ORIGINAL RESEARCH ARTICLE

Increased antibacterial activity of Cinnamon Oil Microemulsionin Comparison with Cinnamon Oil Bulk and Nanoemulsion

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ABSTRACT

Objective(s): Among herbal oils, cinnamon bark oil has several advantages such as anti-inflammatory and antimicrobial activity. It is already reported that particle size of oil droplets affects their properties such as their antibacterial activity. In this study, we investigated inhibitory activity of cinnamon oil products including bulk, microemulsion (ME) and nanoemulsion (NE).

Methods: ME and NE were prepared by low energy methods. Physicochemical characterization of cinnamon oil ME (COME) and NE (CONE) were investigated. Bulk, ME and NE of cinnamon oil were evaluated for antibacterial inhibitory activity against *Escherichia coli* and *Staphylococcus aureus* by microdilution method.

Results: Average particle size of COME and CONE was found to be 2040 nm and 30.4 nm, respectively. Results showed that both CONE and COME had increased inhibitory activity ($p<0.05$) against bacterial infection compared with ablank control group, of which COME had highest antibacterial effects.

Conclusions: Our findings suggested COME and CONE as potential green antibacterial agents.

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INTRODUCTION

Multidrug resistant bacteria have become a major challenge to food preservatives andantibiotics.So, it is necessary to find new substances with antimicrobial properties for use asantibacterial/preservative agents (1, 2). It has been reported that herbal oils (such as cinnamon oil, eucalyptus oil andgarlic oil) as natural antimicrobial substances can effectively inhibit bacterial growth by morphological destruction of bacteria (3, 4). Among these herbal oils, cinnamon oil has various clinical uses such as relieving stomachache, improving general blood circulation as well as treating diarrhea and digestive tract discomforts (5). In food industries, cinnamon oil is used as an additive flavor and aroma with broad-spectrum activity against foodborne pathogenic microorganisms (4, 6-10). Cinnamon oil has shown activity against yeasts, filamentous molds, dermatophytes and bacteria (5). It can also inhibit growth of *Aeromonas* spp. and *Lactococcus* spp (11). Moreover, cinnamon oil nanoemulsion (CONE) has been reported to successfully inhibit growth of *Bacilluscereus* (12).

Herbal oils could be applied as oil phase in emulsion systems to increase their efficacy,for example in case of their antibacterial activity (13). Emulsions are oil based drug delivery systems containing a mixture of two immiscible phases (such as oil and water) that are usually stabilized by surfactants (and co-surfactants). Emulsions

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have different types according to their droplet size including nanoemulsions (NEs), microemulsions (MEs) and coarse (bulk) emulsions. NEs are dispersions of oil and water with droplet size below 100 nm, while droplet size in MEs is above 100 nm, up to few microns.

In drug delivery systems, particle size has shown to be an important factor. For example, Ma *et al*. showed that minimum inhibitory concentration (MIC) of cinnamon oil microemulsion (COME) against *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*was equal to or higher than MIC of bulk cinnamon oil dissolved in ethanol (14). The results indicated that bulk cinnamon oil has higher anitibacterial activity in comparison with COME. In another study, antibacterial activity of bulk and nanoemulsion of peppermint oil was evaluated using an agar dilution method. The results showed that MIC of bulk and nanoemulsion of peppermint oil were similar against *L.monocytogenes* and *S.aureus* (15). However, limited number of such studies, especially in case of cinnamon oil, cannot provide enough data about a comprehensive conclusion about the effect of size of emulsions on their antibacterial activity.

Main objective of the present study was to investigate inhibitory activity of NE, ME and bulk of cinnamon bark oil against *E.coli* and *S.aureus* based on broth microdilution method.

MATERIALS AND METHODS

Materials

Tween 80, Tween 85, ethanol, phosphate buffer saline (PBS), pH strips (pH 0-14) universal indicator, nutrient broth and agar were purchased from Merck Millipore (Germany). Purified cinnamon oil in carrier oil (sesame oil) was purchased from Varona (Tehran, Iran). *E.coli* (ATCC 25922) and *S.aureus* (ATCC 25923) were obtained from Pasteur Institute of Iran (Tehran, Iran).

Preparation of bulk, nano- and microemulsion

Bulk dispersion of cinnamon oil was prepared by dispersing the oil in water at 5% (v/v). COME and CONE were prepared using low-energy method with a magnetic stirrer. Cinnamon oil (oil phase), a mixture of Tween 80 and Tween 85 (surfactant system), ethanol (co-surfactant), and distilled water (water phase) were used to prepare CONE and COME. Briefly, 5% (v/v) oil and 25% (v/v) surfactants were mixed, followed by adding 15% (v/v) ethanol and 55% (v/v) distilled water, and were stirred at 800 RPM for 90 minutes. Composition of NE, ME and bulk of cinnamon oil are given in Table 1.

Particle Size Analysis

Particle size was analyzed using Dynamic Light Scattering (DLS, Scatteroscope I, K-ONE LTD, Korea) at 25°C.

pH Measurement

pH of the formulations was measured by pH indicator at 25°C.

Microbial cultures

Two strains of *E. coli* (gram-negative bacteria) and *S. aureus* (gram-positive bacteria) were selected as representative bacterial species and stored at -20 °C in glycerol 10%. Working cultures were obtained by transferring few stock colonies from nutrient agar into 10 ml sterile nutrient brothby loop, then, cultured at 37°C for 16 h in incubator.

Determination of Inhibitory Activity

Fresh bacterial colonies were dissolved in 10 ml of normal saline by loop to reached 0.5 McFarland (1×10^8cfu/ml) turbidity. According to previous studies with slight modification, 96-well plate microdilution method was used to determine inhibitory activity of cinnamon oil products (16, 17). Serial dilutions of the concentrated cinnamon oil products, ranging from 0.56-1.99 % (v/v), were prepared in PBS. Fifty microliters of diluted samples was added to each well (n=3). Then, 50 µl of sterile nutrient broth (2X) was added and followed by addition of 10 µl of bacterial suspension. Bacteria-free wells served as blanks and cinnamon oil products-free wells served as negative controls

Table 1.Composition of cinnamon oil products.

Samples	Oil% (v/v)	Surfactants% (v/v)		Ethanol% (v/v)	Distilled Water% (v/v)
		Tween 80	Tween 85		
CONE	5	5	20	15	55
COME	5	8	17	15	55
Bulk	5	-	-	-	95

(growth control). Plates were incubated at 37 ºC for 16h. Then, absorption was recorded at 630 nm by microplate reader (BioTek Instruments, Inc., USA). All data were normalized with equation 1:

$$Growth\ (normalized) = \frac{absorbsion\ of\ treatment\ wells}{absorbtion\ of\ negative\ control\ wells} \times 100 \quad (1)$$

Statistical analysis

All results were reported as average ± standard deviation. One-sample T test, One-way ANOVA analysis and least significant difference (LSD) comparison tests between the samples were performed to find the effect of the size on inhibitory activity at significant level of $p<0.05$.

RESULTS

Characterization of CONE formulation

d_{50} (median hydrodynamic diameter) of CONE and COME formulations were 30.4 nm and 2040 nm, respectively. COME showed a degree of turbidity while CONE was optically transparent with a yellowish color (see Fig. 1). pH of the samples was about 5 at 25°C.

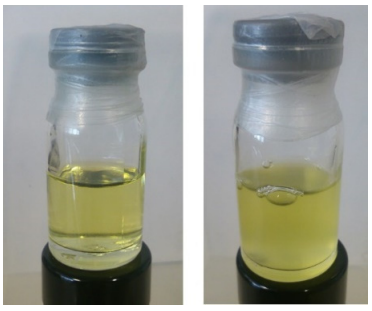

Fig. 1. Picture of CONE (left) and COME (right)

Inhibitory activity of cinnamon oil products

Cinnamon oil products were tested for inhibitory activity on two strains of *E. coli* and *S. aureus* using broth microdilution method (18).

Bulk cinnamon oil

Fig. 2 shows the effect of bulk cinnamon oil on *E.coli* and *S. aureus*. From the details, no important effect from the bulk oil may be suggested on the two bacteria. There is no significant difference ($p>0.05$) between negative control (growth control) with bulk cinnamon oil on inhibitory activity against *E.coli* and *S.aureus* (Table 2).

Cinnamon oil Microemulsion

From Fig. 3 which shows effect of COME on *E. coli* and *S. aureus*, COME shows inhibitory effects on both tested bacteria. This effect is associated with slight fluctuations when the concentration of COME varies. Overall, there are significant differences between negative control and COME on againts *E.coli* ($p<0.01$) and *S.aureus* ($p<0.001$) (Table 2).

Cinnamon oil Nanoemulsion

Fig. 4 illustrates inhibitory activity of CONE against *E. coli* and *S. aureus*. From the data, significant differences between negative control (growth control) and CONE on *E. coli* and *S. aureus* may be observed.

Comparisons of cinnamon oil products

For better understanding the different inhibitory activities between cinnamon oil products, statistical analysis was carried out. One-way

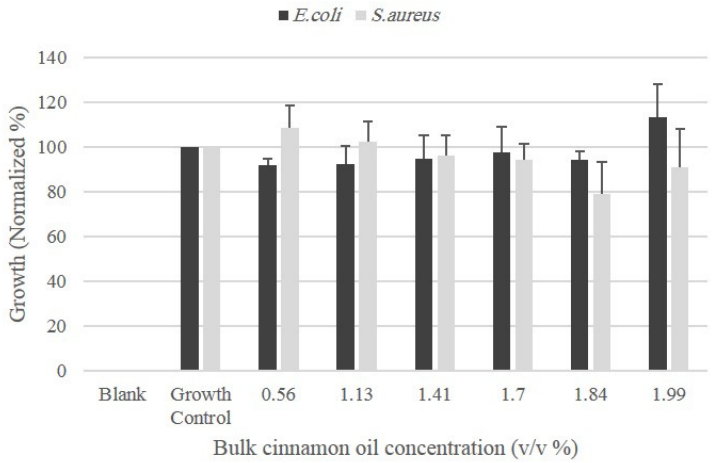

Fig. 2. Mean (SD) inhibitory activity of bulk cinnamon oil against *E. coli* and *S. aureus* (n=3).

Table 2. Results of student's t-test for antibacterial inhibitory of cinnamon oil products.

	Bacterial species	Significant (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
				Lower	Upper
Bulk	*S. aureus*	0.305	-4.78333	-15.5406	5.9740
	E. coli	0.392	-3.33333	-12.4741	5.8074
COME	*S. aureus*	0.000	-58.15000	-63.8036	-52.4964
	E. coli	0.002	-45.83333	-65.9696	-25.6971
CONE	*S. aureus*	0.001	-16.08667	-22.3691	-9.8043
	E. coli	0.023	-35.39000	-63.6291	-7.1509

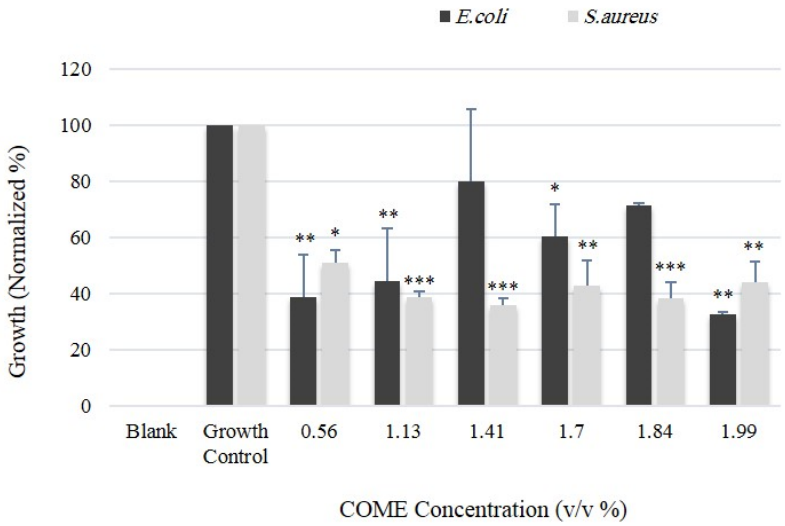

Fig. 3. Mean (SD) inhibitory activity of COME against *E. coli* and *S. aureus* (n=3).*, ** and *** represent $p<0.05$, $p<0.01$ and $p<0.001$, respectively, in treatment group versus blank group.

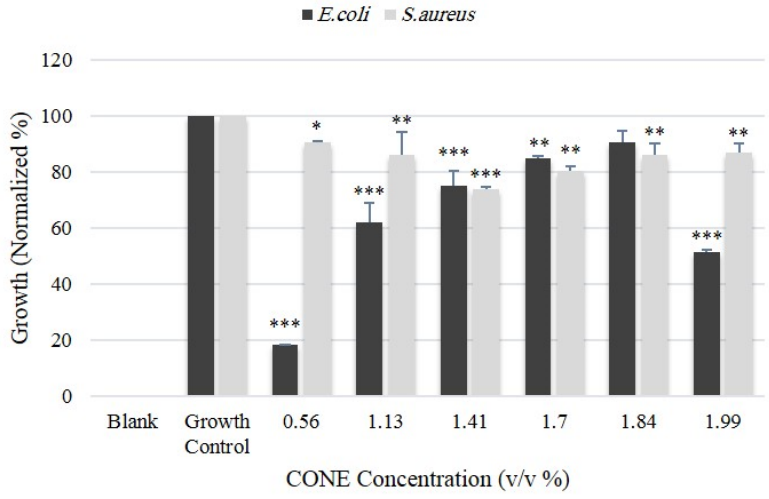

Fig. 4. Mean (SD) inhibitory activity of CONE against *E. coli* and *S. aureus* (n=3).*, ** and *** represent $p<0.05$, $p<0.01$ and $p<0.001$, respectively, in treatment group versus blank group.

Table 3. Multiple comparisons between inhibitory activities of bulk, COME and CONE against *E. coli*.

(I) group	(J) group	Mean Difference (I-J)	Standard Error	Significant	95% Confidence Interval	
					Lower Bound	Upper Bound
Bulk	COME	42.59000*	11.31959	.002	18.4629	66.7171
	CONE	32.58167*	11.31959	.011	8.4545	56.7088
COME	Bulk	-42.59000*	11.31959	.002	-66.7171	-18.4629
	CONE	-10.00833	11.31959	.391	-34.1355	14.1188
CONE	Bulk	-32.58167*	11.31959	.011	-56.7088	-8.4545
	COME	10.00833	11.31959	.391	-14.1188	34.1355

*. The mean difference is significant at the 0.05 level.

Table 4. Multiple comparisons between inhibitory activities of bulk, COME and CONE against *S. aureus*.

(I) group	(J) group	Mean Difference (I-J)	Standard Error	Significant	95% Confidence Interval	
					Lower Bound	Upper Bound
Bulk	COME	53.35333*	4.33805	.000	44.1070	62.5997
	CONE	11.33500*	4.33805	.020	2.0887	20.5813
COME	Bulk	-53.35333*	4.33805	.000	-62.5997	-44.1070
	CONE	-42.01833*	4.33805	.000	-51.2647	-32.7720
CONE	Bulk	-11.33500*	4.33805	.020	-20.5813	-2.0887
	COME	42.01833*	4.33805	.000	32.7720	51.2647

*. The mean difference is significant at the 0.05 level.

ANOVA was performed on the data to find the significant differences between the effect of size on the inhibitory activity of cinnamon oil products at a significance level of $p<0.05$ (Tables 3 and 4).

Bulk vs. Microemulsion

According to the results, antibacterial efficacy of COME is higher than the bulk sample. In comparison to bulk cinnamon oil, COME showed significant inhibitory activity against *E. coli* ($p=0.002$) and *S. aureus* ($p<0.001$).

Bulk vs. Nanoemulsion

Using statistical analysis between NE and bulk of cinnamon oil, CONE showed significant inhibitory activity in comparison with cinnamon bulk oil against *E. coli* ($p=0.011$) and *S. aureus* ($p=0.020$).

Microemulsion vs. Nanoemulsion

COME showed improved antibacterial activity in comparison to CONE but only in case of *S. aureus* ($p<0.001$).

DISCUSSION

Cinnamon oil has several elements such as cinnamaldehyde, pinene andmyrcene (19). Antibacterial activity of cinnamon oil is related to cinnamaldehyde (20). Possible mechanisms for antibacterial activity of cinnamaldehyde are effects on membrane permeability and inhibition of uptake or utilization of glucose (21). There are evidences showing that cinnamon oil can inhibit bacterial activity (i.e., *Porphyromonas gingivalis*, *Listeria monocytogenes*, *Salmonella enterica* and *E. coli*) (9, 14). From our findings, bulk oil did not show antibacterial activity, probably due to low amount of oil which was used in this study.

From our findings, CONE and COME showed significant differences ($p<0.05$) compared to negative control and bulk oil. NEs enter the cell cytoplasm and induce generation of ROS, to make oxidative stress in cells (22). Recently, Topuz *et al.* showed that COME has higher antimicrobial activity compared to bulk anise oil (23). Moghimi *et al.* showed that NEs of essential oils were more effective than bulk essential oils against food-borne bacteria (24). A time- kill kinetic study indicated that NEs have improved long-term antimicrobial activity compared with the bulk essential oils (15). Also, NEs are able to disrupt the rod-shaped bacteria (such as *V. vulnificus* and *E. coli*) in a nonspecific way (25).

Apossible mechanism which explains antibacterial activity of COME and CONE is effect of nonionic surfactants on bacterial cytoplasmic membrane (26). NEs and MEs, because of presence of surfactants, can considerably decrease hydrophobicity of bacterial cell surface (27). This

leads to release of DNA and RNA to extracellular space (27). Surfactants can also affect structure and morphology of cellular membrane and destruct bacterial cell surface (26-28). Another possible mechanism for increasing antibacterial activity in the emulsion systems (i.e., CONE and COME) is related to better dispersion of oil droplets in ME/NE compared with bulk oil, due to presence of surfactants (29). So, higher contact between bacterial cells and oil droplets is expected. Small droplet size might also be suggested as a mechanism for higher antibacterial activities of MEs and NEs. For example, small size of NEs help them better penetrate into the bacterial cells and damage the bacterial cell wall (30). However, this mechanism does not appear to be dominant in our study as COME with larger particle size showed improved antibacterial activity compared with CONE.

The results showed that CONE and COME are more effective than bulk. They are made from similar components, thus, should have similar mode of action against the bacteria. Further studies are required to understand the mechanisms underlying the superior activity of COME in comparison with CONE.

CONCLUSION

In the present study, COME and CONE formulations were obtained with sizes of 2040 and 30 nm, respectively. The inhibitory activity study indicated that COME and CONE had significant inhibitory activity compared with control group ($p<0.05$). Also, higher inhibitory activity observed was from COME. These results indicate that COME and CONE have potential as a green antiseptic agents.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

ABBREVIATION

NE(s): Nanoemulsion(s)
ME(s): Microemulsion(s)
CONE(s): Cinnamon oil Nanoemulsion(s)
COME(s): Cinnamon oil Microemulsion(s)
E. coli: *Escherichia coli*
S. aureus: Staphylococcus aureus

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