%), and beta pinene (0.8%).

2.2. Microorganisms tested

Selected bacterial strains were used to assess the antibacterial activity of the investigated *C. aurantium* EO. Among the Gram-positive bacteria (G^+) group's strains were *Bacillus subtilis* CCM 2217 and *Priestia (Bacillus) megaterium* CCM 2007. *Xanthomonas arboricola* CCM 1441, *Pectobacterium carotovorum* CCM 1008, and *Pseudomonas putida* CCM 7156 were among the Gram-negative (G^-) bacteria. All of the bacterial strains were donated by the Brno, Czech Republic-based Czech Collection of Microorganisms. The bacterial inoculum was grown for a full day at 37 °C in Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK) before examination. The bacterial inoculum's optical density was calibrated to 0.5 McFarland on the day of the experiment.

2.3 Disc diffusion method

The bacterial strains listed above were used in the disk diffusion susceptibility experiment. Mueller Hinton broth (MHB; Merck, Germany) was infected with bacterial strains, and a disc diffusion test was conducted using Mueller Hinton agar (MHA; Merck, Germany; 0.1 mL). After being moistened with 10 µL of the studied CAEO, six-millimeter sterile discs were put 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 tested, 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 microbes. Every measurement experiment was run three times.

2.4 Antimicrobial activity in vapor phase

A variety of bacterial species, including both G^+ and G^- bacteria, were tested *in situ* to see how efficient CAEO was as an antibacterial agent. One kind of model vegetable that was utilized as a substrate to promote bacterial growth was carrots. The experimental approach used in the evaluation follows the guidelines provided by Kačániová et al. [16]. After being dried, the carrot was sliced into 0.5 mm pieces and washed with distilled water. After that, bacteria were put to 60 mm Petri plates with agar-supported substrates that had been produced. 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. Only ethylacetate-exposed filter sheets were utilized as a control.

For seven days, the prefabricated Petri dishes were to be incubated at 37 °C. The assessment of bacterial proliferation *in situ* was done using standard operating procedures. The ImageJ tool was made available by the National Institutes of Health, located in Bethesda, Maryland, USA, in order 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 indicates the places inside the growth substrate's reference space and P indicates the stereological grid points that cross the colonies. The percentage (%) of bacterial growth inhibition (BGI) brought on by the EO

vapor phase therapy is shown here: BGI = (C-T)/C x 100, where the treatment and control groups are denoted by the letters T and C, respectively. The two groups show the bacterial growth as a function of the obtained v/v values; negative values indicate that growth is encouraged.

2.5 Statistical analyses

One-way analysis of variance (ANOVA) was used to evaluate statistically significant variances, and Tukey's significant difference (HSD) test was run at a significance level of $p < 0.05$. Astatsa Anova One Way is an internet tool that was used for this investigation.

3. Results and discussion

Research conducted both *in situ* and *in vitro* indicates that EOs have strong antibacterial action against a variety of bacterial infections. Because of their antioxidant and antibacterial qualities, EOs have been the subject of numerous reviews that have assessed research on their potential to function as natural preservatives in food products [17,18]. It has been determined that all plant-derived EOs, not just those obtained from fruit peels, have the potential to be used as food preservatives.

However, because high dosages are needed to provide good antimicrobial activity and because the quantity, source, and active component profile of the EO to be used in food have not been optimized, their usage in food items has been limited on an industrial scale. When the volatile molecules in EOs interact with other food ingredients, such proteins, they may also create unwanted chemical products. The assessment of these factors is crucial in order to legitimize the usage of EOs in industry [19].

The antibacterial activity of CAEO ranged from 3.33 to 7.67 mm (Table 1). The strongest antibacterial effect of CAEO was found to target the G^- bacteria *P. carotovorum* and the G^+ bacteria *B. subtilis*. *P. carotovorum* and *X. arboricola* were the bacteria most susceptible to the antibiotics gentamycin and chloramphenicol, respectively.

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

Microorganism	Inhibition zone	Gentamycin	Chloramphenicol
Gram positive bacteria			
Bacillus subtilis	7.33±0.58^a	30.33±0.58^a	29.67±0.58^a
Priestia megaterium	3.67±0.58^b	26.67±0.58^b	27.67±0.58^b
Gram negative bacteria			
Xanthomonas arboricola	4.33±0.58^b	30.33±0.58^a	30.67±0.58^a
Pectobacterium carotovorum	7.67±0.58^a	32.33±0.58^c	30.33±0.58^a
Pseudomonas putida	3.33±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, $p \leq 0.05$).

Citrus peel extracts (EOs) from orange, lime, mandarin, and grapefruit peels have been shown to have moderate to high antibacterial activity against *S. aureus, B. subtilis, E. faecalis, E. coli, Neisseria gonorrhoeae*, and *P. aeruginosa*, according to Abd-Elwahab et al. [20]. Lime peel EO outperformed the other citrus EOs in terms of its ability to suppress all six strains of harmful bacteria. Citral, limonene, and linalool in other citrus peel EO [20] and coumarine and tetrazene in lemon peel [21] may have contributed to their antibacterial activity against these microorganisms. Conversely, Shabnam et al. [22] found that the citrus peel EOs from mandarin, tangerine, sweet orange, lime, and grapefruit were the most antimicrobially active. The mandarin peel EO treatments with 10 µL and 5 µL varied in the inhibitory zone for *Salmonella enterica* serovar Typhi, *E. coli, Streptococcus* spp., and *P. fluorescens*, respectively, from 20 to 30 mm. These inconsistent results could be explained by the different citrus peel EO concentrations used in the research [19].

Given its remarkable antibacterial capabilities, an additional aim of this inquiry was to analyze the tested CAEO's antibacterial effects in the vapour

phase. This was in contrast to the antibacterial ability of a variety of natural chemicals, such as EOs. The efficacy of CAEO was evaluated in relation to the G^- and G^+ bacteria (Table 2).

It was discovered that *B. subtilis* exhibited the highest levels of suppression at concentrations of 500 µg/L (75.63%) and that CAEO was most effective against *P. megaterium* at concentrations of 62.5 µg/L (55.44 %) after assessing the inhibitory effects on G^+ bacterial strains in the carrot model. Notably, the vapor phase of CAEO had the highest efficacy against G^- bacteria at a higher dosage (500 µg/L), with reported inhibitory effects of 94.26% against *X. arboricola* in the carrot model.

Table 2. *In situ* analysis of the antimicrobial activity (in %) of *Citrus aurantium* Amara 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	16.00±2.15^a	24.70±1.81^a	65.22±2.39^a	75.63±2.78^a
	Priestia megaterium	55.44±1.60^c	42.63±0.95^c	32.27±1.99^c	23.26±1.64^c
	Gram-negative				
	Pectobacterium carotovorum	93.59±1.11^b	86.26±3.18^b	75.66±2.93^b	34.66±2.16^b
	Pseudomonas putida	3.46±1.03^d	12.82±1.70^d	24.07±2.58^d	33.03±1.45^b
	Xanthomonas arboricola	44.37±2.12^e	33.00±1.47^e	85.19±2.13^e	94.26±2.22^d

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

Chilling, freezing storage, drying, salting, smoking, and fermenting are examples of traditional food preservation techniques [23]. However, because there is a growing need for meals with less salt, consumers are questioning methods like brining, salting, and fermenting [24]. Thus, the possibilities for replacing chemical preservatives with natural substances have garnered more attention lately. Because they can be used in food matrices or food products as a natural bio-preservative and inhibitor, EOs are garnering a lot of attention in this area [25].

As of right now, research has mostly concentrated on essential oils derived from plants and spices. Research on fruit peel essential oils is scarce. Thus, the conversation is expanded to include all EOs originating from plants and their applications in food. The use of rosemary extract in meat [26], the synergistic effect of EO in seafood preservation [27], the use of EO in active packaging [28], and as a food preservative [29] have all been covered in other recent studies.

4. Conclusions

This investigation explores the potential use of EOs on fresh vegetables as a substitute for artificial chemicals used to preserve carrot slices. Although *Citrus aurantium* Amara EO appeared to show promising activity as a preservative in carrots in the *in vitro* study reported in this work, more investigation is required to determine whether using them as natural sanitizing agents in the post-harvest processing of vegetables is technically feasible. Consequently, this plant offers a unique natural source for the extraction of antimicrobial compounds for use in functional foods and pharmaceuticals. To clarify their possible utility, more research on their toxicity, *in vivo* potency, and mode of action is needed.

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(*in vitro, in vivo* and *in situ*) of plant volatile mixtures, their main components and inclusion systems.

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