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The Potential Use of *Citrus aurantifolia* L. Essential Oils for Decay Control, Quality Preservation of Agricultural Products, and Anti-Insect Activity

Lucia Galovičová [1,*], Petra Borotová [2], Nenad L. Vukovic [3], Milena Vukic [3], Simona Kunová [4], Pawel Hanus [5], Przemysław Łukasz Kowalczewski [6], Ladislav Bakay [7] and Miroslava Kačániová [1,8,*]

1 Institute of Horticulture, Faculty of Horticulture and Landscape Engineering, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976 Nitra, Slovakia
2 AgroBioTech Research Centre, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976 Nitra, Slovakia; petra.borotova@uniag.sk
3 Department of Chemistry, Faculty of Science, University of Kragujevac, 34000 Kragujevac, Serbia; nvchem@yahoo.com (N.L.V.); milena.vukic@pmf.kg.ac.rs (M.V.)
4 Institute of Food Sciences, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976 Nitra, Slovakia; simona.kunova@uniag.sk
5 Department of Food Technology and Human Nutrition, Institute of Food and Nutrition Technology, University of Rzeszow, 35-959 Rzeszow, Poland; phanus@ru.edu.pl
6 Department of Food Technology of Plant Origin, Poznań University of Life Sciences, 31 Wojska Polskiego St., 60-624 Poznań, Poland; przemyslaw.kowalczewski@up.poznan.pl
7 Department of Planting Design and Maintenance, Slovak University of Agriculture, Tr. A. Hlinku 2, 94976 Nitra, Slovakia; ladislav.bakay@uniag.sk
8 Department of Bioenergy, Food Technology and Microbiology, Institute of Food Technology and Nutrition, University of Rzeszow, 4 Zelwerowicza St., 35-601 Rzeszow, Poland
* Correspondence: l.galovicova95@gmail.com (L.G.); miroslava.kacaniova@gmail.com (M.K.)

Abstract: The primary objective of the study was to evaluate the biological activity of *Citrus aurantifolia* essential oil (CAEO) with emphasis on antioxidant, antimicrobial, and insecticidal activity, chemical composition, and the antimicrobial effect of its vapor phase *in situ* on various food models. We determined the main volatile components of CAEO as α-phellandrene (48.5%) and *p*-cymene (16.5%). The antioxidant activity was high and reached 74.5 ± 0.5%, which corresponds to 442 ± 2.3 TEAC. The antimicrobial activity in the contact phase was most pronounced against Gram-negative bacteria, with inhibition zones of 12.66–15.33 mm and a minimal inhibition concentration of 2.36–8.26 µL/mL. The antimicrobial activity of the CAEO vapor phase was high at the highest concentration tested (500 µL/mL), but the inhibitory effect was seen at all concentrations tested. The effect was observed on all types of microorganisms and all types of model foods. Based on the findings, CAEO could find use in storing and extending the shelf life of agricultural products. Insecticidal activity reached 10–90% depending on the concentration used. The significant insecticidal effect provides the possibility of using CAEO as a natural insecticidal, larvicidal, or repellent preparation.

Keywords: DPPH; vapor phase; antimicrobial; *in situ*; *Citrus aurantifolia*

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1. Introduction

Plant parts or essential oils obtained from plants have a large number of positive applications. They are used in everyday food preparation as flavorings in the food industry, and are often involved in the production of drugs by the pharmaceutical industry [1]. Essential oils are liquid, lipophilic, volatile extracts obtained from plants by distillation [2]. Essential oils are defined by ISO 4720:2009 and ISO 9235:2013 as the product obtained by steam distillation of plant parts, pressing of fruit and citrus fruits, or dry distillation after separation of the aqueous phase by physical processes [3,4].

Citrus aurantifolia (family *Rutaceae*) native to Southeast Asia is grown mainly in tropical and subtropical regions. It is a perennial, evergreen tree that reaches a height of 3–5 m [5]. It is mainly used as a food and food additive due to its aromatic properties [6]. In addition to the aromatic properties of the volatile compounds contained in the essential oil produced from *Citrus aurantifolia* (CAEO) such as limonene, citral, caryophyllene, linalool, and terpinene, these compounds are also responsible for antimicrobial and antioxidant properties [7]. The traditional and pharmacological use of CAEO is attributed to the presence of secondary metabolites of terpenoids, flavonoids, and coumarins [8]. The chemical composition, as well as the individual properties of the essential oils, can be influenced by various factors, such as the environment in which the plant is grown [9], year of harvest [10], cultivar [11], and geographical area of cultivation [12,13], as well as the essential oil extraction system [14].

Reactive oxygen species (ROS) reduce the stability of food systems, leading to chronic diseases in biological systems. ROS have one or more unpaired electrons, making them very unstable and damaging to other molecules. ROS include superoxide and hydroxyl radicals, hydrogen peroxide, and singlet oxygen, which are formed by-products of biological reactions [15]. The antioxidant potential of phytochemicals has been increasingly recognized in recent years and essential oil studies are therefore underway [16]. In this context, several studies have focused on the antioxidant and antiradical activities of CAEO [17–19].

In the field of food safety, many antimicrobials present in essential oils are of great importance in controlling microbial population by targeting foodborne pathogens [20]. Essential oils are recognized as safe compounds by the United States Food and Drug Administration and are considered a suitable alternative to food preservation techniques, such as natural antimicrobials [21]. Pesticides are chemicals that are commonly used to protect plants from diseases and pests and their use is limited; otherwise, they could have adverse effects on human health and the environment [22]. Natural chemical alternatives are of great importance for reducing the negative impact on the environment [23].

The primary objective of the study was to evaluate the biological activity of *Citrus aurantifolia* essential oil. Emphasis was placed on determining the antioxidant, antimicrobial, and insecticidal activity, as well as the chemical composition of the essential oil. We also focused on the antibacterial and antifungal effect of the *in situ* vapor phase of the essential oil on various food models.

2. Materials and Methods

2.1. Essential Oil

Citrus aurantifolia essential oil was purchased from Hanus, s.r.o. (Nitra, Slovakia). The essential oil was obtained by cold pressing the fruit pericarp. The *Citrus aurantifolia* country of origin was Italy.

2.2. Chemical Composition

CAEO was analyzed by gas chromatography/mass spectrometry (GC/MS) and gas chromatography (GC-FID). 5975B (Agilent Technologies, Santa Clara, CA, USA). An HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) was used. The temperature program was set in the range of 60–150 °C (increase rate 3 °C per minute) and in the range 150–280 °C (increase rate 5 °C per minute). The total duration of the program was 60 min. Helium 5.0 was used as a carrier at a flow rate of 1 mL per minute. The injection volume was 1 µL (sample EO was diluted in pentane), setting the temperature of the split/splitless injector at 280 °C. The analyzed sample was injected in a split mode with a ratio of 40.8:1. Electron impact mass spectrometry (EI-MS; 70 eV) data were obtained in scan mode in the m/z 35–550 range. The MS sources of the ion source and the MS of the quadrupole were 230 °C and 150 °C, respectively. Data acquisition began after a 3-min solvent delay.

GC-FID analyses were performed on an Agilent 6890N gas chromatograph connected to an FID detector. The column (HP-5MS) and chromatographic conditions were the same as for GC-MS. The FID detector temperature was set at 300 °C.

The individual volatile components of the CAEO sample were identified according to their retention indices [18] and compared with reference spectra (Wiley and NIST databases). Retention indices were determined experimentally by a standard method that included retention times of n-alkanes (C6–C34) injected under the same chromatographic conditions [19]. Percentages of identified compounds (amounts greater than 0.1%) were derived from their GC peak areas.

2.3. Determination of Antioxidant Activity

The antioxidant activity of CAEO was determined using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma Aldrich, Schnelldorf, Germany). DPPH stock solution (0.025 g/L dissolved in methanol) was adjusted to an absorbance of 0.7 at 515 nm. A 5 µL sample of the analyzed essential oil was applied to 195 µL of DPPH solution in a 96-well microtiter plate. The reaction mixture was incubated with continuous shaking at 1000 rpm for 30 min in the dark. Antioxidant activity was expressed as a percentage of DPPH inhibition and was subsequently calculated according to the formula $(A0 - AA)/A0 \times 100$, where A0 is the absorbance of DPPH and AA is the absorbance of the sample.

Radical scavenging activity was evaluated against a standard reference substance Trolox (Sigma Aldrich, Schnelldorf, Germany) dissolved in methanol (Uvasol ® for spectroscopy, Merck, Darmstadt, Germany) to a concentration ranging from 0 to 100 g/mL. The total radical scavenging capacity was expressed according to the calibration curve as 1 µg Trolox per 1 mL essential oil sample (TEAC).

2.4. Microorganisms

Gram-negative bacteria (*Azotobacter chroococcum* CCM1912, *Serratia marcescens* CCM 8588), Gram-positive bacteria (*Bacillus* from 2020 *Priestia megaterium* CCM2007, *Micrococcus luteus* CCM 732), and yeast (*Candida tropicalis* CCM 8264, *Candida glabrata* CCM 8270) were obtained from Czech Collection of Organisms (Brno, Czech Republic).

2.5. Determination of Antimicrobial Activity

The antimicrobial activity of CAEO was evaluated by the disk diffusion method. The inoculum was cultured for 24 h on tryptone soy agar (TSA, Oxoid, Basingstoke, UK) at 37 °C for bacteria and Sabouraud dextrose agar (SDA, Oxoid, Basingstoke, UK) at 25 °C for yeast. The inoculum was adjusted to an optical density of 0.5 McFarland standard (1.5×10^8 CFU/mL). 100 µL of conditioned inoculum was micro-pipetted onto a Petri dish (PD) with Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK). Sterile discs (6 mm) were placed on the PD with tweezers. The disks were impregnated with 10 µL of CAEO. The samples were incubated for 24 h at 37 °C for bacteria and 25 °C for yeast. Antibiotics (cefoxitin, gentamicin; Oxoid, Basingstoke, UK) for Gram-negative and Gram-positive bacteria served as positive controls. An antifungal (fluconazole; Oxoid, Basingstoke, UK) was used as a positive control for yeast. Disks impregnated with 0.1% DMSO (dimethyl sulfoxide, Centralchem, Bratislava, Slovakia) were used as a negative control.

An inhibition zone above 10 mm was determined to be very strong antimicrobial activity, an inhibition zone above 5 mm was determined to be mild activity, and an inhibition zone above 1 mm was determined to be weak activity. Antimicrobial activity was measured three times.

2.6. Minimum Inhibitory Concentration (MIC)

The broth microdilution method was used to determine the MIC of bacteria and yeast. The inoculum was cultured and treated as in the disc diffusion method. 100 µL of nutrient medium and 50 µL of inoculum were applied to the wells of a 96-well microtiter plate using a micropipette. Subsequently, CAEO was prepared by serial dilution to a concentration range of 400–0.2 µL/mL in MHB/SDB and mixed thoroughly in the wells. 96-well microtiter plates were analyzed with a Glomax spectrophotometer (Promega Inc., Madison, WI, USA) at 0 h. Subsequently, the bacterial samples were incubated at 37 °C for

24 h. Yeast samples were incubated at 25 °C for 24 h. MHB/SDB with essential oil was used as a negative control and MHB/SDB with inoculum was used as maximal growth control.

2.7. In Situ Antimicrobial Analysis on a Food Model

In situ antimicrobial analysis in the vapor phase on a food model (apple, pear, potato, kohlrabi) was tested on Gram-negative and Gram-positive bacteria, and yeast. Warm SDA for yeast and MHA for bacteria was poured into 60 mm PD and cap. Sliced vegetables and fruits (0.5 mm) were placed on agar. Using a microbiological needle, three inoculum injections were applied to a slice of fruit and vegetables. CAEO was diluted in ethyl acetate to concentrations of 500, 250, 125, and 62.5 µL/L. A sterile filter paper was placed in the lid and applied with a 100 µL micropipette of the appropriate concentration of essential oil. The filter paper was dried for 1 min to evaporate the remaining ethyl acetate; then, the plates were sealed and incubated at 37 °C for bacteria and 25 °C for yeast for 7 days.

Growth inhibition was assessed by stereological methods. Bulk density (Vv) was estimated using ImageJ 1.38e/Java 1.5.0_09 software. The stereological lattice of the colonies (P) and the substrate (p) was calculated. Growth density was calculated in % according to the formula $Vv = P/p \times 100$. Antifungal and antimicrobial activity EO was expressed as growth inhibition $MGI/BGI = [(C - T)/C] \times 100$, where C was the growth density in the control group and T was the growth density in the treated group [24]. Negative results were a stimulus to growth.

2.8. Insecticidal Activity

The insecticidal activity of CAEO was evaluated on a model organism *Pyrrhocoris apterus*. Fifty *P. apterus* individuals were placed in the PD. A ring of sterile filter paper was glued to the lid. Concentrations (100, 50, 25, 12.5, and 6.25%) were prepared by diluting CAEO with 0.1% polysorbate (Sigma Aldrich, Schnelldorf, Germany). 100 µL of the appropriate concentration of CAEO was applied to sterile filter paper. The dishes were closed, sealed around the perimeter with parafilm, and left at room temperature for 24 h. In the control group, 100 µL of 0.1% polysorbate was used. After 24 h, the number of living and dead individuals was evaluated. The experiment was performed in triplicate.

2.9. Statistical Data Processing

One-way analysis of variance (ANOVA) was performed using Prism 8.0.1 (GraphPad Software, San Diego, CA, USA), followed by Tukey's test at $p \leq 0.05$. SAS® software version 8 (SAS Slovakia, s.r.o., Bratislava, Slovakia) was used for data processing. The results of the MIC value (concentration that caused 50% and 90% inhibition of bacterial growth) were determined by logit analysis.

3. Results

3.1. Chemical Composition

We detected α-phellandrene 48.5%, *p*-cymene 16.5%, α-pinene 12.6%, and (*E*,*E*)-α-farnesene 12.6% (Table 1) as the main volatile compounds in CAEO by GC/MS and GC/FID methods.

3.2. Antioxidant and Antimicrobial Activity

CAEO free radical scavenging activity was evaluated by DPPH radical. The antioxidant activity was determined to be 74.5 ± 0.5%, which corresponds to 442 ± 2.3 TEAC. We consider the value of the achieved antioxidant activity to be high. CAEO antimicrobial activity was expressed using inhibition zones and subsequent inhibitory activity based on defined criteria (Table 2). The most pronounced inhibition was observed against Gram-negative bacteria. For Gram-positive microorganisms, we detected strong antimicrobial activity against *P. megaterium* and moderate antimicrobial activity in *M. luteus*. Against yeast, we detected moderate antimicrobial activity against *C. tropicalis*, and in *C. glabrata* we recorded weak antimicrobial activity of CAEO.

Table 1. Chemical composition of CAEO.

RI [a]	Compound [b]	% [c]
926	α-thujene	2.7 ± 0.01
938	α-pinene	12.6 ± 0.03
948	camphene	1.8 ± 0.02
977	sabinene	tr
980	β-pinene	tr
992	β-myrcene	1.6 ± 0.03
1004	α-phellandrene	48.5 ± 0.63
1016	α-terpinene	0.1 ± 0.01
1023	*p*-cymene	16.5 ± 0.05
1028	α-limonene	0.7 ± 0.01
1047	(*E*)-β-ocimene	tr
1060	γ-terpinene	0.3 ± 0.01
1088	α-terpinolene	0.4 ± 0.01
1178	4-terpinenol	tr
1189	α-terpineol	1.4 ± 0.02
1202	n-decanal	tr
1227	nerol	2.2 ± 0.03
1238	neral	1.2 ± 0.01
1256	geraniol	0.3 ± 0.02
1266	geranial	0.9 ± 0.03
1364	neryl acetate	1.6 ± 0.02
1380	geranyl acetate	0.3 ± 0.01
1422	(*E*)-caryophyllene	2.4 ± 0.03
1437	α-trans-bergamotene	2.7 ± 0.04
1506	(*E*,*E*)-α-farnesene	12.6 ± 0.14
1507	β-bisabolene	1.8 ± 0.06
total		95.6 ± 1.22

[a] Values of retention indices on HP-5MS column; [b] identified compounds; [c] tr—compounds identified in amounts less than 0.1%.

Table 2. Antimicrobial activity of CAEO.

Microorganisms	Inhibition Zone (mm)	Activity of EO	MIC 50 (μL/mL)	MIC 90 (μL/mL)	ATB (mm)
Gram-negative bacteria					
Azotobacter chroococcum	15.33 ± 0.58	***	2.36	5.18	26.67 ± 1.15
Serratia marcescens	12.66 ± 0.58	***	6.18	8.26	29.66 ± 0.58
Gram-positive bacteria					
Priestia megaterium	8.67 ± 0.58	**	12.43	14.58	30.67 ± 0.58
Micrococcus luteus	8.33 ± 1.15	**	12.43	15.36	27.67 ± 0.58
Yeasts					
Candida glabrata	3.67 ± 0.58	*	23.45	24.63	28.67 ± 0.58
Candida tropicalis	5.33 ± 0.58	**	12.35	14.38	29.67 ± 0.58

* Weak antimicrobial activity (zone 1–5 mm). ** Moderate inhibitory activity (zone 5–10 mm). *** Very strong inhibitory activity (zone > 10 mm). ATB—antibiotics, positive control (cefoxitin for G−, gentamicin for G+, fluconazole for yeast).

The MIC 50 and MIC 90 of bacteria and yeast were determined by the broth microdilution method (Table 3). Gram-negative bacteria were inhibited at the lowest concentrations (2.36 resp. 5.18 and 6.18 resp. 8.26 μL/mL). Mean values were detected against Gram-positive microorganisms and *C. tropicalis* yeast. We recorded the highest minimum inhibitory concentration in *C. glabrata*.

Table 3. *In situ* analysis of the antibacterial activity of the vapor phase of CAEO in apple.

Apple				
Bacterial Growth Inhibition (%)	**Bacteria**			
Lime EO (µL/L)	***Azotobacter chroococcum***	***Priestia megaterium***	***Serratia marcescens***	***Micrococcus luteus***
62.5	5.78 ± 2.68^{a}	4.15 ± 1.07^{a}	4.69 ± 2.50^{a}	4.01 ± 1.61^{a}
125	11.64 ± 0.94^{b}	$10.73 \pm 2.40^{b,a}$	$14.99 \pm 2.45^{b,a}$	7.47 ± 2.06^{b}
250	$25.07 \pm 2.45^{c,b,a}$	$17.06 \pm 1.56^{c,a,b}$	$30.51 \pm 2.04^{c,a,b}$	$13.71 \pm 1.93^{c,a,b}$
500	$53.46 \pm 2.93^{d,b,a,c}$	$36.23 \pm 1.94^{d,a,b,c}$	$71.35 \pm 1.90^{d,a,b,c}$	$26.96 \pm 2.21^{d,a,b,c}$
Mycelial Growth Inhibition (%)	**Yeast**			
Lime EO (µL/L)	***Candida glabrata***		***Candida tropicalis***	
62.5	25.78 ± 2.68^{a}		27.51 ± 1.79^{a}	
125	$31.98 \pm 0.36^{b,a}$		$15.51 \pm 2.82^{b,a}$	
250	$45.07 \pm 2.45^{c,a,b}$		$13.91 \pm 3.30^{c,a}$	
500	$73.20 \pm 2.58^{d,a,b,c}$		$33.69 \pm 2.88^{d,b,c}$	

Individual letters (a–d) in upper case indicate the statistical differences between the concentrations; $p \leq 0.05$.

3.3. In Situ Antimicrobial Activity in Food Models

The results of the *in situ* evaluation revealed strong antimicrobial activity (inhibition above 50%) of the CAEO vapor phase at the highest concentration (500 µL/L) against the growth of *A. chroococcum*, *S. marcescens*, and *C. glabrata* on apple as a food model. For other microorganisms and test concentrations, inhibitory activity below 50% was observed (Table 3).

The results of the *in situ* evaluation revealed strong antimicrobial activity (inhibition above 50%) of the CAEO vapor phase at the highest concentration (500 µL/L) against the growth of *S. marcescens*, *M. luteus*, and *C. glabrata* on pear as a food model. For other microorganisms and test concentrations, inhibitory activity below 50% was observed (Table 4).

Table 4. *In situ* analysis of the antibacterial activity of the vapor phase of CAEO in pear.

Pear				
Bacterial Growth Inhibition (%)	**Bacteria**			
Lime EO (µL/L)	***Azotobacter chroococcum***	***Priestia megaterium***	***Serratia marcescens***	***Micrococcus luteus***
62.5	30.75 ± 2.40^{a}	1.73 ± 0.56^{a}	7.38 ± 0.87^{a}	9.83 ± 2.75^{a}
125	$19.41 \pm 1.13^{b,a}$	$4.44 \pm 0.72^{b,a}$	$17.29 \pm 0.98^{b,a}$	$22.86 \pm 1.37^{b,a}$
250	$10.78 \pm 1.44^{c,b,a}$	$9.35 \pm 0.85^{c,a,b}$	$38.29 \pm 2.53^{c,a,b}$	$44.21 \pm 1.82^{c,a,b}$
500	$5.05 \pm 0.66^{d,b,a,c}$	$20.60 \pm 0.86^{d,a,b,c}$	$64.73 \pm 3.30^{d,a,b,c}$	$85.72 \pm 1.46^{d,a,b,c}$
Mycelial Growth Inhibition (%)	**Yeast**			
Lime EO (µL/L)	***Candida glabrata***		***Candida tropicalis***	
62.5	12.35 ± 2.05^{a}		17.14 ± 1.55^{a}	
125	13.30 ± 2.01^{b}		$23.39 \pm 1.81^{b,a}$	
250	$4.61 \pm 0.74^{c,a,b}$		$44.71 \pm 2.21^{c,a,b}$	
500	$76.68 \pm 2.22^{d,a,b,c}$		$4.46 \pm 2.02^{d,a,b,c}$	

Individual letters (a–d) in upper case indicate the statistical differences between the concentrations; $p \leq 0.05$.

The *in situ* evaluation results revealed strong antimicrobial activity (inhibition above 50%) of the CAEO vapor phase at the highest concentration (500 µL/L) against the growth of all tested microorganisms. Irregularity was visible in *P. megaterium*, where the lowest tested concentration (62.5 µL/L) on the potato as the food model was the most effective. For other microorganisms and other tested concentrations, inhibitory activity below 50% was observed (Table 5).

Table 5. *In situ* analysis of the antibacterial activity of the vapor phase of CAEO in potato.

Potato				
Bacterial Growth Inhibition (%)	**Bacteria**			
Lime EO (µL/L)	***Azotobacter chroococcum***	***Priestia megaterium***	***Serratia marcescens***	***Micrococcus luteus***
62.5	12.38 ± 0.86 ^{a}	65.59 ± 2.08 ^{a}	7.84 ± 1.42 ^{a}	6.10 ± 1.38 ^{a}
125	25.94 ± 3.07 ^{b,a}	33,37 ± 5.47 ^{b,a}	15.72 ± 2.41 ^{b}	14.25 ± 3.73 ^{b,a}
250	48.09 ± 4.31 ^{c,b,a}	13.38 ± 2.48 ^{c,a,b}	36.96 ± 5.66 ^{c,a,b}	32.04 ± 2.23 ^{c,a,b}
500	98.07 ± 3.21 ^{d,b,a,c}	8.05 ± 2.53 ^{d,a,b}	74.04 ± 2.18 ^{d,a,b,c}	65.81 ± 2.54 ^{d,a,b,c}
Mycelial Growth Inhibition (%)	**Yeast**			
Lime EO (µL/L)	***Candida glabrata***		***Candida tropicalis***	
62.5	16.01 ± 2.11 ^{a}		16.21 ± 2.23 ^{a}	
125	20.65 ± 2.11 ^{b}		25.99 ± 2.02 ^{b,a}	
250	46.21 ± 2.40 ^{c,a,b}		44.73 ± 3.29 ^{c,a,b}	
500	86.25 ± 2.62 ^{d,a,b,c}		76.92 ± 2.24 ^{d,a,b,c}	

Individual letters (a–d) in upper case indicate the statistical differences between the concentrations; $p \leq 0.05$.

The results of the *in situ* evaluation revealed strong antimicrobial activity (inhibition over 50%) of the CAEO vapor phase at 500 and 250 µL/L against *S. marcescens*; at 250 µL/L, we detected inhibition of over 50% against *A. chroococcum* on kohlrabi as a food model. For other microorganisms and other tested concentrations, inhibitory activity below 50% was observed (Table 6).

Table 6. *In situ* analysis of the antibacterial activity of the vapor phase of CAEO in kohlrabi.

Kohlrabi				
Bacterial Growth Inhibition (%)	**Bacteria**			
Lime EO (µL/L)	***Azotobacter chroococcum***	***Priestia megaterium***	***Serratia marcescens***	***Micrococcus luteus***
62.5	6.53 ± 1.10 ^{a}	34.85 ± 3.63 ^{a}	11.66 ± 1.28 ^{a}	2.13 ± 0.91 ^{a}
125	7.38 ± 0.82 ^{b}	27.76 ± 1.04 ^{b,a}	26.26 ± 2.23 ^{b,a}	6.77 ± 1.28 ^{b}
250	54.81 ± 2.52 ^{c,a,b}	23.78 ± 1.95 ^{c,a}	55.61 ± 2.14 ^{c,a,b}	12.68 ± 2.62 ^{c,a,b}
500	10.93 ± 1.28 ^{d,a,c}	35.89 ± 2.41 ^{d,b,c}	99.05 ± 1.09 ^{d,a,b,c}	25.30 ± 3.17 ^{d,a,b,c}
Mycelial Growth Inhibition (%)	**Yeast**			
Lime EO (µL/L)	***Candida glabrata***		***Candida tropicalis***	
62.5	0.66 ± 0.57 ^{a}		8.34 ± 1.70 ^{a}	
125	2.33 ± 0.57 ^{b}		4.27 ± 1.07 ^{b}	
250	4.60 ± 1.44 ^{c,a}		12.68 ± 2.61 ^{c,b}	
500	12.76 ± 1.65 ^{d,a,b,c}		27.64 ± 4.68 ^{d,a,b,c}	

Individual letters (a–d) in upper case indicate the statistical differences between the concentrations; $p \leq 0.05$.

3.4. Insecticidal Activity

We evaluated the insecticidal activity of CAEO as very strong (Table 7). At the highest concentration (100%), the insecticidal effect was up to 90%; at 50%, the effect was 80%, at 25%, the inhibition was 60%, at 12.5%, the insecticidal activity was 20% and at the lowest tested concentration, the insecticidal effect was 10%.

Table 7. Insecticidal activity of CAEO.

Concentration (%)	Number of Living Individuals	Number of Dead Individuals	Insecticidal Activity (%)
100	3	27	90
50	6	24	80
25	12	18	60
12.5	24	6	20
6.25	27	3	10
Control group	30	0	0

4. Discussion

Dao et al. [7] in their study detected limonene (62.17%), α-terpinene (12.36%), and β-pinene (11.72%) as the main compounds of CAEO. In our study, we detected these compounds in significantly lower proportions. Ibrahim et al. [25] report D-limonene (57.84%), neral (7.81%), and linalool (4.75%) as the main components of CAEO. In our work, limonene and neral were detected in much smaller amounts, and linalool was not detected in in any concentration. Asnaashari et al. [6] identified the main components of CAEO as limonene (28.27%), followed by α-terpineol (19.61%), *p*-cymene (8.6%), and β-pinene (5.7%). In our work, we detected these components only in very low amounts. Pathirana et al. [26] in their work detected limonene (56.22%), γ-terpinene (14.31%), and β-pinene (10.96%) as the main compounds. These compounds were also identified only in very low amounts in our study. Majnooni [27] detected limonene, linalool, and trans-β-ocimene as the primary components of CAEO in his analysis. Venkateshwarlu and Selvaraj [28] reported neral, geraniol, and citronellol as the major compounds of CAEO. Based on the results published so far by other authors on the chemical composition of CAEO, we can observe a great diversity in the composition depending on the origin and preparation of the essential oil. Hojjati and Barzegar [29] detected in their work that linalool (30.62%), α-terpineol (14.52%), and linalyl acetate (13.76%) were the main compounds of CAEO. The composition of CAEO from Hanus s.r.o. had significantly different dominant components compared to most CAEOs analyzed so far. These differences could be due to the different origins of the basic raw material, as well as the different ways of producing the essential oil.

Al-Aamri et al. [30] determined that the DPPH scavenging activity of the CAEO was 63.23 ± 0.27%. Al Namani et al. [31] determined the antioxidant activity of CAEO to be 51.91%. Kuljarachanan et al. [32] detected antioxidant activity of CAEO of 90.7 ± 0.47% in their study. Babbar et al. [33] found free radical scavenging activity of 83%. Patil et al. [34] found the DPPH radical scavenging activity to be 85.4%. Moosavy et al. [35] detected antioxidant activity of CAEO by DPPH at 55.09%. These results can confirm our finding that the antioxidant activity of CAEO is high. Tundis et al. [36] detected the antioxidant activity of CAEO IC_{50} 201.3 µg/mL. Loizzo et al. [37] determined the free radical scavenging activity as IC_{50} with value 78.3 ± 1.8 µg/mL. Lin et al. [38] determined the IC_{50} of CAEO at 2.36 mg/mL. Chi et al. [39] determined the antioxidant activity of CAEO by expressing the IC_{50} at 1.21 mg/mL. Despite the inconsistency of methodological procedures and expressions of antioxidant activity, the authors agree that CAEO has high antioxidant activity.

Onyeagba et al. [40] evaluated the antimicrobial effect of CAEO against *Bacillus* spp. (17 mm), *Staphylococcus aureus* (17 mm), *Escherichia coli* (11 mm), and *Salmonella* spp. (13 mm). Julaeha et al. [41] analyzed the antimicrobial activity of CAEO against *S. aureus* (34 mm), *S. epidermidis* (38 mm), *E. coli* (12 mm), and *K. pneumoniae* (37 mm). Al-Aamri et al. [30] analyzed the effect of CAEO at a concentration of 10 µL per disc and identified inhibition zones for *S. aureus* (7.9 mm) and *E. coli* (3.1 mm). Compared to our findings of higher efficacy on Gram-negative microorganisms, the authors observed that the effect is more pronounced against Gram-positive bacteria. Akinnibosun and Edionwe [42] detected inhibition zones of 18.7 mm for *C. albicans* using methanol extract and 11.7 mm using acetone extract. Chi et al. [39] determined CAEO inhibition zones for *S. aureus* (20.1 mm), *B. cereus* (21.1 mm), *S. typhi* (20.1 mm), and *P. aeruginosa* (14.7 mm). Ben Bnina et al. [43] evaluated the antibacterial activity of CAEO against eight pathogenic bacteria (*S. epidermidis, P. aeruginosa, S. aureus, M. luteus, E. coli, S. typhimurium, L. monocytogenes*, and *E. faecium*), with maximum inhibition zones ranging from 6 to 16 mm for all strains at a concentration of 7 µL per disc. However, the authors came to the same conclusion that the antimicrobial effect is significantly lower compared to antibiotics. Our CAEO had a very different chemical composition compared to the oils analyzed so far, which may have contributed to the difference in antimicrobial effect. The different findings may also have been affected by other microorganisms that were used for analyses.

Sánchez Aldana et al. [20] found minimum inhibitory concentrations for *E. coli* and *S. typhimurium* to be 1500 mg/L, *B. cereus* 750 mg/L, *S. aureus* 1000 mg/L, and *L. monocytogenes* 500 mg/L. These values are significantly higher than the amounts determined in our study. Costa et al. [44] set the MIC_{50} and MIC_{90} values for *C. albicans* at 0.125 and 0.125 mg/mL, respectively. In our study, we detected significantly higher MIC values. Mulyanti et al. [45] set the MIC for *S. mutans* at 5.2 mg/mL. In our work, significantly lower values of the minimum inhibitory concentration were detected. Safaeian Laein et al. [46] detected MICs for *E. coli* (20 mg/mL), *P. aeruginosa* (20 mg/mL), *S. typhimurium* (40 mg/mL), *S. aureus* (5 mg/mL), *B. cereus* (5 mg/mL), and *L. monocytogenes* (10 mg/mL). The values determined by the authors are multiple times higher than those observed in the CAEO we tested. Chi et al. [39] evaluated the MIC for *S. aureus* (21 mg/mL), *B. cereus* (10.5 mg/mL), *S. typhi* (21 mg/mL), and *P. aeruginosa* (42 mg/mL) in their study. Compared to our study, most authors report higher minimum inhibitory concentrations. These differences may be due to the different chemical composition of the essential oil compared to the other authors.

Various studies have confirmed that the vapor phases of EO have higher antimicrobial activity than their corresponding liquid phases [47–50]. Křůmal et al. [51] noted a significant effect of CAEO in the vapor phase on spore inhibition in their work. Kloucek et al. [52] evaluated the effect of the vapor phase of CAEO on a food model and found that none of the tested concentrations inhibited the growth of bacteria or filamentous microscopic fungi. This finding contradicts our results, which may have been affected by the composition of CAEO. While in all mentioned studies, limonene was the dominant component, in our case α-phellandrene was the main component. Parichanon et al. [53] examined the positive effect of CAEO in the vapor phase on the cold storage of vegetable salads ready for consumption. In their study, they also focused on the inhibition of *L. monocytogenes* by the essential oil in vapor phase. In both cases, they recorded a positive effect of CAEO in the vapor phase without disturbing the sensory properties of the consumer.

Essential oils obtained from various plants are used as natural acaricides and insecticides, growth regulators, repellents, and inhibitors [54–56]. Sarma et al. [57] have reported the ovicidal effect of CAEO. Sanei-Dehkordi et al. [58] found in their study that CAEO had larvicidal activity against *Anopheles stephensi.* Laarif et al. [59] confirmed the effect of CAEO against *Spodoptera littoral* and *Tuta absoluta* in their work. Abdelgaleil et al. [60] confirmed a very good insecticidal effect of essential oils including CAEO against *Spodoptera littoralis.* Hamid et al. [61] observed very high repellent effects of CAEO against *Aedes aegypti.* These authors' findings are in line with our observations of the good insecticidal effect of CAEO; moreover, these authors suggested both larvicidal and repellent effects of CAEO.

5. Conclusions

Our study shows that CAEO produced in Slovakia has good biological activity. Better antimicrobial activity was found against g\Gram-negative bacteria. Medium to weak activity was detected in Gram-positive bacteria and yeast. The values of the MIC correlated with the results of the disk diffusion method. The antimicrobial effect of the vapor phase of CAEO was the most pronounced at the highest tested concentration, but an inhibitory effect was observed at all tested concentrations in all microorganisms and on all model foods. Our findings suggest that CAEO could be suitable for future use in extending shelf life or protecting agricultural products by steam application. The high insecticidal activity offers the possibility of future use for the preparation of natural insecticidal, larvicidal, or repellent preparations.

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