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In Vitro Vibriocidal Activity of Coriander (*Coriandrum sativum* L.) and Aniseed (*Pimpinella anisum* L.) Essential Oils

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ABSTRACT

Vibriocidal activity of coriander (*Coriandrum sativum*) and aniseed (*Pimpinella anisum*) essential oils are reported against *Vibrio cholerae*, *V. cholerae* O139 and *V. alginolyticus* by primary screening techniques through DDM. The minimum inhibitory concentrations (MIC) of coriander and aniseed oils ranged between 1.95-62.5 and 7.81-125 µl/ml against the strains, respectively. The activity of the oils was retained, even after heating at 100°C for 1h. Vibriocidal activities reported at 4, 32 and 37°C indicate temperature-independent activity of the oils. The activities compared well with standard antibiotics (Ciprofloxacin, Polymyxin-B, Ampicillin, Erythromycin and Trimethoprim). The bactericidal activity of the two oils against *V. cholerae* and *V. cholerae* O139 speculated to be multidimensional, whereas, against *V. alginolyticus* could be due to cell wall and/or protein synthesis inhibition. Coriander oil revealed better vibriocidal activity in comparison to aniseed oil. Essential oils, being natural in origin, could be a novel agent of antimicrobial compounds against antibiotic-resistant vibrios.

Keywords: bactericidal activity, essential oils, food preservation, minimum killing time, minimum inhibitory concentration, possible mode of action

INTRODUCTION

In this modern era of chemotherapeutics, the herbal medicinal system has drawn the attention of both academicians as well as researchers for the development of new drugs, use in cosmetic industries, food preservation etc. This could be attributed to the development of resistance among pathogens towards antimicrobial compounds, because of their prolonged use, different side effects, and their non-biodegradability nature. Among the plant products, essential oils (EOs) have proved to be a suitable antimicrobial agent. Similarly, a large number of reports are available in the literature regarding the drug resistance of *Vibrio cholerae* against the commonly used antibiotics. Moreover, vibrios of marine origin are the major source of contamination of marine food and food products and pose a threat to human society. We have already reported antibacterial activity of different EOs (Rath *et al*. 1999, 2001; Baswa *et al*. 2001; Gupta *et al*. 2004; Rath *et al*. 2005a, 2005b) from our laboratory. However, very little work has been carried out to test the antibacterial potentiality of coriander and aniseed oil against vibrios, and this prompted us to study the vibriocidal activity of these two oils. In the present investigation the issues addressed are: (i) determination of minimum inhibitory concentration (MIC) of the two oils; (ii) study of nature of toxicity i.e. bactericidal/bacteriostatic; (iii) determination of minimum killing time; (iv) effect of temperature on the activity of the oils; (v) comparison of the activity of the oils with standard antibiotics and determining the possible mode of action.

MATERIALS AND METHODS

Bacteria

Vibrio cholerae (Ogawa), *V. cholerae* (0139), and *V. alginolyticus* were procured from the National Institute of Cholera and Enteric Diseases, Kolkotta, India and Central Institute of Fisheries and Aquaculture, Bhubaneswar, India, and maintained in our laboratory at room temperature on Nutrient Agar (NA) slants.

Oils

Coriander (*Coriandrum sativum*) and aniseed (*Pimpinella anisum*) were obtained from the local market, Kolkatta, India.

Media

Nutrient broth (NB), Nutrient Agar (NA), Tween-20 (T-20), and antibiotic discs were obtained from Hi-Media, Mumbai, Ltd. NB/NA and supplemented with 0.75% T-20 to facilitate the solubility of the oils in the medium. Media with T-20 and without oil served as controls through out the study.

Initial screening

For initial screening the disc diffusion method as described previously by Pattnaik *et al*. (1995, 1997) was followed with slight modifications. Briefly, NA plates were swept with freshly grown cultures of the test pathogens by the help of a presterilized cotton swab. Sterile filter paper discs (5 mm diameter) were kept on the above plates at equidistance. Varying volumes (2, 5 and 10 µl) of coriander and aniseed oils were loaded over the sterile filter paper discs. The plates were incubated at 37°C 18-24 h, and observed for a zone of clearance around the discs which indicated positive vibriocidal activity of the oils. All the experiments were carried out in triplicate.

Determination of Minimum Inhibitory Concentrations (MIC)

Minimum inhibitory concentration of the oils was determined by the tube dilution method (Rath *et al*. 1999). Briefly, two-fold dilution of the oil was done with NBT (Nutrient broth supplemented with 0.75% of Tween-20) to give oil concentrations of 1.95 to 500 µl/ml. Fifty microlitres of overnight growth of the test organisms

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in NB was inoculated into 1 ml of NBT containing various concentrations of the oils. The tubes were incubated overnight at 37°C and the lowest concentration inhibiting bacterial growth (no turbidity) was noted as MIC.

Test for bactericidal effect

In order to evaluate the effect (bactericidal/bacteriostatic) of the oils one loopful from the MIC tube was subcultured onto NA plates which were then incubated at 37°C overnight to check whether the oils merely inhibited the growth of the vibrios (bacteriostatic) or had bactericidal activity, i.e. no growth on subculturing.

Determination of minimum killing time (MKT)

This experiment was designed to determine the time required to kill the bacteria *in vitro* by the oils. One milliliter of (NBT), NB supplemented with 0.75% of T-20 at MIC level of the oil was prepared and inoculated with 0.1 ml freshly grown test organisms and incubated at 37°C. One loopful of the sample from the above test tubes were sub-cultured onto NA plates at 0, 5, 10, 15, 30, 45, 60, 75, 90, 120, 180 min intervals and incubated overnight. Two sets of tubes were incubated for each test organism from which subculturing was carried out alternatively (to avoid time lapse during sub-culture). The activity was observed after overnight incubation of the plates at 37°C. No growth on the streaking line was considered to be the time required by the oil to kill the organism.

Determination of effect of heat on the activity of the oils

This experiment was designed to study the effect of high temperature on antimicrobial activity of these oils. The oils were heated at 40, 60, 80, and 100°C for 1 h in a water bath, in presterilized screw cap vials separately. The vibriocidal activity was tested by the disc diffusion method described above, by loading MIC level of the oils in the discs.

Determination of possible mode of action of the oils

Possible mode of action of the oils was determined through antibiotic sensitivity studies (studying antibiogram with group specific antibiotics) following the method described previously (Rath *et al*. 2001). Group of specific antibiotics [Ciprofloxacin: 5 µg, Ampicillin: 10 µg, Polymyxin-B: 300U, Trimetrhoprim: 25 µg, and Erythromycin: 15 µg] with specified concentrations were procured from Hi-Media, Ind. Ltd., and used to study the antibiogram of the isolates.

Statistical analyses

Each experiment was carried out in triplicate. The data were statistically analysed using SPSS 10.0. A least significant difference ($LSD_{0.05}$) was used to test the effects of EOs through a general linear model. The test was statistically significant at $p<0.05$.

RESULTS

Coriander and aniseed EOs are colourless but they differ in their optical rotation, specific gravity and refractive indices. These oils are with spicy aromatic odour, insoluble in water and soluble in alcohol and organic solvents. The physico-chemical properties of the two oils are reported in **Table 1**.

The chemical constituents of the oils differ to a great extent (Cantore *et al*. 2004). Hydrocarbons common in both oils are α-pinene and camphene. The percentage of α-pinene and camphene were 0.28-8.5 and 1-4, and 0.17 and 0.07 in case of coriander and aniseed oil, respectively. Other hydrocarbons which were present in coriander seed oil are g-terpinene (1-8%), *p*-cymene (3.5%), limonene (0.5-4%) and myrcene (0.2-2%) while β-pinene (0.01%) is present in aniseed oil only. The predominant constituents in coriander and aniseed oils were observed to be monoterpenes (60-80%) and *trans*-anethole (85%), respectively. However, the chemical constituents of the two oils contain also alcohols, esters, and terpenoids, at varied concentrations.

From the preliminary screening studies by disc diffusion method, it was observed that the test pathogens were susceptible to both oils. However, a difference in zone sizes were observed at different concentrations used (**Table 2**). Coriander oil showed better activity against *V. cholerae* followed by *V. alginolyticus*, and least activity against *V. cholerae* O139. In comparison, aniseed oil showed highest activity against *V. alginolyticus*. The minimum inhibitory concentration of coriander and aniseed oils ranged between 1.95-62.25, and 7.81-125 µl/ml, respectively. Though, a variance was observed in the zones of inhibition and the MIC values, the three test pathogens were killed immediately (0 min) when they come in contact with coriander oil at their respective MICs. In contrast, a difference in killing time was observed in the case of aniseed oil. We noted that *V. cholerae* O139 and *V. cholerae* were killed at 15 min and 24 h, respectively, while *V. alginolyticus* was killed immediately (**Table 3**). The activity was observed to be temperature independent in both cases. Occurrence of bactericidal activity at 4°C further indicates the energy independent nature of activity of coriander and aniseed oil.

Surprisingly, an increased zone of inhibition was observed, when heat treated oils were tested for their vibriocidal potential by disc diffusion method (loading MIC level of oils per disc) in comparison to unheated oils. This increased activity of the oils after heat treatment could be attributable to the thermoactivation of the molecules present in the oil. Both *V. cholerae* and *V. cholerae* 0139 were sen-

Table 1 Physico-chemical properties of coriander and aniseed oils.

Characters	Oil	
	Coriander	**Aniseed**
Colour and appearance	Colourless/pale yellow	Colourless
Odour	Sweet, woody, spicy and Slightly musky scent	Sweet spicy
Specific gravity	0.86300-0.87500	0.847-0.881
Refractive index	1.46330	1.479-1.481
Optical rotation	+9.00	NA
Acid number	NA	NA
Solubility	Insoluble in water, soluble in organic organic solvents	Insoluble in water, soluble in organic organic solvents

Table 3 Minimum Killing Time (MKT) of coriander and aniseed oils.

Organisms	Killing Time					
	Coriander oil			Aniseed oil		
	4°C	**37°C**	**RT**	**4°C**	**37°C**	**RT**
V. cholerae	0 min	0 min	0 min	24 hrs	24 hrs	24 hrs
V. cholerae (O139)	0 min	0 min	0 min	15 min	15 min	15 min
V. alginolyticus	0 min	0 min	0 min	0 min	0 min	0 min

Table 2 Antibacterial activity of coriander and aniseed oil by DDM and their Minimum Inhibitory Concentrations (MICs).

Organism	Mean zone sizes in mm by DDM						MIC (µl/ml)	
	Coriander			Aniseed			Coriander	Aniseed
	2.5 µl	**5 µl**	**10 µl**	**2.5 µl**	**5 µl**	**10 µl**		
V. cholerae	28.16 ± 0.35 a	36.00 ± 0.94 a	50.33 ± 0.72 a	8.66 ± 0.13 c	12.00 ± 0.94 b	13.16 ± 0.36 b	1.95 ± 0.00 a	7.81 ± 0.00 a
V. cholerae (O139)	6.33 ± 0.27 c	4.00 ± 0.46 c	4.66 ± 0.72 c	7.00 ± 0.00 c	12.33 ± 0.72 b	12.33 ± 0.27 b	62.50 ± 0.00 b	15.62 ± 0.00 c
V. alginolyticus	9.00 ± 0.00 c	18.33 ± 0.72 b	28.00 ± 0.94 a	18.00 ± 0.00 b	18.00 ± 0.94 b	24.00 ± 1.41 a	62.50 ± 0.00 b	12.50 ± 0.00 b

Values within the table with a different letter are statistically different according to the LSD test ($P<0.05$).

Table 4 Antibiogram of the test pathogens in comparison to coriander and aniseed oils.

Organisms	Zone sizes in mm						
	Antibiotics/Oils used*						
	Cf	A	Pb	Tr	E	Coriander Oil	Aniseed Oil
V. cholerae	17.00 ± 0.00 b	20.00 ± 0.00 b	17.00 ± 0.00 b	26.00 ± 0.00 a	23.33 ± 0.46 a	36.66 ± 0.98 a	19.33 ± 0.27 b
V. cholerae (O139)	32.00 ± 0.00 a	30.00 ± 0.00 a	17.00 ± 0.00 b	28.00 ± 0.00 a	28.00 ± 0.00 a	35.33 ± 0.27 a	15.33 ± 0.27 b
V. alginolyticus	31.00 ± 0.00 a	16.00 ± 0.00 b	R	R	19.00 ± 0.00 b	34.00 ± 0.46 a	28.00 ± 0.00 a

Cf: Ciprofloxacin, A: Ampicillin, Pb: Polymyxin-B, Tr: Trimethoprim, E: Erythromycin.
R = Resistance, *Oils loaded at MIC level/Disc
Values within the table with a different letter are statistically different according to the LSD test (P<0.05).

sitive to all the antibiotics tested although the zones of inhibition were less compared with those of oils. In contrast, *V. alginolyticus* showed high resistance against Polymyxin-B and Trimethoprim (**Table 4**).

DISCUSSION

The results of this study suggest that the antimicrobial activity of the EOs of coriander and aniseed is bactericidal. The two oils were significantly effective ($p<0.05$) against the test pathogens. Bacterial susceptibility towards both the oils was observed at 2.5 µl per disc but higher concentrations showed larger zones of inhibition, when tested by agar plate technique. The results were highly significant for all the treatments, determining MIC_s MKT_s and even when the activities compared with standard antibiotics. In general, there seemed to be overall agreement between the size of inhibition zones obtained by the disc diffusion method (DDM) and the minimum inhibitory concentration (MIC) values, i.e. larger zones of inhibition correlated with lower MIC values. This relationship between inhibition zones and MIC values has been reported in literature while studying the antibacterial activity of EOs (Lis-Balchin *et al.* 1998; Rath *et al.* 1999; Baswa *et al.* 2001; Rath *et al.* 2001, 2002; Gupta *et al.* 2004; Senatore *et al.* 2004; Rath *et al.* 2005b). Better activity of coriander oil could be due to presence of high percentage of monoterpenes (60-80%) in comparison to aniseed oil, where the major constituents observed is transanethol (85%) (Cantore *et al.* 2004). The activity of the oils is concluded to be vibriocidal/bactericidal, as no growth was observed on subculture onto NA plates from the MIC tubes after treatment. The killing of the test pathogens immeditely by coriander oil implies that the oils result in an irreverible damage to the bacterial cell, when come in contact with the bacterial cell. Similar effects of the EOs on pathogenic bacteria have been reported earlier (Lis-Balchin *et al.* 1998; Rath *et al.* 2001). The constancy and increased activity of the oils at high temperature indicate the thermostability/ thermoolerance of the active principles present in both coriander and aniseed oil. Further, this could be attributed to the increase in the synergistic effect of the components on heat treatment. Synergistic effect of EOs and their components against bacteria have been well recorded in literature (Rath *et al*, 2002; Gupta *et al.* 2004; Senatore *et al.* 2004). But in contrast to these findings (Rath *et al.* 2001) reported a reduction in zones of inhibition of heat treated turmeric leaf and rhizome oils at 100°C against *E. coli*. This reduction in activities was due to the increase in viscosity causing a poor difusibility due to polymerization of the components of the oils over the agar medium at high temperature.

Since, *V. cholerae* and *V. cholerae-O139* were sensitive to all the groups of specific antibiotics studied, the vibriocidal activity of the oils could be attributed as multidimensional. Whereas, the killing of *V. alginolyticus* may be due to cell wall synthesis/protein synthesis inhibition as it showed a resistance to Polymyxin-B and Trimethoprim. Antibacterial activity of EOs (Pattnaik *et al.* 1995) and fixed seed (Baswa *et al.* 2001) oils of *Azadirachta indica* (Neem) and *Pongamia pinnata* (Karanj) through inhibition of cell wall synthesis has been reported against both $Gram^+$ and $Gram^-$ bacteria. Pattnaik *et al.* (1997) have also stated that the antibacterial activity of *Eucalyptus* and Lemongrass oil against *E. coli* was due to inhibition of cell wall synthesis.

Previous studies have already shown the growth inhibition activity of *C. sativum* EO on different microorganisms. However, this is the first time that the bactericidal activity of *C. sativum* and *P. anisum* EOs have been demonstrated against pathogenic vibrios. The results appear promising, for possible use of these oils as vibriocidal agents, more particularly in preservation of food items those are susceptible to be contaminated by vibrios, such as marine foods and food products, including foods rich in animal proteins. The common methods of preservation of these food items include mainly use of different physical methods and chemical preservatives. Preservation by chilling temperature is one of the most common physical method used in most parts of the world. Salting or marination of fish by dry salt is an effective method of preservation of these food items. Benzoic acids and benzoates have been moderately successful as chemical preservatives. In addition to this sodium and potassium nitrites and nitrates have been reported to lengthen the keeping time and permitted in some countries. Furthermore, chemical preservatives along with different antibiotics such as chlorotetracyclin, oxytetracycline and chloramphenicol at low concentrations have been used to some extent in preserving these food items. These chemicals have been proved to be poorly effective and carcinogenic/mutagenic, in addition, now-a-days consumers are concerned about the use of synthetic chemicals such as colourants and preservatives in food and there is a resulting trend towards less processed foods (Soomro *et al.* 2002); moreover, people prefer foods with less chemical preservatives (Daeschel 1993; Brewer *et al.* 1994). When safety of synthetic additives and/or preservatives are questioned natural compounds of plant origin may appeal to the public. The antimicrobial activity of some EOs and their components against food-borne pathogens, including mycotoxin-producing fungi, has also been tested (Bullerman *et a*l. 1977; Kim *et al.* 1995; Ultee *et al.* 2000; Senhaji *et al.* 2007). Probably in our investigation, for the first time we have documented the antibacterial activity of coriander and aniseed EOs against vibrios. Furthermore, EOs of coriander and aniseed proved to have vibriocidal properties at low concentrations, and most of the EOs possess antioxidant properties (Barrata *et al.* 1998a, 1998b) holds a promise as an alternate to expensive, harmful chemical preservatives against these pathogens. Of course, other studies are highly necessary to study the toxicity of these oils in order to set an appropriate formulation for this purpose.

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