



Assessment of antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) essential oil combined with EDTA and polyethylene glycol in yogurt

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ABSTRACT. Essential oils (EOs) are technological options that may be employed in natural foods due to their antimicrobial activities. However, restrictions exist when high EOs concentrations are required which, in their turn, affect sensory qualities. Technological alternatives, such as combination of EOs with chelating and dispersing agents, have been proposed in the literature. Current research determined the antimicrobial activity of cinnamon EO against microbial spoilage in yogurt when added at the highest acceptable sensory EO concentration, alone or associated with ethylenediaminetetraacetic acid (EDTA) and/or polyethylene glycol. Cinnamon EO's chemical analysis was performed by gas chromatography-mass spectrometry (GC-MS). Sensory analysis was conducted to define the highest acceptable sensory concentration of cinnamon EO in yogurt, stipulated at 0.04% cinnamon EO. Antimicrobial activity in yogurt was then evaluated for aerobic mesophiles, psychrotrophic microorganisms, yeasts and molds counts. Treatments comprised (1) control, (2) 0.04% EO, (3) 0.04% EO + 0.01% EDTA, (4) 0.04% EO + 0.02% polyethylene glycol; (5) 0.04% EO + 0.01% EDTA + 0.2% polyethylene glycol, in triplicates. Concentration 0.04% of cinnamon EO, alone or associated with EDTA and/or polyethylene glycol, failed to show any antimicrobial activity against aerobic mesophiles, yeasts and molds.

Keywords: natural preservatives, microbial spoilage, dispersing agents.

Determinação da atividade antimicrobiana do óleo essencial de canela (*Cinnamomum zeylanicum*) combinado com EDTA e polietilenoglicol no iogurte

RESUMO. Óleos essenciais (OEs) são opções tecnológicas que podem ser empregados em alimentos naturais pelas suas atividades antimicrobianas, mas isso é restrito pela necessidade de elevadas concentrações, interferindo nos atributos sensoriais. Alternativas tecnológicas são propostas na literatura, incluindo a combinação de OEs com agentes quelantes e dispersantes. Este estudo objetivou determinar ação antimicrobiana de OE de canela contra deteriorantes microbianos em iogurte, quando adicionado na maior concentração aceitável sensorialmente de OE, isolado ou associado com etilenodiaminotetracético (EDTA) e/ou polietilenoglicol. A análise química do OE de canela foi realizada por cromatografia gasosa-espectrometria de massa (CG-EM). Primeiramente foi feita a análise sensorial para definir a porcentagem como a maior concentração aceitável sensorialmente de OE de canela em iogurte, estipulada em 0,04% de OE de canela. Foi avaliada a atividade antimicrobiana do OE de canela em iogurte, pela contagem de aeróbios mesófilos, psicrotóxicos e bolores e leveduras. Os tratamentos foram (1) controle, (2) 0,04% de OE, (3) 0,04% de OE + 0,01% de EDTA, (4) 0,04% de OE + 0,2% de polietilenoglicol e (5) 0,04% de OE + 0,01% de EDTA + 0,2% de polietilenoglicol, em triplicatas. A concentração de 0,04% de OE de canela, isolado ou combinado com EDTA e/ou polietilenoglicol não apresentou atividade antimicrobiana contra aeróbios mesófilos e bolores e leveduras.

Palavras-chave: conservantes naturais, deteriorantes microbianos, agentes dispersantes.

Introduction

Foods must be protected against microbial spoilage during their shelf life and should be pathogen-free. Since the demand for safe and natural products, without chemical preservatives, has increased among

consumers, research evaluating alternative techniques to preserve their microbiological quality and keeping their nutritional and sensory properties is on the increase (GOÑI et al., 2009).

Essential oils (EOs) are new technological options that may be employed as preservatives. They

have a broad-spectrum activity against bacteria, viruses, fungi, parasites and insects and may be potentially applied in pharmaceutical, sanitary, cosmetic, agriculture and food industries. Due to their extraction procedure, generally by steam distillation, they contain a variety of volatile molecules such as terpenes, terpenoids, aromatic compounds derived from phenol, and aliphatic components (BAKKALI et al., 2008).

Cinnamon (*Cinnamomum zeylanicum* Blume), Lauraceae family, has been used for flavoring for millennia. It is mentioned in the Bible and was employed as a balsamic fluid in Ancient Egypt. Its application is related to antibacterial, fungicidal and antioxidant activities (BARCELOUX, 2009). Cinnamon EO contains cinnamaldehyde as its major compound, coupled to β -caryophyllene, linalool and other terpenes. It is widely used as a food additive and flavoring agent and qualified as 'generally recognized as safe' (GRAS) by the Food and Drug Administration (BARCELOUX, 2009; TZORTZAKIS, 2009).

EO and resins may be extracted from the leaves and bark of the cinnamon plant. Singh et al. (2007) reported that EO from leaves and barks had antifungal action, whereas EO and resin from leaves had bactericidal activities. The same authors also mentioned that resins, from either leaves or barks, at 0.02% concentration, had excellent inhibitory activity against primary and secondary oxidation in mustard oil.

Kim et al. (2004) isolated cinnamic aldehyde from *Cinnamomum cassia* B. and the minimal inhibitory concentration (MIC) for *Escherichia coli* O157: H7. growth was 250 mg mL⁻¹, and 26.2 μ g mL⁻¹ against *Mycobacterium avium* subsp. *paratuberculosis* strain with cinnamon EO (WONG et al., 2008). The MIC of cinnamon EO ranged between 0.5 and 1% against *Staphylococcus epidermidis* strains isolated from human specimens (NURYASTUTI et al., 2009). MIC was 3.2 mg mL⁻¹ for *S. aureus*; > 1.6 mg mL⁻¹ for *Bacillus subtilis*; 3.2 mg mL⁻¹ for *Klebsiella pneumoniae*; > 1.6 mg mL⁻¹ for *Proteus vulgaris*; > 0.8 mg mL⁻¹ for *Pseudomonas aeruginosa*; > 1.6 mg mL⁻¹ for *Escherichia coli* (PRABUSEENIVASAN et al., 2006).

Several *in vitro* studies have been published on EOs and food-borne pathogenic bacteria mainly employing disk diffusion and micro dilution methods. Few studies have been conducted on EO's antimicrobial activity on foods, such as minimally processed vegetables (LANCIOTTI et al., 2004; GUTIERREZ et al., 2009), broccoli juice (MUÑOZ et al., 2009), barley (MOOSAVY et al., 2008), sausage (BUSATTA et al., 2007, 2008),

ground beef burger (BARBOSA et al., 2009), cheese (SMITH-PALMER et al., 2001), fermented milk (MORITZ et al., 2012) and yogurt (EVRENDILEK; BALASUBRAMANIAM, 2011; SINGH et al., 2011).

Although EOs and their compounds are highly efficient against pathogens and food-originated spoiling microorganisms, the same effect has been found only when higher EO concentrations are used in foods. However, this may also comprise an organoleptic impact exceeding the thresholds of acceptable flavor (BURT, 2004).

Busatta et al. (2007) reported that oregano (*Origanum vulgare*) EO in sausages may have a promising bacteriostatic effect, but sensory acceptance decreased with increasing oil concentrations. Although marjoram (*Origanum majorana*) EO in sausages (BUSATTA et al., 2008) had a bacteriostatic effect in *in vitro* MIC and a bactericidal effect at higher concentrations, changes in taste has been detected in the food.

Whereas Smith-Palmer et al. (1998) reported an *in vitro* bactericidal effect in less than 0.1% concentrations of EOs, Smith-Palmer et al. (2001) showed that EOs concentrations between 0.5 and 1% were required to inhibit food contamination. Furthermore, the use of insufficient concentrations allowed the restoration of non-lethally injured cells. When the chemical composition was verified in relation to fat, the food matrix proved to be an important factor in determining EOs' efficiency. Thus, the necessity of concentrations of essential oils in food higher than *in vitro* tests for antimicrobial activity may be assumed since the former is a complex environment protecting microbial cells.

Gutierrez et al. (2008, 2009) reported that different compounds have influenced the antimicrobial activity of EOs. In model food systems, EOs were more efficient against pathogenic bacteria when applied to media of high protein content and acidity. Moreover, low fat and carbohydrate contents and moderate levels of simple sugars were required.

Smith-Palmer et al. (2001) described the technological proposals to enhance the use of EOs as natural preservatives when adding them to products that already have a strong aroma, thereby masking their presence. Suggestions comprise the application of some major active components instead of the whole oil; the development of synergistic combinations between two EOs or between an EO and another antimicrobial; the combination of EOs with chelating agents, such as ethylenediaminetetraacetic acid

(EDTA) (although EDTA is not a preservative, it may potentiate other antimicrobials); the employment of EOs with dispersing agents, such as polyethylene glycol, to increase contact with microbial cells, especially in food with high lipid contents.

Ojagh et al. (2010) applied a solution of chitosan (2% v v⁻¹) and cinnamon EO (1.5% v v⁻¹) coating to trout and reported inhibition of lipid oxidation and increase of shelf life from 12 days (control) to 16 days of storage at 4°C. A sensory analysis was performed after three days of storage: the samples were prepared by steaming for 10-20 min at 98°C and 1.5% of salt were added. Results showed that coatings went unperceived and the cinnamon EO coating did not produce any undesirable sensory properties.

Current investigation determined the antimicrobial activities of cinnamon (*Cinnamomum zeylanicum* B.) EO against microbial spoilage (aerobic mesophiles, psychrotrophic microorganisms, yeasts and molds) in yogurt samples when added at the higher acceptable sensory concentration, alone or associated with ethylenediaminetetraacetic acid (EDTA) and/or polyethylene glycol.

Material and methods

Extraction and chemical analysis of cinnamon EO

Cinnamon (*Cinnamomum zeylanicum*) EO was achieved by steam distillation in a Clevenger-type apparatus (MA480 - Marconi) and its density was evaluated by weighing a volume of 1 mL (FONSECA; LIBRAND, 2008). Chemical characterization was done by gas chromatography-mass spectrometry (GC-MS) (QP5050A - Shimadzu), with a CBP-5 capillary column of 50 m length, 0.25 mm internal diameter and 0.25 µm film thickness. The injector temperature was 250°C; the interface temperature was 250°C; the detector operated in the electron impact (EI) mode at 70e V; the carrier gas was He. Chromatographic conditions were: initial temperature 60°C; heating up to 160°C at a rate of 3°C min.⁻¹; heating up to 220°C at a rate of 15°C min.⁻¹, with the maintenance of the latter temperature for 20 min. Identification of EO components were based on NIST (National Institute of Standards and Technology, MD, USA) for mass spectrum analysis and on data available in the literature (ADAMS, 1989).

Sensory analysis

Sensory analysis test was performed by 28 non-trained panelist of both sexes, aged between 20 and 50 years. The 9-point Hedonic Scale test, ranging

from *Like Extremely* (9) to *Dislike Extremely* (1) (POSTE et al., 1991), was applied. Current study was approved by the Ethics Committee in Research (Process 3126/2009 – CEP FMB/UNESP) of São Paulo State University 'Júlio de Mesquita Filho' and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All individuals gave their informed consent prior to their inclusion in the study.

Yogurt was obtained by inoculating pasteurized milk with Thermophilic Yoghurt Culture Yo-Flex® (Christian Hansen-Brazil). It was then incubated at 42°C until 0.80% lactic acid was formed and homogenized with 20% fruit pulp prepared with banana *in natura* and 10% sugar. Treatments were performed by adding cinnamon EO at concentrations 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2 and 0.4% (w w⁻¹).

After processing for 48h, two sensory analysis sessions, between 9:00 and 11:00 a.m. and between 3:00 and 5:00 p.m., were carried out to avoid panelists' sensory fatigue. Four samples of interspersed concentrations were served at each period.

Results were subjected to analysis of variance (ANOVA) and to Tukey's test by Statistical Analysis System (SAS, 1992) Minimum score 7.0, equivalent to *Like Moderately*, revealed the acceptable sensory concentration limit of cinnamon EO.

Cinnamon EO's antimicrobial activity

Cinnamon EO concentration used in current investigation was 0.04% w w⁻¹, which corresponded to the highest acceptable concentration established during sensory acceptance assays.

Yogurt was obtained as previously described and analytic units of 200 g were separated into aseptic flasks. The following treatments were established: (1) control; (2) addition of 0.04% EO; (3) addition of 0.04% EO associated with 0.01% (w.w⁻¹) EDTA; (4) addition of 0.04% EO associated with 0.2% (w w⁻¹) polyethylene glycol; and (5) addition of 0.04% EO associated with 0.01% EDTA and 0.2% polyethylene glycol (both w.w⁻¹). The analytical units were kept under refrigeration at 4°C for 48h until microbiological analyses and after serial dilutions were plated in the appropriate media. Total count was performed by surface plating for aerobic mesophiles (Plate Count Agar-Difco and 37°C / 48h), psychrotrophic microorganisms (Plate Count Agar-Difco and 7°C / 10 days), and yeasts and molds (Potato Dextrose-Difco and 25°C / 3 to 5 days) (DOWNES; ITO, 2001).

The experiment was performed in triplicates and data underwent analysis of variance (ANOVA) and Tukey's test by SAS (1992).

Results and discussion

Chemical characterization of cinnamon EO

Cinnamon EO has a density of 1.0049 g mL⁻¹. Figure 1 shows its chemical composition by GC-MS: 89.58% of the compounds were identified and its main compound was cinnamaldehyde, followed by cinnamyl acetate and cineole.

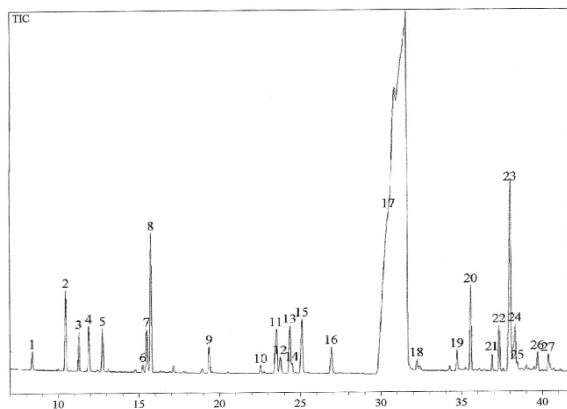


Figure 1. Chromatogram obtained for cinnamon essential oil sample used in the identification of compounds by Gas Chromatography-Mass Spectrometry (GC-MS); 88.59% compounds were identified. 2: α -pinene (1.62%); 3: camphene (0.79%); 5: β -pinene (0.93%); 6: cymene (0.17%); 7: limonene (1.11%); 8: cineole (3.31%); 9: linalool (0.55%); 12: borneol (0.50%); 13: terpinen-4-ol (1.22%); 15: α -terpinene (1.43%); 17: cinnamaldehyde (67.58%); 19: eugenol (1.82%); 22: caryophyllene (0.88%); 23: cinnamyl acetate (6.49%); 25: α -humulene (0.19%).

According to Domadia et al. (2007), the bactericidal effect of cinnamaldehyde occurs during cell division when the aggregation reaction of filamentation temperature-sensitive protein Z (FtsZ) decreases and GTP hydrolysis, necessary for FtsZ polymerization, is inhibited. FtsZ is a prokaryotic homolog of tubulin and regulates cell division by assembling into the Z-ring at the site of cell division. Cinnamaldehyde binds FtsZ, disrupts the cytokinetic Z-ring formation and inhibits its assembly dynamics, and consequently the bacterial cell division.

Cinnamaldehyde's activity in cell division may explain the bacteriostatic activity that cinnamon EO has on the bacterial contaminant in yogurt, which would result in lower population and consequently

longer shelf life in samples treated with cinnamon EO, when compared to control.

Sensory analysis

Yogurt acceptance by panelists decreased with increasing concentration of cinnamon EO in the food. According to Table 1, among the lowest concentration of cinnamon EO, 0.04%, there was no statistical difference in the average grades assigned, and the mean 6.28 was equivalent to *Like Slightly*, according to the Hedonic Scale. However, the median was 7.00 in the case of this concentration and might explain the 50% data for the option *Like Moderately*.

Table 1. Statistical analysis for yogurt scores in the sensory evaluation according to the Hedonic Scale.

Concentrations of cinnamon EO (% w w ⁻¹)	Mean
0.01	7.82 ^a ± 0.92
0.02	6.89 ^{ab} ± 1.75
0.04	6.28 ^{abc} ± 1.82
0.06	5.57 ^{bc} ± 2.25
0.08	4.57 ^{cd} ± 2.14
0.10	4.28 ^{cd} ± 2.26
0.20	2.61 ^{de} ± 1.91
0.40	1.92 ^e ± 1.43

The same letters in the same column indicate that means are statistically equal at $p < 0.05$.

Although studies have shown the antimicrobial action of EOs against food pathogens and spoilage, the concentrations established are high and cause sensory failures of the product. Assays start with the sensory acceptance concentration, followed by technological options that enhance the antimicrobial activity of EOs at low concentrations.

Assays on cinnamon EO's antimicrobial activity

The concentration of 0.04% of cinnamon EO was established to check the antimicrobial action in yogurt. Table 2 shows the results of the microbial counts assays.

In the case of microbial counts of psychrotrophic microorganisms, no growth lower than 2 log mL⁻¹ occurred in all treatments and control. It is worth mentioning that analyses were carried out after 48 hours of product's preparation, insufficient for the development of psychrotrophic contaminants.

Table 2. Microbiological analyses for different treatments of yogurt with the addition of cinnamon EO, EDTA and polyethyleneglycol. Means are expressed as log mL⁻¹.

Treatments	Mesophiles	Yeasts and molds	Psychrotrophilics
1: control	3.85 ^a ± 0.04	2.52 ^a ± 0.07	< 2 ^a ± 0.0
2: 0.04% cinnamon EO	3.91 ^a ± 0.05	2.64 ^a ± 0.18	< 2 ^a ± 0.0
3: 0.04% cinnamon EO + 0.01% EDTA	3.78 ^a ± 0.04	2.45 ^a ± 0.53	< 2 ^a ± 0.0
4: 0.04% cinnamon EO + 0.2% polyethylene glycol	3.73 ^a ± 0.03	2.20 ^a ± 0.35	< 2 ^a ± 0.0
5: 0.04% cinnamon EO + 0.01% EDTA + 0.2% polyethylene glycol	3.63 ^a ± 0.55	2.59 ^a ± 0.26	< 2 ^a ± 0.0

The same letters in the same column indicate that means are statistically equal at $p < 0.05$.

According to the standard counts of aerobic mesophiles and yeasts and molds, there was no statistically difference among treatments.

According to Smith-Palmer et al. (2001), the polyethylene glycol's dispersing activity may have improved oil and cells contact, facilitating the antagonist action, whereas the chelating action of EDTA on cations, present in the cytoplasmic membrane's destabilizing action, improved EO and caused cell death by lysis.

However, the association of cinnamon EO with the coadjuvants tested, EDTA and polyethylene glycol, didn't have a statistically significant reducing effect for aerobic mesophiles and for yeasts and molds.

Singh et al. (2011) applied oil and anise oleoresin in yogurt at concentrations 0.1 and 1.0 g L⁻¹, in both treatments. They observed that after 5 days of storage no significant difference occurred in total viable mesophilic counts and in yeasts and molds when compared to control. During storage time, counts in the control treatment tended to increase significantly and differed from treatments with oil and anise oleoresin.

Assay showed that at first no log reduction in aerobic mesophilic counts and in yeasts and molds occurred. Nevertheless, a bacteriostatic effect was perceived with the passage of storage time.

Besides the use of chelating agents and dispersants, there is also the possibility of combining the use of EOs with other preservative methods, as suggested by Evrendilek and Balasubramaniam (2011). These researchers combined 0.1% mint EO with high pressure processing which appeared to be a promising technique for preserving microbiologically-safe food with no significant impact on product quality.

Conclusion

The concentration of 0.04% cinnamon EO, the higher acceptable concentration for sensory analysis in yogurt, alone or associated with EDTA and/or polyethylene glycol, failed to show antimicrobial activity against aerobic mesophiles and yeasts and molds. Although EOs may be used as antimicrobial agents in natural food, in addition to their flavoring function, great technological challenges exist to adapt the above concentration to avoid food organoleptic failure and simultaneously preserve the antimicrobial property.

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