Original

Inhibitory effect of the essential oil from *Cinnamomum zeylanicum* Blume leaves on some food-related bacteria

Efeito inibitório do óleo essencial das folhas de Cinnamomum zeylanicum Blume sobre bactérias de interesse em alimentos

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Abstract

Cinnamomum zeylanicum Blume, Lauraceae, has long been known for having many biological properties. This study aimed to identify the constituents of the essential oil from *C. zeylanicum* leaves using GC-MS and to assess its inhibitory effect on *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* based on MIC and MBC determination and kill-time study. Eugenol (73.27%) was the most prevalent compound in the essential oil followed by trans-β-cariophyllene (5.38%), linalool (3.31%), and alcohol cinamic acetate (2.53%). The results showed an interesting antibacterial activity of the oil with MIC ranging from 1.25 to 10 μL.mL^{-1}. MBC values were in the range of 20 – 80 μL.mL^{-1}. A concentration of 10 and 40 μL.mL^{-1} of the essential oil caused a fast and steady decrease in viable cell count (2 to 5 log cycles) of all assayed strains along 24 hours. A concentration of 40 μL.mL^{-1} of the oil provided a total elimination of the initial inocula of *S. aureus* after 2 hours. These results show the possibility of regarding the essential oil from *C. zeylanicum* leaves as alternative sources of antimicrobial compounds to be applied in food conservation systems.
Keywords: *Cinnamomum zeylanicum Blume; essential oil; antibacterial effect.*

Resumo

Cinnamomum zeylanicum Blume, Lauraceae, é uma espécie vegetal reconhecida, a longo tempo, como possuidora de muitas propriedades biológicas. Este estudo objetivou identificar os constituintes do óleo essencial das folhas de *C. zeylanicum*, utilizando CG-EM, e avaliar seu efeito inibitório sobre *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus* e *Pseudomonas aeruginosa* através da determinação da CIM e CBM, bem como através da análise do tempo de morte bacteriana. Eugenol (73,27%) foi o composto mais prevalente no óleo essencial, seguido por trans-β-cariofileno (5,38%), linalol (3,31%) e álcool acetato cinâmico (2,53%). Os resultados mostraram uma interessante atividade antibacteriana do óleo essencial com CIM, variando de 1,25 a 10 μL.mL^{-1}. Os valores de CBM oscilaram entre 20 e 80 μL.mL^{-1}. O óleo essencial na concentração de 10 e 40 μL.mL^{-1} causou uma rápida e constante diminuição (2 a 5 ciclos logarítmicos) na contagem de células viáveis de todas as cepas ensaiadas ao longo de 24 horas. O óleo essencial na concentração de 40 μL.mL^{-1} causou uma total eliminação do inóculo inicial de *S. aureus* depois de 2 horas. Estes resultados mostram a possibilidade do uso do óleo essencial extraído das folhas de *C. zeylanicum* como uma fonte alternativa de compostos antimicrobianos para aplicação em sistemas de conservação de alimentos.
Palavras-chave: *Cinnamomum zeylanicum Blume; óleo essencial; efeito antibacteriano.*

1 Introduction

Food conservation is based on an intermittent search for foods that have high nutritional quality and microbial stability, and it involves controlling the growth/survival of spoiling and pathogen foodborne microorganisms. The improvement of the shelf-life of foods has an important economic impact by reducing losses attributed to spoilage and allowing the products to reach distant and new markets. Consumers demand play a major role in the modifications of food supply and its demand, currently driven towards natural, safe, and additive free foods (LEMAY et al., 2002; LEUSCHNER; ZAMPARINI, 2002).

Chemicals are used to inhibit the microbial growth in/on foods, but the negative consumer perception of chemical preservatives directs attention toward the use of natural alternatives (RASOOLI; OWLIA, 2005). Recently, the antimicrobial potential of essential oils has been of great interest in both academia and food industry since their possible use as natural additives emerged from a growing tendency to replace synthetic additives (SAMAPUNDO et al., 2007; TZORTZAKIS; ECONOMAKIS, 2007). The antimicrobial effect of plants and derivatives has been scientifically proven in assays with essential oils, extracts, and isolated phytochemicals (SKANDAMIS; TSIGARDIA; NICHAS, 2002; RHAYOUR et al., 2003; SOUZA et al., 2007). Plants are characterized for possessing wide variation of volatile compounds, and many have been

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designated as Generally Recognized as Safe – GRAS (OZCAN; ERKMEN, 2001; VALERO; SALMERÓN, 2003).

In the Lauraceae family, the *Cinnamomum* genus (cinnamon) is a very popular spice throughout the world. *Cinnamomum zeylanicum* Blume originates from Ceylon, but it also native to South-East India. Its sensorial qualities are slightly sweet, pleasant, warm, and bitter, besides being strongly aromatic (BENARROZ et al., 2008).

C. zeylanicum has showed many biological applications due to its analgesic, antiseptic, antispasmodic, aphrodisiac, astringent, carminative, haemostatic, insecticidal, and parasiticide property. Barks from branches without the epidermis and subereous layer, is marketed as the commercial cinnamon which has a long use in perfumery, culinary, and native medicine systems (GAYOSO et al., 2005; SAMARASEKERA; KALHARI; WEERASINGHE, 2005). Previous studies have revealed interesting antimicrobial efficacy in essential oils obtained from *C. zeylanicum* branches and barks (VELLUTI et al., 2003; LIMA et al., 2005); however there has been a lack of studies focusing on the chemical composition and antimicrobial properties of essential oils obtained from its leaves.

This study aimed to analyze the chemical composition of the essential oil obtained from *C. zeylanicum* leaves and assess its inhibitory effect on the growth/survival of some food-related bacteria.

2 Materials and methods

2.1 Essential oil

The essential oil from *C. zeylanicum* leaves was obtained from Ferquima Ind. e Com. Ltda. (Vargem Grande Paulista – SP, Brazil), and its quality parameters (appearance, color, purity, odor, density –20 °C, and refraction index –20 °C) were described in a accompanying technical report. The extracts essential oils are extracted by the supplier on an industrial scale using the hydrodistillation procedure. The essential oil was assayed at concentrations ranging from 160 to 0.62 μL.mL^{-1}, and the solutions were prepared according to Souza et al. (2007).

2.2 Essential oil chemical analysis

Essential oil chemical composition was analyzed using a gas chromatograph (GC) fitted to a mass spectrophotometer (MS) (GC-MS, Shimadzu QP-5000, Kyoto, Japan) operating in electron-impact (70 eV, *m/z* 40 – 450) mode; the fused-silica capillary column used was an OV–5 with diameter of 30 m long., 0.25 mm i.d., 0.25 μm film thickness (Ohio Valley Special Chemical Inc., USA). The chromatographic conditions were as follows: sample preparation 1μL in 1 mL of hexane; injection volume 1 μL; split ratio 1:55; helium flow rate 1.0 mL/minutes; temperature programme ramp from 60 to 240 °C with a gradient of 3 °C/minutes (holding the initial and final temperature for 10 minutes); injector temperature 240 °C; and detector temperature 230 °C.

The identification of the essential oil components was performed by retention indexes and by comparing their mass spectra with a data bank (System GC-MS, Nist. 62 lib) and the literature (ADAMS, 1995; McLAFFERTY; STAUFER, 1989). Retention indexes were obtained by co-injection with a hydrocarbons (C_9-C_{24}) standard mixture using the van den Doll equation (van den DOOL; KRATZ, 1963).

2.3 Bacteria

Escherichia coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and *Salmonella enterica* ATCC 6017 strains were used as test microorganisms. The strains were supplied by National Institute of Quality in Health, FIOCRUZ, Rio de Janeiro, Brazil and Institute of Antibiotics, Federal University of Pernambuco, Recife, Brazil. Bacteria were kept on Nutrient Agar (NA) slants at 4 °C. Inocula were obtained from overnight cultures grown on NA slants at 37 °C and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10^6 count forming unit per mL (cfu.mL^{-1}) adjusted according to the turbidity of 0.5 McFarland scale tube.

2.4 Minimum inhibitory concentration and minimum bactericidal concentration

The microplate bioassay was used to determine the MIC of *C. zeylanicum* essential oil. 96-well plates were prepared by dispensing 100 μL of double strength Nutrient Broth (NB) inoculated with the bacterium inoculum into each well prior to the assay. An aliquot (100 μL) of the essential oil solutions at their respective concentrations was transferred into seven consecutive wells. The final volume in each well was 200 μL. The solution having the highest essential oil concentration was added into the first well and the one having the smallest concentration was added into the penultimate well. The last well, containing 200 μL of the NB inoculated with the bacteria suspension, was used as the positive control. The microplate was aseptically sealed, followed by mixing on a plate shaker (300 rpm) for 30 seconds and incubated at 37 °C for 24 hours (VILJOEN et al., 2003; SAHIN et al., 2004).

The antibacterial activity was detected using the colorimetric method by adding 200 μL of resauzurin staining (0.1 g.100 mL^{-1}) aqueous solution in each well at the end of the incubation period. MIC was defined as the lowest essential oil concentration able to inhibit the bacterial growth as indicated by resauzurin staining (dead cells were not able to change the staining color by visual observation – blue to red) (BURT; REINDERS, 2003).

2.5 Kill time assay

The Kill time assay was carried out with the oil at 10 and 40 μL.mL^{-1} and the viable cells count method was used. 5 mL of double strength NB was inoculated with 1 mL of the bacterial suspension. After that, 4 mL of the essential oil solution was added to the system and followed by shaking for 30 seconds using vortex. The system was incubated at 37 °C. At different time intervals (0, 1, 2, 4, 8, 12 and 24 hours) of exposure, 1 mL of the suspension was serially diluted (10^{-1} – 10^{-5}) in the sterile peptone water (0.1 g.100 mL^{-1}) and inoculated onto NA Petri dishes for 24 hours at 37 °C (VILJOEN et al., 2003). In the control

assay the essential oil solution was replaced by the sterile distilled water. At the end of the incubation period, the mean number of the colonies (cfu.mL^{-1}) was counted and compared with that found in the control assay. The results were expressed in log of cfu.mL^{-1}. All assays were performed in duplicate and the results were expressed as the average of the two parallel assays.

2.6 Statistical analysis

Statistical analysis was performed to determine significant differences ($p < 0.05$) by Tukey test using the SPSS 14.0 software.

3 Results and discussion

The composition of the essential oil from *C. zeylanicum* leaves was analyzed using GC-MS leading and employing the comparison of the relative retention times and the mass spectra of oil components with those of authentic samples and mass spectra from data library. As shown in Table 1, GC-MS of the oil resulted in the identification of 16 compounds making 95.95% of the oil. Eugenol (73.27%) was the most prevalent compound, followed for trans-β-cariophyllene (5.38%), linalool (3.31%), and alcohol cinamic acetate (2.53%). Other compounds such as α-pinene (1.31%), α-humullene (1.01%), *p*-cymene (1.24%), eugenol acetate (1.06%), and safrole (1.76%) were found in minor percentage. The oil showed to be constituted of different monoterpenes (e.g. α-pinene, β-pinene, phellandrene), terpene alcohol (linalool), sesquiterpene (humullene, caryophyllene), and phenols (eugenol).

The composition of the essential oils may change based on the differences of cultivation, origin, vegetative state, and growing seasons of the plants. However, eugenol and cinnamaldehyde have been reported as major compounds of the essential oil extracted from *C. zeylanicum* bark and branches of different origin. Cinnamaldehyde is related to be found in higher amount in the essential oil obtained from the leaves of the plant (SAMARASEKERA; KALHARI; WEERASINGHE, 2005; FICHI et al., 2006).

To our knowledge there are few studies regarding the chemical composition of the essential oil from *C. zeylanicum* leaves since most studies are addressed to the investigation of the chemical composition and biological properties of the essential oil obtained from its bark and branches. In accordance with the study by Fichi et al. (2006) on the composition of the oil from *C. zeylanicum* leaves, we found eugenol (76.10%), trans-β-caryophyllene (6.7%), linalool (3.7%), eugenol acetate (2.8%), and benzyl benzoate (1.9%). Lima et al. (2005) studying the chemical composition of the oil from the leaves and branches of *C. zeylanicum* noted a qualitative and quantitative different profile of constituents between the analyzed oils. Eugenol (60%) was the major compound found in the essential oil from leaves, whereas the oil from branches showed a higher range of compounds with linalool (10.6%), α-pinene (9.9%) and α-phellandrene (9.2%) as prevalent compounds.

The microdilution assay was used to find MIC and MBC of the oil from *C. zeylanicum* leaves against some food-related bacteria (Table 2). The oil exhibited marked inhibitory activity against all assayed strains. MIC and MBC values were in range of 1.25 – 10 μL.mL^{-1} and 20 – 80 μL.mL^{-1}, respectively. The smallest MIC (1.25 μL.mL^{-1}) value was found against *S. aureus* while the highest one was against *P. aeruginosa*.

López-Díaz et al. (2002) noted an interesting antimicrobial effect of the essential oil from *C. zeylanicum* leaves toward some pathogen bacteria. The authors reported that the oil was more effective in inhibiting the growth of Gram-negative bacteria in comparison to Gram-positive ones. Still, it was noted that *P. aeruginosa* was the least sensitive tested bacteria. Smith-Palmer, Stewart and Fyfe (1998) found a static effect of the essential oil from *C. zeylanicum* bark against *Campylobacter jejuni, Salmonella enteridis, E. coli, S. aureus* and *Listeria monocytogenes*.

The results of the effect of the essential oil from *C. zeylanicum* leaves at 10 and 40 μL.mL^{-1} on the cell viability (kill time) of some food-related bacteria are shown in Figures 1, 2, and 3. The oil provided significant reduction ($p < 0.05$) in viable cell count of the assayed strains with respect to the control assay. For all bacteria, the oil provided a steady killing-rate characterized for a concentration and time exposure dependent antibacterial effect. The oil caused a decrease in the viable cell count ranging from 2 to 5 log cycles along the evaluated times in comparison to the control assay.

Table 1. GC-MS analysis of the essential oil from *C. zeylanicum* leaves (results expressed in percent of oil total mass).

Peaks	Retention time (minute)	Compound	% of the oil
1	5.644	α–pinene	1.31
2	6.017	Camphene	0.45
3	6.218	Benzaldheyde	0.25
4	6.792	β–Pinene	0.48
5	7.641	α-phellandrene	1.29
6	8.293	*p*-cymene	1.24
7	8.471	β – phellandrene	1.57
8	11.164	Linalool	3.31
9	14.217	4-terpineole	0.12
10	19.133	Safrole	1.76
11	23.633	Eugenol	73.27
12	25.217	Trans-β-cariophyllene	5.38
13	26.133	Alcohol cinamic acetate	2.53
14	26.498	α–humullene	1.01
15	29.511	Eugenol acetate	1.06
16	31.708	Cariophyllene oxide	0.92

Table 2. MIC and MBC of the essential oil from *C. zeylanicum* leaves against some food related bacteria.

Bacteria	MIC (μL.mL^{-1})	MBC (μL.mL^{-1})	Strain viability*
E. coli ATCC 8739	2.5	80	+
P. aeruginosa ATCC 9027	10	-	+
S. aureus ATCC 6538	1.25	20	+
S. enterica ATCC 6017	5	40	+

*Capability of the bacterial strain to grow in NB without adding essential oil; (-): no bactericidal effect was noted.

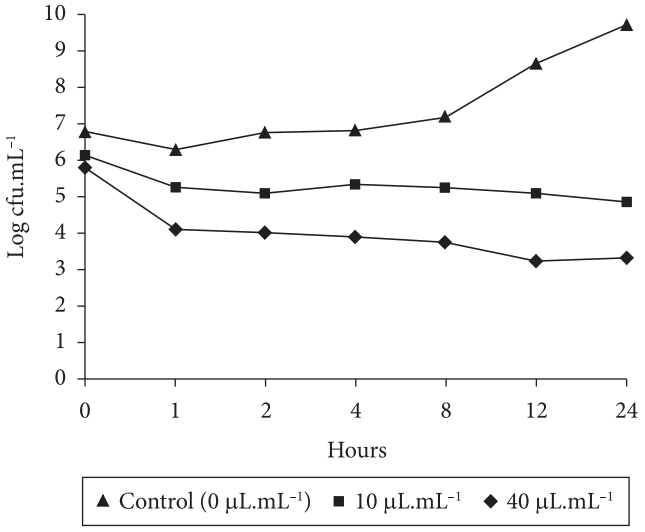

Figure 1. Survivors curves for *E. coli* ATCC 8739 in nutrient broth at different concentration of *C. zeylanicum* leaves essential oil at 37 °C.

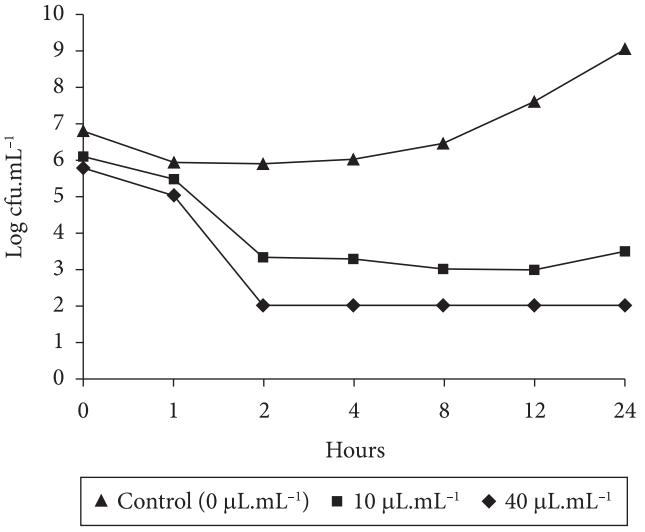

Figure 3. Survivors curves for *S. aureus* ATCC 6538 in nutrient broth at different concentration of *C. zeylanicum* leaves essential oil at 37 °C.

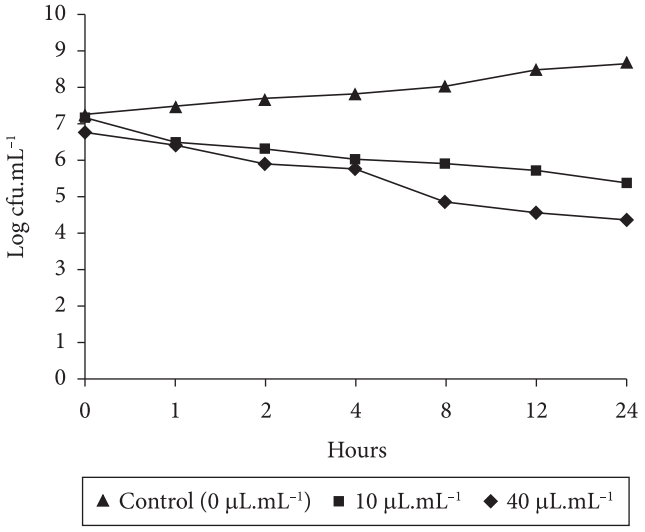

Figure 2. Survivors curves for *P. aeruginosa* ATCC 9027 in nutrient broth at different concentration of *C. zeylanicum* leaves essential oil at 37 °C.

The inhibition of the bacterial viability was rapidly exhibited and the viable count of *E. coli* and *S. aureus* was considerably lowered after 1 hour of exposure. For *P. aeruginosa,* the oil provided a significant drop in cell count just after 2 hours of exposure. Small regrowth was noted only for *S. aureus* after 12 hours to 10 µL.mL^{-1}, and it was the most sensitive bacteria showing a count around 2 log cycles after 2 hours of exposure to 40 µL.mL^{-1} of the oil.

Oxygenated and hydrocarbon terpenes (e.g. *p*-cymene, α-pinene, β-pinene, linalool and 4-terpineole) found in *C. zeylanicum* essential oil are believed to accumulate in the bacterial membrane and cause a loss of the membrane integrity, leakage of cytoplasmic content, dissipation of the proton motive force, cell lysis, and cell death (SIKKEMA; DE BONT; POOLMAN, 1995; GUSTAFSON et al., 1998). This action mode is similar to that found in membrane active disinfectants which cause protein denaturation and disruption of membrane structure. This action mode is similar to that found in membrane active disinfectants which cause protein denature and disruption of membrane structure (GARDNER; PELL, 1991). Plasma membrane permeability is integral to the maintenance of the cell energy status, other membrane-coupled-energy-transduction-processes, solute transport, regulation of metabolism, and control of turgor pressure (TRUNPOWER; GENIS, 1994; COX et al., 2000).

Eugenol, a phenolic found as the major compound of the assayed oil, is believed to exhibit antibacterial property by the inhibition of extracellular enzymes synthesis, disruption of the cell wall structure resulting in lack of cytoplasm, cytoplasm granulation, cytoplasm hyperacidity, and depletion of intracellular ATP pool (BURT, 2004).

The present study confirmed the antibacterial effect of the essential oil obtained from *C. zeylanicum* leaves based on MIC, MBC, and kill-time study against some food-related bacteria. Regarding our results, the essential oil from *C. zeylanicum* leaves presents a fast and steady antibacterial effect supporting its rational use as an alternative to be applied in the biocontrol of spoiling and pathogen bacteria in foodstuffs.

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