Cinnamomum cassia and *Cananga odorata* in the Vapor Phase

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Abstract
The aim of this study was to compare the effectiveness of contact and steam application of essential oils *Cinnamomum cassia* and *Cananga odorata*. Using the disk diffusion method, we found that *Cinnamomum cassia* essential oil achieved good effects against gram-positive, gram-negative bacteria and yeasts in contact application. Very weak efficacy was found against filamentous microscopic fungi. *Cananga odorata* essential oil showed very weak antibacterial effects after contact application. Inhibition of yeast and filamentous microscopic fungi was weak to moderate. In the vapor phase, *C. cassia* essential oil achieved very good antibacterial effects and its effect on the inhibition of filamentous microscopic fungi was significantly increased. *C. odorata* essential oil showed significantly better inhibitory effects against gram-negative and gram-positive bacteria. Its effectiveness against filamentous microscopic fungi was higher than with contact application. These findings suggest that essential oils with a higher proportion of volatile compounds may increase their effectiveness when vapor is applied. Although there is still no standard methodology for determining the activity of essential oils in the vapor phase, results reported thus far are encouraging and suggest possible applications in food preservation. With higher efficiency, lower amounts of essential oils are sufficient, which reduces the impact on sensory properties.

Keywords: essential oil, *Cinnamomum cassia*, *Cananga odorata*, antimicrobial activity, food model

1. Introduction

Aromatic plants and their essential oils have been used since ancient times in perfumery, as flavourings, spices in culinary arts, in medicines, antimicrobial agents and for insecticidal use or protection of stored products [1–3].
Cinnamomum cassia is a tall, aromatic, evergreen tree belonging to the family *Lauraceae*. It is known for its versatile use in the pharmaceutical and food industries [4]. It is used in the pharmaceutical industry for its strong antimicrobial and antioxidant effects. In the food industry, it is mainly used as a flavouring [5]. The Food and Drug Administration has recognized cinnamon as a safe food additive [6].
Cananga odorata is also known as "Ylang Ylang" is a fast-growing tree native to India. It belongs to the family *Annonaceae*. It is used in the pharmaceutical industry for its anti-inflammatory, antifungal, and antioxidant effects and in the food industry as a flavouring agent and adjuvant [7,8].
The antimicrobial efficacy of essential oils, when applied by direct contact, has been demonstrated and extensively studied [9]. However, the vapor phase and volatile components present in the essentials have not been thoroughly investigated;

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there are a small number of published reports on the antimicrobial activity of essential oils in the vapor phase [10].

This study aimed to analyse the antibacterial and antifungal efficacy of contact application of essential oils *Cinnamomum cassia* and *Cananga odorata* using the disk diffusion method. To monitor the effect of essential oils in the vapor phase *in situ* against fibrous microscopic fungi *Penicillium citrinum* and *Penicillium rubrum* and the bacteria *Serratia marcescens* and *Micrococcus luteus* on food models. Based on the findings, the effectiveness of contact and steam application in inhibiting microorganisms was compared.

2. Materials and methods

The essential oil *Cinnamomum cassia* (CCEO) and *Cananga odorata* (COEO) were obtained from Hanus, s.r.o. (Nitra, Slovakia). CCEO was obtained by steam distillation of fresh leaves and shoots. COEO was obtained by steam distillation of fresh flowers.

Gram-positive bacteria (*Micrococcus luteus* CCM 2563, *Bacillus subtilis* CCM 1999, *Staphylococcus aureus* subsp. *aureus* CCM 8223, *Enterococcus faecalis* CCM 4224), Gram-negative bacteria (*Pseudomonas aeruginosa* CCM 3955, *Yersinia enterocottica* CCM 720, *Salmonella enterica* subsp. *enterica* ser. Enteritidis CCM 4420, *Serratia marcescens* CCM 8588), and yeast (*Candida krusei* CCM 8271, *Candida albicans* CCM 8261, *Candida tropicalis* CCM 8223, *Candida glabrata* CCM 8270) were obtained from the Czech collection of microorganisms (Brno, Czech Republic). Bacteria were identified by sequencing 16S rRNA and MALDI-TOF MS Biotyper. *Penicillium citrinum* and *Penicillium rubrum* fungi were obtained from grape samples and identified by sequencing 16S rRNA and MALDI-TOF MS Biotyper.

The antimicrobial activity of essential oils was determined by disk diffusion method. The microbial inoculum was cultured 24 hours for bacteria on Tryptone soy agar (TSA, Oxoid, Basingstoke, UK) at 37 °C and Sabouraud dextrose agar (SDA, Oxoid, Basingstoke, UK) at 25 °C was used for yeast. The inoculum was adjusted to an optical density of 0.5 McFarland standard (1.5 x 10^8 CFU / ml) and 100 μl was added to Mueller Hinton agar plates (MHA, Oxoid, Basingstoke, UK). Sterile 6 mm disks were saturated with 10 μl of CCEO and COEO and placed on a layer of agar with a microbial suspension. The samples were incubated for 24 hours at 37 °C for bacteria and 25 °C for yeast. Two antibiotics (cefoxitin, gentamicin; Oxoid, Basingstoke, UK) and one antifungal (fluconazole; Oxoid, Basingstoke, UK) were used as positive controls for gram-negative, gram-positive bacteria and yeast. Discs impregnated with 0.1% DMSO (dimethyl sulfoxide, Centralchem, Bratislava, SK) served as a negative control. An inhibition zone above 15 mm was determined to be a very strong antimicrobial activity, an inhibition zone above 10 mm was determined to be a mild activity, and an inhibition zone above 5 mm was determined to be weak activity. Antimicrobial activity was measured in triplicate.

The antifungal and antibacterial effect of the vapor phase of EO was evaluated in 0.5 l sterile glass containers (Bormioli Rocco, Italy) on bread used as a food model. Antimicrobial activity was tested against *S. marcescens* and *M. luteus* and microscopic filamentous fungi of the genus *Penicillium*. Fungi were cultured for 5 days on SDA at 25 °C and bacteria for 24 h on TSA at 37 °C. Cultures were plated on bread slices (15 × 15 × 1.5 cm) with three stabs. 6 cm sterile filter paper was placed on the lid of the vessel and 100 μl of EOs (62.5, 125, 250, and 500 μL/L diluted in ethyl acetate) was applied. The control group was left untreated. The glass containers were hermetically sealed and incubated in the dark for 14 days at 25 °C for fungi and bacteria for 7 days at 37 °C**.**

Antifungal and antibacterial analysis *in situ* in the vapor phase on carrots was tested against *S. marcescens* and *M. luteus* and microscopic filamentous fungi of the genus *Penicillium*. Warm MHA was poured into 60 mm Petri dishes (PD) and lids. Chopped carrots (0.5 mm) were placed on agar. The inoculum was prepared as described above in section 2.3. EOs were diluted by 1/2 serial dilution in ethyl acetate to 500, 250, 125, and 62.5 μL/L and applied on sterile filter paper. The filter paper was placed in a lid and allowed to ventilate for 1 minute to evaporate the remaining ethyl acetate. PDs were sealed and incubated at 37 °C for 7 days for bacteria and fungi at 25 °C for 14 days.

An inhibition of the fungal growth was evaluated by stereological methods. A volume density (Vv) of fungi was estimated using ImageJ software.

The stereological grid points of the colonies (P) and substrate (p) were counted. The density of fungal growth was calculated in % according to the formula Vv = P/p × 100. The antifungal activity of EO was expressed as mycelial growth inhibition in % (MGI): MGI = [(C − T)/C] × 100, where C was the density of the fungal growth in the control group and T was the density of the fungal growth in the treatment group [11,12].

In situ bacterial growth was determined using stereological methods. In this concept, the volume density (Vv) of bacterial colonies was firstly estimated using ImageJ software and counting the points of the stereological grid hitting the colonies (P), and those (p) falling to the reference space (growth substrate used). The volume density of bacterial colonies was consequently calculated as follows: Vv (%) = P/p. The antibacterial activity of EO was defined as the percentage of bacterial growth inhibition (BGI) BGI = [(C − T)/C] × 100, where C and T were bacterial growth (expressed as Vv) in the control group and the treatment group.

Bacterial growth inhibition by essential oil was expressed as a percentage of inhibition compared to control, where control represented 0 % inhibition. Inhibition of more than 50 % was considered effective.

All analyses were performed in triplicate. Statistical variability of the data was processed using Microsoft-Excel® software.

3. Results and discussion

3.1 Antimicrobial activity in contact application of essential oil

From the results of the disk diffusion method (**Error! Reference source not found.**), mild to very strong antimicrobial activity against gram-positive microorganisms was observed in CCEO. The effect against gram-negative bacteria was evaluated as mild activity. The weak activity was found against candida yeasts. No efficacy was observed in evaluating the effect of contact application of CCEO against microscopic fibrous fungi of the genus *Penicillium*. Yang et al. [13] in their work found for *S. aureus* and *P. aeruginosa*, inhibition zones with a range of 24-42 mm and 17-27 mm values are higher than those detected in our work which may be caused by different plant growing conditions and subsequent preparation of essential oils. Firmino et al. [14] evaluated CCEO as very effective against gram-negative, gram-positive bacteria and yeast. Cinnamon oil has been shown to have inhibitory effects against many bacteria [15] and microscopic filamentous fungi as well as yeasts involved in mycoses and candidiasis of the airways [16]. Mild to strong antimicrobial effects in this study support the findings on the antimicrobial activities of CCEO.

Table 1. Antimicrobial activity CCEO of contact application

Microorganism	Zone inhibition (mm)	Activity of CCEO
Micrococcus luteus	11.67±0.58	**
Bacillus subtilis	20.33±1.53	***
Staphylococcus aureus	19.67±1.15	***
Enterococcus faecalis	12.33±0.58	**
Serratia marcescens	10.33±1.15	**
Pseudomonas aeruginosa	13.00±1.00	**
Yersinia enterocolitica	14.33±0.58	**
Salmonella enterica subs. *enterica* ser. Enteritidis	15.67±0.58	***
Candida albicans	7.66±0.58	*
Candida krusei	9.33±1.15	*
Candida tropicalis	8.15±0.33	*
Candida glabrata	8.67±0.58	*
Penicillium citrinum	4.33±0.58	-
Penicillium rubrum	5.00±1.00	-

An inhibition zone above 15 mm was determined to be a very strong antimicrobial activity, an inhibition zone above 10 mm was determined to be a mild activity, and an inhibition zone above 5 mm was determined to be weak activity. Antimicrobial activity was measured in triplicate.

In the COEO evaluation (Table 2), significantly lower inhibitory activity was observed than with CCEO. The results show that the essential oil achieved none to weak antibacterial activity and mild antifungal activity. Weak inhibitory activity was found in only one representative of the gram-positive microorganism *P. aeruginosa* and against yeast of genus *Candida*. No inhibitory activity based on criteria was observed for gram-negative microorganisms. We recorded a slight inhibitory activity in the tested microscopic fibrous fungi. Overall, different types of *C. odorata* extracts and essential oils showed better antibacterial activities against gram-positive bacteria than gram-negative bacteria and showed remarkable antifungal activity [8,17]. The very weak to weak antibacterial effects and mild antifungal effects of COEO in this study support the potential use of this essential oil as an antifungal agent.

Table 2. Antimicrobial activity COEO of contact application

Microorganism	Zone inhibition (mm)	Activity of CCEO
Micrococcus luteus	2.00±1.00	-
Bacillus subtilis	0.00±0.00	-
Staphylococcus aureus	4.33±2.08	-
Enterococcus faecalis	1.33±0.58	-
Serratia marcescens	3.33±0.58	-
Pseudomonas aeruginosa	6.00±2.00	*
Yersinia enterocolitica	3.00±1.00	-
Salmonella enterica subs. *enterica* ser. Enteritidis	1.67±0.58	-
Candida albicans	6.33±2.52	*
Candida krusei	5.33±1.15	*
Candida tropicalis	5.67±0.58	*
Candida glabrata	5.33±0.58	*
Penicillium citrinum	12.00±0.00	**
Penicillium rubrum	13.33±1.15	**

An inhibition zone above 15 mm was determined to be a very strong antimicrobial activity, an inhibition zone above 10 mm was determined to be a mild activity, and an inhibition zone above 5 mm was determined to be weak activity. Antimicrobial activity was measured in triplicate.

3.2 Antimicrobial activity of the vapor phase of essential oil

The antimicrobial activity of CCEO in the vapor phase was very effective in all tested microorganisms except *S. marcescens* (**Error! Reference source not found.**). It was occurred at all tested concentrations. *S. marcescens* was inhibited by CCEO only at higher concentrations (250 µL/Land 500 µL/L). Kačániová et al. [5] in their work, evaluated the effect of CCEO in the vapor phase as very good and potential for use in practice as a natural inhibitor of bacterial growth and microscopic filament fungi on foods. Jiang et al. [18] in their work showed that when the vapor phase is used, the minimum inhibitory concentration against *Sclerotinia sclerotiorum* was sixteen times lower than using contact application.

Table 3. Antimicrobial activity CCEO of the vapor phase in carrot

	Bacterial growth inhibition [%]			
Microorganisms	**62.5 µL/L**	**125 µL/L**	**250 µL/L**	**500 µL/L**
S. marcescens	13.39±3.54	20.33±1.15	91.86±0.74	98.70±1.30
M. luteus	58.42±3.76	76.29±1.51	90.45±53	97.64±0.81
	Mycelial growth inhibition [%]			
Microorganisms	**62.5 µL/L**	**125 µL/L**	**250 µL/L**	**500 µL/L**
P. citrinum	57.14±0.65	75.38±1.66	90.42±1.67	97.63±1.26
P. rubrum	66.31±1.23	87.43±0.97	90.84±1.42	99.69±0.31

Bacterial growth inhibition by essential oil was expressed as a percentage of inhibition compared to control, where control represented 0 % inhibition. Inhibition of more than 50 % was considered effective.

The antimicrobial activity of COEO in the vapor phase (**Error! Reference source not found.**) was effective from a concentration of 62.5 µL/L in *P. citrinum*. From a concentration of 125 µL/L, we observed inhibition against *S. marcescens* and *P. rubrum*. The inhibitory effect against *M. luteus* was manifested at a concentration of 250 µL/L. These findings suggest a more pronounced effect of COEO in the vapor phase. Jiang et al. [19] found that vapor phase essential oils represent a suitable alternative to antimicrobials in the food industry due to the need of lower concentrations of EO than using the contact effect of the liquid phase. Mani López et al. [20] found that essential oils in the vapor phase inhibited the growth of microscopic fibrous fungi of the genus *Penicillium* on bread and it would be appropriate to verify the effect on sensory properties.

Table 4. Antimicrobial activity COEO of the vapor phase in carrot

	Bacterial growth inhibition [%]			
Microorganisms	**62.5 µL/L**	**125 µL/L**	**250 µL/L**	**500 µL/L**
S. marcescens	32.43±0.79	58.43±1.33	86.78±0.97	98.66±1.22
M. luteus	22.46±1.14	45.51±1.27	72.91±0.51	92.34±0.49
	Mycelial growth inhibition [%]			
Microorganisms	**62.5 µL/L**	**125 µL/L**	**250 µL/L**	**500 µL/L**
P.citrinum	51.27±1.12	65.64±1.62	94.54±0.32	98.67±1.22
P.rubrum	33.63±1.74	85.69±0.78	95.64±1.18	99.94±0.06

Bacterial growth inhibition by essential oil was expressed as a percentage of inhibition compared to control, where control represented 0 % inhibition. Inhibition of more than 50 % was considered effective.

4. Conclusions

The results of our work show that the essential oil *Cinnamum cassia* exhibited good effects against gram-negative, gram-positive bacteria and yeasts in the contact application. Its effectiveness against fibrous microscopic fungi was very weak. In the vapor phase application, the effectiveness was also very high against microscopic fibrous fungi. In the disc diffusion method test, *Cananga odorata* essential oil showed very weak effects against bacteria and moderate against fibrous microscopic fungi. In the vapor phase, its efficiency was significantly higher in all tested microorganisms. There is a possibility of future use of the tested essential oils in extending the shelf life of bakery products and could find application in the storage of root vegetables. The vapor phase has a weaker effect on the sensory properties than the contact phase. In the future, it would be necessary to evaluate the influence of essential oil in the vapor phase on the sensory properties of model foods.

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