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Inactivation of *Cronobacter sakazakii* in reconstituted infant milk formula by plant essential oils

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Summary

This study aimed to screen the *in vitro* antimicrobial activity of 10 plant essential oils against 4 *Cronobacter sakazakii* strains, and use these oils or their combination to control *C. sakazakii* cocktail at low (3 $\log_{10}$ CFU/ml) and high (6 $\log_{10}$ CFU/ml) contamination levels in reconstituted infant milk formula (RIMF). Cinnamon and fir oils were the most inhibitory to *C. sakazakii* strains with inhibition zone of 32 to 40 mm at 20 µl/disc (the minimum inhibitory concentrations were 0.16 and 0.625 µl/ml, respectively). The addition of each of cinnamon or fir oil at 1 % (v/v) reduced the *C. sakazakii* numbers in RIMF by 0.7-0.8 $\log_{10}$ CFU/ml when inoculated with high contamination level and by 2.5-3.1 $\log_{10}$ CFU/ml when inoculated with low contamination level. However, the combination of cinnamon and fir oils reduced *C. sakazakii* numbers at both inoculum levels to undetectable levels after 3 h of incubation at 37 °C. The results of the current study indicated that a combination of cinnamon and fir oils has a potent antimicrobial activity which may potentially be used to control *C. sakazakii* in RIMF.

Introduction

Cronobacter sakazakii is an opportunistic pathogen that may cause bacteremia, sepsis, meningitis and necrotizing enterocolitis, mainly in neonates, preterm and low birth weight infants, and immunocompromised infants and adults with case fatality rate of 10 to 80 % (Nazarowec-White and Farber, 1997a; Yan et al., 2012). *C. sakazakii* has the ability to adapt several environmental stresses including chilling, heating, drying and osmotic stresses and natural antibiotics. This characteristic enables the organism to survive in foods and environments to cause foodborne illnesses (Lee and Jin, 2008; Osaili et al., 2008).

Although *C. sakazakii* has been isolated from several food sources including dairy products, meat products, vegetable origin food, water and other foods (Chap et al., 2009; Friedmann, 2007; Shaker et al., 2007), the *C. sakazakii* infection has been linked epidemiologically to the consumption of contaminated powdered infant milk formula (PIMF). *C. sakazakii* can be inactivated during milk pasteurization; however, post-processing contamination of PIMF is mainly responsible for the presence of *C. sakazakii* in these products. Furthermore, *C. sakazakii* has the ability to colonize on the surfaces of rehydrated infant milk formula (RIMF) preparation equipment and utensils such as brushes, bottles, and spoons (Nazarowec-White and Farber 1997a, b).

The microbiological safety of PIMF is very critical because infants are known to be particularly more vulnerable to foodborne infections (Drudy et al., 2006). To reduce the risk of *C. sakazakii* infections, Food and Agriculture Organization and World Health Organization (FAO and WHO, 2004) recommended using hot water (≥70 °C) in reconstitution of PIMF before feeding infants. However, the improper storage of RIMF may permit a significant growth of *C. sakazakii* which has a short lag time and generation time in RIMF (Nazarowec-White and Farber, 1997b). Therefore, incorporation of effective antimicrobials may have the potential to reduce the infection risk of *C. sakazakii* in infants by contaminated RIMF (Nair et al., 2004).

Increasing bacterial resistance to drugs has created problems in the treatment of several diseases. Since there is increased concerns regarding the safety of synthetic compounds and overuse of antibiotics as preservatives, interest in the development of effective natural antimicrobials as food preservatives has been increased recently (Abee et al., 1995; Nair et al., 2004). Several studies investigated the inhibitory effects of natural antimicrobials, such as animal-, plant-, and microorganism-derived antimicrobials against foodborne pathogens (Tajkarimi et al., 2010; Lee and Jin, 2008). Natural antimicrobial agents derived from plant sources such as essential oils have been recognized and used for centuries in food preservation. Edible, medicinal and herbal oils are potent antimicrobials against foodborne pathogens such as *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Shigella dysenteriae*, *Bacillus cereus* and *Staphylococcus aureus* (Tajkarimi et al., 2010). However, few studies have investigated the inhibitory effects of carvacrol, thymol, eugenol and cinnamic acid (Lee and Jin, 2008) or trans-cinnamaldehyde (Amalaradjou et al., 2009) against *C. sakazakii*. Other individual or combined essential oils may have a potential to reduce *C. sakazakii* in RIMF. Therefore the objectives of the current study were to: i) screen the antimicrobial activity of 10 medicinal plant essential oils against 4 strains of *C. sakazakii,* ii) determine the minimum inhibitory concentration (MIC) of cinnamon and fir oils against *C. sakazakii* strains, and iii) use these oils or their combination to control *C. sakazakii* cocktail at low (3 $\log_{10}$ CFU/ml) and high (6 $\log_{10}$ CFU/ml) contamination levels in RIMF.

Materials and methods

Preparation of *C. sakazakii* cultures

Four strains of *C. sakazakii* isolated from infant formula in Jordan were obtained from the bacterial culture collection of the Department of Nutrition and Food Technology at Jordan University of Science and Technology: CS1; CS14; CS15 and CS19 (Shaker et al., 2007; Chap et al., 2009). Each strain of *C. sakazakii* was kept in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK) containing 20 % glycerol at -40 °C. The strains were individually maintained on BHI agar slants at 4 °C and transferred bi-weekly to maintain viability. To prepare the working cultures, one loopful of each strain was transferred to 10 ml BHI broth and incubated at 37 °C for 24 h. A volume of 100 µl of the overnight strain cultures was transferred to 10 ml BHI broth and incubated for 20-24 h at 37 °C. A cocktail of *C. sakazakii* strains was prepared by mixing equal proportions of each of the four strains into a sterile container and diluted with a sterile 0.1 % peptone water (Oxoid, Basingstoke, UK) to obtain the desired inoculum level.

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Medicinal plants and essential oils extraction

The essential oils of 10 different medicinal plants (cinnamon, anise, eucalyptol, chamomile, fenugreek, fennel, fir, pooh, qysoom and rosemary) were obtained from a local extraction facility in Amman, Jordan. Essential oils were extracted by using hydro-distillation procedure. The oils were kept in a sealed dark glass vial at 4 ºC until required.

Screening of antimicrobial activity of medicinal plant essential oils

Disc-diffusion method was used to determine the antimicrobial activity of the 10 essential oils against 4 *C. sakazakii* strains. Briefly, 20 µl of each essential oil was dispensed onto 6 mm-diameter sterile filter paper discs which were dried by air. Each disc was placed on tryptic soy agar (TSA, Oxoid, Basingstoke, UK) plates which were previously inoculated with approximately 5.0 $\log_{10}$ CFU/ml of the individual *C. sakazakii* strains. Plates were incubated at 37 °C for 24 h and the inhibitory zones were measured in millimeter using a caliper. The synergistic activity of essential oils mixture that showed the maximum antibacterial activity (cinnamon and fir oils) was prepared by mixing equal volumes of each essential oil, and then 20 µl of the mixture was applied onto 6 mm-diameter sterile filter paper (Awaisheh, 2013).

Determination of the minimum inhibitory concentration of cinnamon and fir oils against *C. sakazakii* strains

MICs of cinnamon or fir oils were determined using 96-wells Micro-Dilution method. A 100 µl of overnight culture containing 6.0 $\log_{10}$ CFU/ml of each *C. sakazakii* strain and 100 µl of cinnamon and fir oils dilution were added with 200 µl total volume in each well. A two-fold serial dilution was used to yield concentrations of 0.08 to 100 µl/ml essential oils. Then, the sealed plates were incubated for 24 h at 37 °C and *C. sakazakii* growth in each well was measured by reading the respective absorbance at 600 nm using a microplate reader (ELx 800, Biotek, Highland Park, VT, USA). Absolute dimethyl sulfoxide (DMSO) containing essential oils added to sterile media and DMSO added to the bacterial suspension were used as negative and positive controls, respectively. MIC of the essential oils is the lowest dilution which inhibits the bacterial growth (Awaisheh, 2013).

Relative antimicrobial activity of cinnamon and fir oils combination to selected antibiotics

The inhibitory efficacy of cinnamon and fir oils combination at 1% (v/v) against *C. sakazakii* cocktail was compared with selected antibiotics using disc-diffusion method as described above. Antibiotic discs (6 mm in diameter) of 5 reference antibiotics including: amikacin (30 µg), azithromycin (15 µg), ceftriaxone (30 µg), gentamicin (10 µg), and nalidixic acid (30 µg) were obtained from ARCOMEX medical supplies company (Amman, Jordan). The ratio of the inhibition zone (mm) produced by the cinnamon and fir oils combination and the reference antibiotics was used to express the relative antimicrobial activity of cinnamon and fir oils combination.

Inactivation of *C. sakazakii* in reconstituted infant milk formula model by cinnamon and fir oils or their combination

Commercial PIMF was reconstituted according to the manufacturer's instructions with sterile distilled water. Under aseptic conditions, approximately 7.7 g PIMF was reconstituted with 60 ml water and mixed using a magnetic stirrer at 350 rpm for 25 min at 100 °C until completely dissolved. A cocktail culture of 4 strains *C. sakazakii* by mixing equal volume from each strain at 4.0 log or 7.0 log CFU/ml was inoculated into 10 ml of RIMF (control) or RIMF containing either 1% (v/v) of cinnamon oil, fir oil, or cinnamon and fir oils mixture. The samples were incubated at 37 °C for 6 h and the *C. sakazakii* numbers at 0, 3 and 6 h were carried out as described by Osaili et al. (2010) with minor modification. A 100 µl of each sample was spread on plates of Violet Red Bile Agar (VRBA, Oxoid, Basingstoke, UK) supplemented with 2% glucose which were incubated at 37 °C for 24 h.

Statistical analysis

The experiments were replicated 4 times using two samples per experiment (N=8). All data represents the mean value ± standard deviation (SD). One-way ANOVA and multiple range tests were used to evaluate differences among means using the least significance difference (LSD) test at a significance level of $P < 0.05$.

Results and discussion

Antimicrobial activity of 10 medicinal plant essential oils

The antimicrobial activities of essential oils obtained from 10 different plant species against 4 *C. sakazakii* strains were determined (Tab. 1). These plants showed to have some antimicrobial properties against wide range of foodborne pathogens (Awaisheh, 2013). The average zone of inhibition with 20 µl of essential oils ranged from 0.0 to 40.0 mm. Cinnamon and fir oils were the strongest antimicrobials against tested *C. sakazakii* strains with a range of inhibition zone of 32 to 40 mm. However, chamomile and rosemary oils showed weak antimicrobial activities against *C. sakazakii* strains with inhibition zone range of 4.5 to 6.0 and 3.0 to 8.0, respectively. While other plant (anise, eucalyptol, fennel, fenugreek, pooh, and qysoom) essential oils did not exhibit antimicrobial activity against tested *C. sakazakii* strains. Al-Nabulsi et al. (2009) also found that herbal infant teas such as chamomile and fennel supported growth of *C. sakazakii* at 37 and 21 °C. The antimicrobial activity tested essential oils (except cinnamon oil) in the present study against

Tab. 1: Antimicrobial activity of 10 medicinal plant essential oils (20 µl/disc) against 4 *C. sakazakii* strains using disc diffusion method at 37 °C.

C. sakazakii strains	Inhibition zone diameter (mm) [1, 2]									
	Anise	Cinnamon	Chamomile	Eucalyptol	Fennel	Fenugreek	Fir	Pooh	Rosemary	Qysoom
C. sakazakii CS1	0.0±0.0^{c}	32.0±3.1^{a}	5.0±0.2^{b}	0.0±0.0^{c}	0.2±0.1^{c}	0.2±0.1^{c}	34.0±2.1^{a}	2.0±0.1^{c}	3.0±0.2^{bc}	2.0±0.1^{c}
C. sakazakii CS14	1.0±0.1^{c}	35.0±2.9^{a}	5.0±0.1^{b}	0.2 ± 0.1^{c}	0.5±0.1^{c}	1.0±0.1^{c}	32.0±2.1^{a}	1.5±0.1^{c}	3.0±0.2^{bc}	1.5±0.1^{c}
C. sakazakii CS15	0.5±0.1^{c}	40.0±3.4^{a}	4.5±0.2^{b}	0.5 ± 0.1^{c}	1.0±0.1^{c}	0.0±0.0^{c}	40.0±1.8^{a}	1.0±0.1^{c}	4.5±1.0^{bc}	1.0±0.1^{c}
C. sakazakii CS19	1.0 ±0.1^{c}	38.0±2.1^{a}	6.0±0.2^{b}	0.5 ± 0.1^{c}	1.0±0.1^{c}	1.0±0.1^{c}	36.0±2.4^{a}	1.0±0.1^{c}	8.0±0.4^{b}	1.0±0.1^{c}

[1] Values are mean of 4 replicates ± standard deviation

[2] Values with different letters in the same row are significantly different ($P < 0.05$).

C. sakazakii have not been investigated as they did not show inhibitory effect against *C. sakazakii* strains. However, several studies reported their inhibitory activity against other foodborne pathogens (BAĞCI and DIĞRAK, 1996; KON and RAI, 2012). The findings of the current study are consistent with those of PRABUSEENIVASAN et al. (2006) and KON and RAI (2012) who found that cinnamon oil had the strongest inhibitory activity among 21 to 35 plant essential oils against Gram-negative and Gram-positive bacteria. BAĞCI and DIĞRAK (1996) also found that essential oil from different species of fir had antimicrobial activity against *Bacillus spp., Pseudomonas aeruginosa, L. monocytogenes, Klebsiella pneumoniae, Enterobacter aerogenes and S. aureus*.

Minimum inhibitory concentration of cinnamon and fir oils against C. *sakazakii* strains

Based on the results of disc diffusion method for screening of antimicrobial activity of 10 plant essential oils against *C. sakazakii* strains, cinnamon and fir oils showed the highest zone of inhibition and these were selected to determine their MIC values against *C. sakazakii* strains (Tab. 2). The MICs of cinnamon and fir oils were 0.16 and 0.62 µl/ml, respectively, against all tested *C. sakazakii* strains. The MICs of essential oils such as carvacrol, thymol, eugenol and cinnamic acid was found to be 1.25, 1.25, 5.0 and >5.0 mmol/L, respectively against 2 *C. sakazakii* strains (LEE and JIN, 2008). The results of current study are similar to those reported by GHOSH et al. (2013) who found that MIC of cinnamaldehyde was 0.263 mg/ml against C. *sakazakii*. Similarly, KON and RAI (2012) found that MIC of cinnamon oil was 0.2 mg/ml against *S. aureus*. The MIC of fir oil in the current study was slightly lower than those reported by OH et al. (2007) who found that the MICs of fir oil against *E. coli* and *Staphylococcus epidermidis* were 2.05 and 3.81 mg/ml, respectively.

Tab. 2: Minimum inhibitory concentration (MIC) values of cinnamon and fir oils against 4 *C. sakazakii* strains at 37 °C.

C. sakazakii strain	MIC (µl/ml)[1]	
	Cinnamon	Fir
C. sakazakii CS1	0.160	0.625
C. sakazakii CS14	0.160	0.625
C. sakazakii CS15	0.160	0.625
C. sakazakii CS19	0.160	0.625

[1] Values are mean of 4 replicates

Relative antimicrobial activity of cinnamon and fir oils combination to selected antibiotics

The effectiveness of cinnamon and fir oils combination on inhibition of *C. sakazakii* cocktail was compared to the standard antibiotics (Tab. 3). The cinnamon and fir oils combination showed higher antimicrobial activity against *C. sakazakii* than the reference antibiotics. The combination of cinnamon and fir oils significantly inhibited the growth of *C. sakazakii* cocktail by 38.5 mm on plates. However, ceftriaxone had the strongest inhibitory effect (20.5 mm) among antibiotics tested against *C. sakazakii* cocktail, while gentamicin showed the weakest activity (8.0 mm). The relative antimicrobial activity of cinnamon and fir oils combination to gentamicin, nalidixic acid, amikacin, azithromycin and ceftriaxone were 482%, 412%, 405%, 385% and 188%, respectively. Although the inhibitory effect of cinnamon and fir oil combinations against *C. sakazakii* was obvious, it should be noted that the concentration of the oil mixtures (20 µl, approximately 20 mg) was greater than the concentrations of the standard reference antibiotics (10 to 30 µg). Previous studies have demonstrated the antimicrobial resistance of *C. sakazakii* to the antibiotics (AL-NABULSI et al., 2011; FARAJNIA et al., 2009; SAAED and MUSSALAM, 2011). Therefore, using natural antimicrobial agents such as cinnamon and fir oils may be used as alternative to overcome emerging of antibiotics resistance thus reducing the risk associated with *C. sakazakii* infection.

Inactivation of *C. sakazakii* in RIMF by cinnamon and fir oils or their combination

The inhibitory effects of cinnamon and fir oils or their combination against a 4 strains *C. sakazakii* cocktail at high (6.0 $\log_{10}$ CFU/ml) or low (3.0 $\log_{10}$ CFU/ml) inocula in RIMF during 6 h of incubation at 37 °C was investigated (Tab. 4). In the control samples (without essential oils), *C. sakazakii* gradually increased from 3.1 and 6.1 $\log_{10}$ CFU/ml to reach 6.3 and 8.4 $\log_{10}$ CFU/ml, respectively after 6 h at 37 °C. The addition of 1% cinnamon or fir oils alone reduce the viability of *C. sakazakii* with low inoculum level by 3.1 and 2.5 $\log_{10}$ reduction of *C. sakazakii*, respectively, compared to the control. Similarly, with higher initial inoculum level (6.0 $\log_{10}$ CFU/mL), cinnamon or fir oils reduced the numbers of *C. sakazakii* by 0.7 and 0.8 $\log_{10}$ CFU/ml, respectively, after 6 h. However, the *C. sakazakii* cells were not detected when RIMF incorporated with 1% of cinnamon and fir oils mixture after 3 h of incubation at both inoculum levels. This demonstrates that the combination of cinnamon and fir oils could be used as a practical approach to reduce the risk of *C. sakazakii* in RIMF and to improve the safety of these consumable products. The antimicrobial effect of cinnamon oil has been well studied against *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* (OUSSALAH et al., 2007). But there are still limited studies on

Tab. 3: Relative antimicrobial activity for combination of cinnamon and fir oils (20 µl/disc) to selected antibiotics against a 4 strains *C. sakazakii* cocktail using disc diffusion method at 37 °C.

C. sakazakii Cocktail	Cinnamon and fir oils mixture (20 µl/disc)[1]	Reference antibiotics (concentration (µg/disc))[1]				
		Amikacin (30 µg)	Azithromycin (15 µg)	Ceftriaxone (30 µg)	Gentamicin (10 µg)	Nalidixic acid (30 µg)
Inhibition zone diameter (mm) [2, 3]	38.5±3.5[a]	9.5±1.5[c]	10.0±2.1[c]	20.5±2.2[b]	8.0±1.5[c]	9.0±1.4[c]
Relative antimicrobial Activity[4]	100%	405%	385%	188%	482%	412%

[1] Concentration of the oil mixtures is greater than concentrations of the standard reference antibiotics.
[2] Results are means of 4 replicates ± standard deviation.
[3] Values with different letters in the same row are significantly different ($P < 0.05$).
[4] Relative antimicrobial activity of cinnamon and fir oils mixture = (inhibition zones of cinnamon and fir oils mixture ÷ inhibition zones of antibiotic) x 100%

Tab. 4: *C. sakazakii* numbers (log_{10} CFU/ml) in RIMF in the presence of 1% (v/v) of cinnamon oil, fir oil, or their combination at 37 °C for 6 h.[1, 2]

Treatments	*C. sakazakii* numbers (log_{10} CFU/ml) at low level			*C. sakazakii* numbers at (log_{10} CFU/ml) at high level		
	0 h	3 h	6 h	0 h	3 h	6 h
Control	3.08±0.22[a]	4.70±0.50[a]	6.26±0.40[a]	6.08±0.34[a]	7.11±0.61[a]	8.37±0.43[a]
Cinnamon oil	3.04±0.25[a]	3.00±0.19[b]	3.18±0.25[b]	6.04±0.45[a]	6.07±0.55[b]	7.67±0.51[b]
Fir oil	3.08±0.15[a]	3.18±0.23[b]	3.78±0.21[b]	6.04±0.50[a]	6.20±0.45[a]	7.56±0.66[b]
Cinnamon and fir oils Mixture	3.05±0.12[a]	ND	ND	6.08±0.52[a]	ND	ND

[1] Values are means of 4 replicates ± standard deviation.
[2] Values with different letters in the same column are significantly different ($P < 0.05$).
ND = Not detected (Detection level was < 1 log_{10} CFU/ml)

essential oils against *C. sakazakii* in RIMF. The results of current study are similar to those reported by Amalaradjou et al. (2009) who found that 0.5% trans-cinnamaldehyde reduced the *C. sakazakii* numbers to undetectable levels (> 6 log_{10} CFU/ml reduction) by 4 h at 37 or 23 °C and by 10 h at 8 or 4 °C). It has been reported that mechanisms of cinnamaldehyde (the major component of cinnamon oil) action is related to inhibition of different enzymes involved in cytokinesis, action as an ATPase inhibitor and/or disturb the cell membrane (Hyldgaard et al., 2012). Kim et al. (2009) also reported that muscadine seed extracts reduced *C. sakazakii* numbers (6 log_{10} CFU/ml) to undetectable levels by 1 h at 37 °C. Osaili et al. (2009) found that *C. sakazakii* had lower growth in wheat-based infant follow-on formulas reconstituted with grape or apple juices compared to those reconstituted with water or milk 25 and 37 °C. The potent antimicrobial activity of these plant compounds is attributed due to the presence of several phenolic compounds, organic acids, and variety of active components in essential oils (Kim et al., 2009; Amalaradjou et al., 2009). The phenolic compounds may cause bacterial membrane damage and disrupt the cell wall peptidoglycan. Also, the hydroxyl group in phenolic compounds may bind the active sites of enzymes and change their substrate affinity. Moreover, the lipid solubility of phenolic compounds and their degree of steric hindrance may also contribute to their overall antimicrobial activity (Ceylan and Fung, 2004).

Conclusions

Among 10 plant essential oils, cinnamon and fir oils showed the strongest antimicrobial activity against *C. sakazakii* strains with a range of inhibition zone of 32 to 40 mm. The relative antimicrobial activity (inhibition zone ratio) of 1% cinnamon and fir oils mixture compared to amikacin, azithromycin, ceftriaxone, gentamicin and nalidixic acid ranged from 188% to 482% against *C. sakazakii* cocktail. Cinnamon and fir oils combination at 1% (v/v) eliminated the *C. sakazakii* numbers (> 6 log_{10} CFU/ml reduction) by 3 h at 37 °C. FAO/WHO (2004) recommended that RIMF should be stored at room temperature for less than 2 h. It is evident from the results of this study that combination of cinnamon and fir oils caused a strong and rapid antimicrobial effect against *C. sakazakii*, therefore cinnamon and fir oils combination may have a potential to control *C. sakazakii* in RIMF. However, effect of cinnamon and fir oils on the sensory attributes of RIMF is required.

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