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Original Article

Antibacterial Activity of Cinnamaldehyde and Carvacrol against Foodborne Pathogens and Spoilage Bacteria

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The excessive use of chemical preservatives is a serious problem worldwide, which necessitates the discovery of new classes of antimicrobials from other sources like herbs and spices for preservation of food and/or food products. In this study, antibacterial activity of essential oils (EOs) such as cinnamaldehyde and carvacrol were tested against four foodborne pathogens viz *Staphylococcus aureus* ATCC 25923, *Shigella dysenteriae-I* MJ-84, *Escherichia soli* ATCC 25922, *Vibrio cholerae* ATCC 6395 and two food spoilage bacteria viz *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella rhizophila* ATCC 13882, respectively, using disc diffusion method. These EOs were found to inhibit both categories of bacteria. Cinnamaldehyde and carvacrol showed maximum 30.0 ± 0.2 and 27.5 ± 0.5 zones of inhibition, respectively against *S. aureus*. *Pseudomonas aeruginosa* was found resistant against carvacrol. Cinnamaldehyde and carvacrol was determined. Both showed antibacterial activity after treatment at 100°C for 30 min suggesting that high temperature does not affect the activity. Both the EOs have a broad pH range and exhibited highest activity at pH 7.0, suggesting that they remain un-dissociated at pH change. The MIC and MBC values of cinnamaldehyde ranged between 0.125 and .0 %, and 0.25 and 2.0 %, respectively and MIC and MBC values of carvacrol ranged between 0.125 and 0.5%, and 0.25 and 1.0 %, respectively. The results of this study confirmed the possibility of using cinnamaldehyde and carvacrol in preventing the growth of foodborne pathogens and spoilage bacteria and extend the shelf life of foods.

Key note: Antibacterial activity, cinnamaldehyde, carvacrol, foodborne pathogens, spoilage bacteria

Introduction

Worldwide it is considered that foodborne diseases are mainly caused by foodborne pathogens either by infection and/or by intoxication and frequently reported potent pathogens are *Staphylococcus aureus, Salmonella* spp., *Escherichia* spp. (Various serotypes), and *Bacillus cereus*[1]. Short shelf life of food products due to spoilage is one of the major problems of the food industry, which includes mainly *Pseudomonas aeroginosa, Bacillus subtilis, and Alcaligenes faecalis* etc. [2, 3].

Food poisoning caused by *Listeria monocytogenes, Staphylococcus aureus, Salmonella* sp. *B. cereus, Aeromonas* spp. and *Escherichia coli* 0157:H7 is worldwide serious problem, especially in developing countries. It has been reported that, among the various diarrheagenic serotypes, enterohemorrhagic *E. coli* 0157:H7 is implicated in large number of food borne outbreaks in many parts of the world[4]. *Shigella dysenterae* and *V. cholerae* are serious problem in Bangladesh. It is the most virulent *Shigella* with low infectious dose, high attack rate, and high mortality. *Vibrio cholerae,* 0139, spread rapidly in Bangladesh, producing disease in adults as well as children. It is mainly food and water borne pathogen but can survive for prolong time in water, possibly in blue-green algae and replicating in finfish and shellfish[5].

Food preservation by chemical preservatives for quality food is now global concern, because chemical preservatives at high concentration are health hazardous, for which food safety has recently led to the development of natural antimicrobials to control foodborne pathogens and spoilage bacteria. Essential oils from spices, and antimicrobials from plants and herbs may be the alternative of the chemical preservatives, because of their ability to inhibit the growth of both Gram positive and Gram negative foodborne pathogens and spoilage bacteria [6-16]. Thymol, cinnamon oil and carvacrol have previously been demonstrated as a broad spectrum antimicrobials[17,18].Cinnamaldehyde, thymol, carvacrol and eugenol were most active against *E. coli* serovars, *Salmonella enterica and L. monocytogenes*[19]. Essential oils from cloves and cinnamon can kill all cells of *E. coli* O157:H7 and *Listeria monocytogenes* in vivo within 60 minutes [8,20]. Cinnamaldehyde [8,21,22] and carvacrol[23,-25] are recognized as a potent growth inhibitor of common foodborne pathogens and spoilage bacteria.

The objective of this study is to assess i) Screening of the cinnamaldehyde and carvacrol for antibacterial activity against food borne pathogens and spoilage bacteria, ii) Determination of the effect of temperatures and pH on antibacterial activity of these essential oils, and iii) Determination of the Minimum

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Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of these essential oils.

Materials and Methods

Essential oils

Cinnamaldehyde and carvacrol (EOs) wwere used in this study. Cinnamaldehyde was purchased from Nacalai Tesque, Co (Kyoto, Japan) and carvacrol from Wako Pure Chemical Industries Limited (Osaka, Japan).

Reference strain

Total 6 strains of food borne pathogens were used in the study (Table 1). Of the 6 organisms studied, first 4 were food borne pathogens and the rest of 2 were food spoilers. The long-term stock cultures of the test organisms in 20% glycerol in cryogenic vials were kept at -70°C. Working cultures were kept at 4°C on Trypto Soy Agar (TSA) slants and were periodically transferred to fresh slants.

Table 1 . *Test organisms used in this study*

Organisms	No. of type culture	Source
Staphylococcus aureus	ATCC 25923	ICDDR'B
Vibrio cholerae	ATCC 6395	ICDDR'B
Escherichia coli	ATCC25922	Shishu Hospital
Shigella dysenterae	MJ-84	University of Dhaka
Pseudomonas aeroginosa	ATCC 27853	Shishu Hospital
Klebsiella rhizoplila	ATCC13882	Shishu Hospital

Media used

Mueller-Hinton Agar (MHA) (Dickinsm and Company, France), Trypto Soya Agar (TSA) (NISSUI, Japan), Mueller-Hinton Broth (MHB) (Becton, Dickinsm and Company, France).

Standard antibiotics

Antibiotic and its disc potency used was gentamycin (10 µg) (NISSUI Pharmaceuticals Co. Ltd. Japan).

Preparation of stock solutions of essential oils

The crude sample contained 100 % cinnamaldehyde and carvacrol, from which 10 % stock solution of cinnamaldehyde and carvacrol was made with 95 % ethanol. The stocks were made aliquot in 5 ml volumes and kept at refrigeration temperature until use.

Impregnation of filter paper discs

Discs (8 mm in diameter) made of Whatman filter paper no, I (ADVANTEC; Toyo Roshi Kaisha Ltd., Japan) were impregnated with 50 µl of each 3 % cinnamaldehyde and carvacrol made from 10 % stock solutions and were then dried at 40°C for 1 hour in hot air oven (Barnstead Labline, USA) and were stored at 4°C until use. Negative control (without the essential oil) was prepared in 95 % ethanol.

Preparation of inocula

One loopful of inoculum of each test organism from cryogenic vial was transferred into 9 ml of sterile Trypto Soya Broth (TSB) and grown at 37 °C for 24 hours. One loopful of the TSB culture was then streaked into the TSA plate and grown at 37 °C for 24 hours. The inocula of the test organisms were prepared by transferring 3 to 4 colonies of the cultures on TSA into 9 ml of sterile MHB and incubated at 37°C for 5-6 hours, if necessary 12 h to 18 h was considered. The MHB culture was compared with McFarland 0.5 turbidity standards (10^8 CFU/ml)[26]

Preparation of the McFarland standard

0.05 ml of 0.048 M $BaC1_2$ was added to 9.95 ml of 0.18 M H_2SO_4 in a test tube with constant stirring. The tube was then sealed tightly and stored in the dark at room temperature.

Inoculation of inoculated plates

After adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time.

Application of discs to inoculated agar plates

The essential oil impregnated discs were dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. For each plate 5 discs were placed. Discs for negative control were prepared using the same solvent without the essential oil. The plates are inverted and placed in an incubator at 37 °C for 24 hours.

Evaluation of antibacterial activity

Antibacterial activity was evaluated by measuring the zones of inhibition in mm (including the 8 mm disc) with slide calipers near the agar surface and the results were recorded. The endpoint was taken as complete inhibition of growth as determined by the naked eye. Each essential oil was tested in triplicates and assay in this experiment was repeated thrice.

Effect of temperature on antimicrobial activity of essential oils

The effects of temperature on antibacterial activity of essential oils were determined by the methods as described by Lee Ching-Fu *el al.,* (2004)[27].

The vials with the essential oil and the negative control were incubated in water bath set at 25, 37, 50, 75, and 100 °C, respectively for 30 min. After the temperature treatment, the antibacterial activity of the cinnamaldehyde and carvacrol was carried out against the test organisms (4 food borne pathogen and 2 food spoiler). The antibacterial activity was assayed by the disc diffusion methods by Bauer *et al.* (1966)[28].

Effect of pH on antibacterial activity of essential oils

The effect of pH on the antibacterial activities of cinnamaldehyde and carvacrol were assayed by using methods reported previously with slight modification[29,30]. The buffer solutions used were 50 mM citrate buffer (pH 7.0), 50 mM Phosphate buffer (pH 7.0) and 20 mM Tris-HC1 buffer (pH 9.0). All the buffers were sterilized through 0.45 µm of membrane filter, stored at 4°C and used within 30 min. The pH of the essential oil was determined with sterile pH paper strip. At 10 % concentration, pH of the cinnamaldehyde was 5.0 and for carvacrol it was 6.0.

The discs impregnated with 50 µl of 3 % cinnamaldehyde were then placed on the MHA plates previously seeded with the test organisms[28]. Negative controls were prepared using the different buffer solution without the essential oil. Three discs with the same pH were placed on each plate. The plates were kept at 4°C for 30 min for better absorption of the sample and then inverted and placed in an incubator set to 37°C for 24 hours. Antibacterial activity was evaluated by measuring the zones of inhibition in mm (including the 8 mm disc) for different pH with slide calipers near the agar surface and the results were recorded. Each essential oil was tested in triplicates.

Determination of the MIC and MBC of essential oils

The MIC of cinnamaldehyde and carvacrol was determined by tube dilution techniques in MHB medium[31]. The range of concentration for cinnamaldehyde and carvacrol used was 4-0.0625 % (v/v). The MIC was done at 37°C and at optimum pH 7.0. 'Stock solutions of the cinnamaldehyde and carvacrol were diluted in 50mM phosphate buffer (pH 7.0) to make concentrations of 8 %. Further dilutions for the MIC / MBC were done by the two fold dilution method in the buffer to make 4 %, 2 %, I %, 0.5 %, 0.25 %, 0.125 % and 0.0625 %.

0.9 ml of the MHB was taken in each of the sterile and dry glass vials appropriately labeled with concentrations of cinnamaldehyde and carvacrol. 1.0 ml of the respective essential oil concentrations was dispensed into the respective vials and 100 µl of the each test organisms were added to the vials with their names labeled to make sure that each of the organisms faced a different concentration of the cinnamaldehyde and carvacrol. So, the final reaction volume became 2 ml. A positive control was made with MHB and sterile distilled water plus culture of test organisms. A negative control was made with essential oil and MHB but no test organisms. All the prepared vials were then incubated at 37°C for 24 hours. For determination of MIC or MBC, l0 µl inoculum from each test vial was transferred onto MHA plate and incubated at 37 °C for 24 hours.

Statistical analysis

The inhibition zones were calculated as means ± S.D. (n = 3). The significance among different data was evaluated by analysis of variance (ANOVA) using Microsoft excel program. Significant differences in the data were established by least significant difference at the 5 % level of significance.

Results

Screening of the antibacterial activity of essential oil

Antibacterial activity of cinnamaldehyde and carvacrol are summarized in Tables 2. Cinnamaldehyde showed better inhibitory activity against the 6 selected food borne pathogens and spoilage bacteria with zones of inhibition ranged between 14.5 and 30.0 mm with maximum zone of inhibition for *S. aureus* (30.0 mm) which was larger than gentamycin (10 µg/disc) and minimum for *Pseudomonas aeruginosa* (14.5 mm).

Carvacrol was found to be active against all the test organisms except *Pseudomonas aeruginosa* with zones of inhibition ranged between 21.0 and 27.5 mm. Carvacrol showed maximum zone of inhibition for *S. aureus* (27.5 mm), which was larger than those observed against the antibiotic tested.

Effect of temperature on antibacterial activity of essential oils

The effect of the temperatures on the antibacterial activity of cinnamaldehyde and carvacrol against all the test bacteria were shown in Table 3. The antibacterial activity of cinnamaldehyde and carvacrol was found at all temperatures employed (25 º, 37º, 50º, 75º and 100º C), suggesting that cinnamaldehyde and carvacrol were not destroyed at high temperature even at 100 °C for 30 min treatment. Moreover, the antibacterial activities were found to increase with the increasing temperature. Highest activity was found at 100 °C and lowest activity was found at 50 °C for the test bacteria.

All the values are mean ± standard deviation of three determinations. Mean ± S.D. mm (n=3), $P < 0.05$

Effect of pH on antibacterial activity of cinnamaldehyde and carvacrol

The antibacterial activity was not affected at pH 5.0, and no significant decrease in inhibition was found (Table 4). The pH 7.0 and 9.0 enhanced the antibacterial activities of cinnamaldehyde and carvacrol against most of the organisms tested, where the highest activities were found at pH 7.0(Table 4). Both the EOs showed the higher activity against *S. aureus* at pH 7.0 compared to pH 5.0 and 9.0.

MIC and MBC of cinnamaldehyde and carvacrol

MIC values of cinnamaldehyde against the test bacteria ranged between 0.125 % and 1.0 % (0.25 and 2.0 % MBC) and for carvacrol 0.125 to 0.5%, (0.25 and 1.0 MBC) (Table 5).

The MIC of cinnamaldehyde showed the highest inhibition for *S .aureus* (0.125 %) and followed by *K. rhizophila* (0.25 %) and *V. cholerae* (0.25 %). However, lowest MIC value was found for *P. aeruginosa* (1%). The MIC of carvacrol showed the highest inhibition *S. aureus* (0.125 %) and followed by *S. dysentarae* (0.25 %), *K. rhizophila* (0.25 %) and *V. cholerae* (0.25 %).

Table 2. *The screening of antibacterial activities of cinnamaldehyde (Cinn) and carvacrol (Carv)*

Organisms	Zones of inhibition (mm)		
	Cinn (3 %)	Carv (3 %)	Gentamycin(I 0 µg/disc)
Staphylococcus aureus	30.0±0.2	27.5±0.5	23.5 ±1.0
Vibrio cholerae INC2	26.5±1.0	26.0±0.45	24.0±0.64
Escherichia coli	27.0±0.1	21.0±0.6	21.0±0.42
Shigella dysenteriae -1	24.0±0.8	22.5±1.12	24.0±1.1
Pseudomonas aeruginosa	14.5±0.2	Resistant	26.5±0.18
Klebsiella rhizophila	29.5±0.4	24.2±0.09	28.0±0.50

Mean ± S.D. mm (n = 3), $P < 0.05$

Table 3. *Effect of temperatures on antibacterial activity of cinnamaldehyde (Cinn.) and carvacrol (Carv.) at 3 % concentration*

	Zones of inhibition (mm) Temperatures									
	25°C		37°C		50°C		75°C		100	
	Cinn	Carv.	Cinn	Carv.	Cinn	Carv.	Cinn	Carv	Cinn	Carv.
Staphylococcus aureus	26.5±0.2	24.5±0.2	29.4±0.1	27.4±0.1	24.8±0.5	23.0±0.5	26.0±0 .3	28.0±0.3	31.0± 0.1	29.0±0.1
Vibrio cholerae	25.0±0.8	24.5±0.8	27.0±1.0	26.0±1.0	23.2±0.5	22.0±0.5	27.0±0.1	26.0±0.1	28.0±0.3	27.3±03
Escherichia coli	22.0±0.3	18.5±0.3	25.0±0.7	21.0±0.7	22.2±0.6	17.0±0.6	24.3±0.2	22.5±0.2	26.0±0.1	24.0±0.1
Shigella dysenteriae -1	21.5±0.4	21.5±0.4	24.0±1.1	24.0±1.1	19.5±0.6	20.5±0.6	24.8±0.2	24.0±0.2	26.0±0.1	25.2±0.1
Pseudomonas aeruginosa	11.9± 1	R	13.8±0.2	R	11.0±0.1	R	14.5±0.2	R	16.0±0.6	R
Klebsiella rhizophila	24.2±0.4	23.0±0.4	28.5±0.7	24.0±0.7	22.3±0.2	21.5±0.2	27.5±0.4	25.5±0.4	29.5±0.1	27.0±0.1

Table 4. *Effect of pH on antibacterial activity of cinnamaldehyde (Cinn) and carvacrol (Carv) at 3 % concentration*

Organisms	Zones of inhibition (mm) pH					
	5.0		7.0		9.0	
	Cinn	Carv	Cinn	Carv	Cinn	Carv
Staphylococcus aureus	25.0±1.0	23.0±0.2	29.6±0.57	26.5±0.6	28±0.1	24.3±1.0
Vibrio cholerae	24.0±10	21.5±0.5	27.3±0.57	24.0±0.57	26.8±0.4	22.8±0.4
Escherichia coli	22.5±0-5	20.6±0.3	24.0±0.63	21.2±0.63	24.8±1.0	23.0±1.0
Shigella dysenteriae -1	19.5±0.8	18.2±0.8	23.3±0.9	23.0±0.9	26.2±0.6	20.0±0.5
Pseudomonas aeruginosa	13.8±0-5	R	14.5±1.0	R	15.3±1.1	R
Klebsiella rhizophila	21.2±0-7	21.5±0.5	28.3±0.9	24.0±0.9	27.8±0.5	22.5±1.0

All the values are mean ± standard deviation of three determinations. Mean ± S.D. mm (n = 3), $P < 0.05$

Table 5. *MIC and MBC values of cinnamaldehyde (Cinn) and carvacrol (Carv)*

Test Organisms	MIC (%)		MBC (%)	
	Cinn	Carv	Cinn	Carv
Staphylococcus aureus	0.125	0.125	0.25	0.25
Vibrio cholerae	0.5	0.25	1.0	0.5
Escherichia coli	0.5	0.5	1.0	1.0
Shigella dysenteriae -1	0.25	0.25	0.5	0.5
Pseudomonas aeruginosa	1.0	-	2.0	-
Klebsiella rhizophila	0.25	0.25	0.5	0.5

Discussion

The results presented in Table 2 showed that the oils under investigation exhibited marked antibacterial activity as evidenced by their zones of inhibition. The findings of cinnamaldehyde correlated with the findings of Hoque. *et al.* (2007)[8] studied on essential oils of cloves and cinnamon against food borne pathogen and spoilage bacteria. The essential oils under this study consist of phenolic components, which render them effective against the tested microorganisms. This was confirmed by Farag *et al.* (1989)[32]. The antibacterial property of carvacrol matched with that of the findings of Friedman *et al*. (2002)[19] who worked on carvacrol and found the carvacrol exhibited significant bactericidal activities. These observations also correlated with Ultee *et al.* (1998)[33] who exhibited the antibacterial activity of carvacrol against foodborne pathogens.

The antibacterial activity of cinnamaldehyde and carvacrol was found at all temperatures employed (25°, 37º, 50º, 75º and 100 °C) suggesting that cinnamaldehyde and carvacrol were not destroyed at high temperature even at 100 ºC for 30 min treatment. A little increase in activity of both cinnamaldehyde and carvacrol was observed at 100 ºC which may be due to the partial exhaustion of solvent (ethanol) in oils at high temperature above 60 ºC. Similar type of findings was reported by Hoque. *et al.* (2007)[8].

The antibacterial activity of cinnamaldehyde and carvacrol against most of the tested organisms was slightly decreased at pH 5.0, but increased at pH 7.0 and 9.0 with maximum inhibition at pH 7.0. These findings supported the findings of Hoque *et al.* (2007)[8], they reported higher activity of cinnamon oil against cocktail of S. *aureus* at pH 7.0 compared to pH 5.0 and 9.0.

MICs of cinnamaldehyde and carvacrol were determined by the broth dilution method at 37°C and at pH 7.0. MIC values of cinnamaldehyde against the test bacteria ranged between 0.125 and 1.0 % and for carvacrol 0.125 and 0.5%. The MIC of cinnamaldehyde showed the highest inhibition of *S. aureus* (0.125 %) and followed by *S. dysentarae* (0.25 %), *K. rhizophila* (0.25 %) and *V. cholerae* (0.5 %). However, lowest inhibition was found for *P. aeruginosa* (1.0 %). The MIC of carvacrol showed the highest inhibition for *S. aureus* (0.125 %), followed by *S. dysentarae* (0.25 %), *K rhizophila* (0.25 %) and *V. cholerae* (0.25 %).

Conclusion

The essential oil cinnamaldehyde and carvacrol possess antibacterial property that can be fruitfully used as antibacterial as alternatives of antibiotics to control foodborne pathogens and spoilage bacteria. These essential oils act through natural inhibitory mechanisms by either inhibiting or killing the pathogens completely. Cinnamaldehyde may be a better choice as preservatives for foods. In developing countries like Bangladesh, where spices are produced and used as food additives, their use as antibacterial agents and potential preservatives can be extremely useful without health risk.

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