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Original Article

In Vitro Inhibition of Cholera Toxin Production in *Vibrio cholerae* by Methanol Extract of Sweet Fennel Seeds and Its Components

Shruti Chatterjee[1**†], M. Shamim Hasan Zahid[1†], Sharda Prasad Awasthi[1], Nityananda Chowdhury[1***], Masahiro Asakura[1], Atsushi Hinenoya[1], T. Ramamurthy[3], Emiko Iwaoka[2], Shunji Aoki[2], and Shinji Yamasaki[1*]

[1]*Graduate School of Life & Environmental Sciences, Osaka Prefecture University, Osaka;*
[2]*Faculty of Pharmacy, Hyogo University of Health Sciences, Hyogo, Japan; and*
[3]*National Institute of Cholera and Enteric Diseases, Kolkata, India*

SUMMARY: A newly emerged *Vibrio cholerae* O1 El Tor variant strain with multidrug resistance is considered a threat to public health. Recent strategies to suppress virulence factors production instead of bacterial growth may lead to less selective pressure for the emergence of resistant strains. The use of spices and their active constituents as the inhibitory agents against cholera toxin (CT) production in *V. cholerae* may be an alternative approach to treat cholera. In this study, we examined the potential of sweet fennel seed (*Foeniculum vulgare* Miller var. *dulce*) methanol extract to inhibit CT production in *V. cholerae* without affecting viability. The methanol extract of sweet fennel seeds significantly inhibited CT production in various *V. cholerae* strains, regardless of serogroup or biotype. Interestingly, *trans*-anethole and 4-allylanisole, essential oil components of sweet fennel seeds, also demonstrated similar effects. Here, we report that sub-bactericidal concentrations of sweet fennel seed methanol extract and its major components can drastically inhibit CT production in various *V. cholerae* strains.

INTRODUCTION

Diarrheal disease remains a leading global health issue. Some enteric pathogens, including *Vibrio cholerae*, *Escherichia coli*, and *Shigella* spp., produce protein toxins that are the primary cause of diarrhea (1). Cholera toxin (CT) is a principal virulence factor produced by toxigenic *V. cholerae*, the causative agent of the dreadful disease cholera. Although more than 200 O serogroups of *V. cholerae* have been identified so far, only O1 (El Tor and classical biotypes) and O139 are responsible for cholera epidemics (2). Serogroups other than O1 and O139 (non-O1/non-O139) are associated with sporadic cases of diarrhea (3). A variant of *V. cholerae* O1 El Tor biotype, possessing some attributes of the classical biotype, has emerged recently and was isolated from hospitalized patients with diarrhea more severe than that caused by typical El Tor strains (4,5). These hypervirulent strains have been characterized and designated as O1 El Tor variants (6).

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*Corresponding author: Mailing address: Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku Ourai-kita, Izumisano, Osaka 598-8531, Japan. Tel/Fax: +81-72-463-5653, E-mail: shinji@vet.osakafu-u.ac.jp

**Present address: CSIR-Central Salt and Marine Chemicals Research Institute, G.B. Marg, Bhavnagar, Gujarat-364002, India.

***Present address: Department of Chemical Engineering, Pennsylvania State University, University Park, PA 16802-4400, USA.

†These authors contributed equally to this work.

Administration of antimicrobial agents is the most common treatment strategy for bacterial infections. However, it is sometimes very difficult to treat cholera patients with proper antimicrobial agents due to the rising problem of multidrug resistance (MDR) in *V. cholerae* strains via mutations, horizontal gene transfer, etc. (7). A recent study has shown that acquisition of the MDR phenomena in O1 El Tor variant strains is increasing (8). Conventional antimicrobial agents are generally bactericidal or bacteriostatic, which may foster the development of MDR strains (9). Hence, novel therapeutic approaches are required to combat problems related to MDR in some pathogens, especially those that can cause devastating epidemics like cholera. Screening of natural compounds, which can specifically target bacterial virulence like toxin production without affecting viability, is one such approach and could be used to discover novel therapeutic interventions.

The use of natural products as therapeutics has increased dramatically in the last two decades (10). Spices and their constituents are generally recognized to be safe because of their traditional use without any documented detrimental impact (11). Since ancient times, natural products like spices and herbs have been considered effective against diarrheal diseases (12). Spices like cinnamon, cardamom, clove, turmeric, different peppers, red chili, fennel seeds, ginger, and garlic, or their extracts have been reported to possess medicinal properties against infectious pathogens including toxigenic *V. cholerae* (8,12,13). However, there is very limited information regarding the effect of any particular spice or its components on virulence factors production in *V. cholerae*.

Spices are inexpensive and used almost daily by South Asian people among whom cholera is endemic. Thus, we hypothesized that there may be some spices that can be used for medicinal purposes to treat diarrheal dis-

eases, such as cholera in the cholera endemic area. As an approach to search for anti-CT compounds, but not antimicrobial agents, we selected common spices, such as red chili (*Capsicum annuum*) and sweet fennel seeds (*Foeniculum vulgare*). Our preliminary studies indicated that among 6 species (red chili, sweet fennel seeds, white pepper, red pepper, cassia bark, and star anise), red chili showed the highest inhibitory activity against CT production followed by sweet fennel seeds. We have already reported that sub-bactericidal concentrations of extracts of red chili and capsaicin (a well-studied active component of red chili) can effectively suppress CT production in toxigenic *V. cholerae* (14,15). In this study, therefore, the methanol extract of sweet fennel seeds, which showed the second highest inhibitory activity against CT production, was examined for its effects on growth and CT production in toxigenic *V. cholerae* strains belonging to various serogroups and biotypes, such as O1 El Tor variants, O1 classical, O139 (classical and El Tor CT producer), and non-O1/non-O139 strains. Further, two major components of sweet fennel seeds, *trans*-anethole and 4-allylanisole, were examined for activity against CT production in *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains and culture conditions: In total, 17 clinical toxigenic *V. cholerae* strains were used in this study as described in Table 1. Among the *V. cholerae* strains, O1 El Tor variants and O139 serogroups were grown in AKI media (pH 7.4) at 37°C (16,17), whereas O1 classical and non-O1/non-O139 serogroups were grown at 30°C and 37°C, respectively in Luria–Bertani (LB) broth (pH 6.6; Difco, Lawrence, KS, USA) (18) for 8 h.

Preparation of methanol extracts of sweet fennel seeds: Sweet fennel seeds purchased at a local market in Guangzhou, China were extracted with 99.9% methanol (Nacalai Tesque, Kyoto, Japan) using an evaporator (R-210; Buchi, Flawil, Switzerland) under reduced pressure. The dried methanol extracts of sweet fennel seeds were preserved at 4°C, and appropriate amounts were dissolved in 99.9% methanol to make suitable concentrations before use.

Chemical components of sweet fennel seeds: For the major components of sweet fennel seeds, *trans*-anethole (purity ≥98%) was purchased from LKT Laboratories (St. Paul, MN, USA), and 4-allylanisole (Purity 98%) was from Sigma-Aldrich (St. Louis, MO, USA). The components were dissolved in 99.9% methanol to appropriate concentrations before use.

Purification of CT: CT was purified as described by Uesaka et al. (19). In brief, the *E. coli* strain MC1061 transformed with a plasmid containing the *ctx* genes was cultured in LB-broth supplemented with 100 μg/ml ampicillin at 37°C for 18 h with vigorous shaking. The cells were harvested by centrifugation, suspended in 10 mM Tris-HCl buffer (pH 8.6) containing 0.9% NaCl, and disrupted by sonication. The cell lysate was fractionated by ammonium sulfate to 65% saturation and thoroughly dialyzed against TEAN buffer (50 mM Tris-HCl, 0.2 M NaCl, 3 mM NaN_3, and 1 mM EDTA [pH 7.4]). The dialyzed sample was applied to an immobilized D-galactose column (Thermo Fisher Scientific, Waltham, MA, USA), eluted, and the concentration of CT was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The purity of the eluted CT was determined by SDS-PAGE.

Analysis of bacterial growth and quantification of CT production by bead-enzyme-linked immunosorbent as-

Table 1. Details for *Vibrio cholerae* strains and CT inhibition (%) by methanol extract of sweet fennel seeds

No.[1)]	Strain	Serogroup/Biotype	Country, isolation year	CT production Base line[2)]	CT production with SF extract[3)]	% inhibition[4)]
1	CO533	O1 El Tor variant	India, 2000	297 ± 53.4	18.7 ± 1.53	94
2	CRC27		India, 2000	166 ± 26.0	9.00 ± 0.51	95
3	CRC41		India, 2000	230 ± 23.9	13.9 ± 0.91	94
4	CRC87		India, 2000	160 ± 16.4	15.7 ± 3.01	90
5	CRC86		India, 2000	71.3 ± 7.51	7.65 ± 0.87	89
6	569B	O1 Classical	India, 1948	207 ± 24.3	101 ± 7.26	51
7	CL362		Bangladesh, 1963	56.3 ± 6.43	24.5 ± 5.27	57
8	CL432		India, 1969	44.0 ± 4.77	19.3 ± 2.52	56
9	CL614		India, 1970	36.7 ± 4.25	12.7 ± 1.60	65
10	CL507		India, 1970	135 ± 9.93	24.3 ± 4.65	82
11	SG24	O139	India, 1992	7.75 ± 0.57	5.26 ± 0.34	32
12	CO756		India, 1994	5.76 ± 0.88	5.01 ± 0.34	13
13	CRC142		India, 2000	272 ± 26.0	15.6 ± 0.68	94
14	PG234		India, 1998	56.5 ± 9.37	5.63 ± 0.70	90
15	AS507		India, 1997	6.33 ± 0.87	4.73 ± 0.38	25
16	CRC127	Non-O1/non-O139	India, 2000	15.5 ± 1.05	5.91 ± 0.75	62
17	RC1239		India	4.20 ± 0.76	2.86 ± 0.19	32

[1)]: Serial number.
[2)]: Quantification of CT production (ng/ml) in *V. cholerae* strains without sweet fennel (SF) seeds methanol extract measured by bead-ELISA as described in Oku et al. (20). Data are presented as the average ± SD of 3 test samples (ng/ml).
[3)]: Quantification of CT production (ng/ml) in *V. cholerae* strains with SF seeds methanol extract measured by bead-ELISA as above.
[4)]: CT inhibition (%) by SF methanol extracts (100 μg/ml) in different serogroups of *V. cholerae*.

say (ELISA): Initially, the effect of methanol extract of sweet fennel seeds on the growth of *V. cholerae* strains was determined. In brief, the optical densities (ODs) at 600 nm (OD_{600}) of *V. cholerae* cultures (using the aforementioned conditions) were adjusted to 1.0 and further diluted 100-fold with fresh culture medium before being incubated for 8 h either in the presence or absence of sweet fennel methanol extract. The methanol extract was added to the culture samples at sub-bactericidal concentrations ($\leq 100\ \mu g/ml$). After incubation, a portion of the culture was withdrawn and serially diluted with phosphate-buffered saline (PBS, pH 7.0). The diluted culture was spread onto LB-agar (Difco) plates to measure the CFU/ml in each sample.

Quantification of CT production in *V. cholerae* strains, either in the presence or absence of sweet fennel methanol extract, *trans*-anethole, or 4-allylanisole, was carried out by bead-ELISA as described previously (20). After 8 h of incubation, the remaining culture was used for preparation of cell free supernatant (CFS). CFS was prepared by centrifugation of the bacterial culture at 12,000 × *g* for 10 min, followed by filtration through a 0.22 μm filter (AGC Techno Glass, Tokyo, Japan). The CFS was adjusted to the appropriate dilutions with PBS (pH 7.0). Dilutions of purified CT of known concentrations were used to estimate the amount of CT in the CFSs of the cultures (14). Since the extract and pure chemicals were dissolved in methanol, the final concentration of methanol was always adjusted to 0.2% in cultures. Methanol (0.2%) alone was also added in a control study to check its effect on bacterial growth and CT production.

Assessment of dose-dependent effects of methanol extract of sweet fennel seed and its chemical components: Among the *V. cholerae* strains used in this study, 4 O1 El Tor variant strains were selected to analyze the dose-dependent effects of sweet fennel seed methanol extract and its chemical components under the same conditions as mentioned above. Sweet fennel methanol extract was added to the culture of 4 *V. cholerae* O1 El Tor variant strains (CO533, CRC27, CRC41, and CRC87) at different concentrations (10, 50, and 100 μg/ml), and quantification of CT production was carried out as described previously. In all cases, a control bacterial cultures was kept without adding any compounds, but contained 0.2% methanol. Furthermore, the pure chemical components of sweet fennel seeds, namely *trans*-anethole and 4-allylanisole were analyzed for their dose-dependent effects on the growth and CT production of an El Tor variant strain CO533.

Statistical analysis: All the experiments were conducted in triplicate, and mean values with standard deviation (SD) were calculated.

RESULTS

Efficiency of sweet fennel as an inhibitory agent against CT production: Initially, 17 clinical *V. cholerae* strains including 5 O1 El Tor variant (CO533, CRC27, CRC41, CRC87, and CRC86), 5 O1 classical (569B, CL362, CL432, CL614, and CL507,), 5 O139 (SG24, CO756, CRC142, PG234, and AS507), and 2 non-O1/non-O139 (CRC127 and RC1239) were selected randomly from a laboratory strain library (Table 1). We checked the effects of sweet fennel seed methanol extract against CT production in the above mentioned strains. Since 100 μg/ml of methanol extract of sweet fennel seed did not affect the growth of the *V. cholerae* strains tested in this study (data not shown), the strains were analyzed for CT production levels both in the absence and presence of sub-bactericidal concentrations of the spice extract. We observed that in the presence of spice extract the amounts of detectable CT in the culture supernatants of different *V. cholerae* serogroups were significantly reduced (Table 1). Despite belonging to different *V. cholerae* serogroups and biotypes, CT production (ng/ml) was significantly repressed in most of the bacterial strains tested in this study. Table 1 shows the percent inhibition of CT production in the 17 *V. cholerae* strains in the presence of sweet fennel seed methanol extract. Although all of the 5 O1 El Tor strains variant showed higher production (71.3–297 ng/ml) of CT in the absence of the extract, addition of the methanol extract caused drastic reductions in CT production (varied from 89–95% of CT production inhibition). In O1 classical strains, the inhibition of CT production varied from 51–82% in the presence of spice extract; whereas, the percent inhibition of CT production by the spice extract varied widely (13–94%) among strains belonging to the O139 serogroup. In contrast, in non-O1/non-O139 strains, CT production was inhibited by 32–62% (Table 1) by the methanol extract of sweet fennel seeds. Methanol (0.2%) alone, which was used as a control, did not show any inhibitory effect on the growth or CT production in *V. cholerae* strains compared to the without-methanol control (data not shown).

Dose-dependent inhibitory effect of methanol extract of sweet fennel seeds on CT production by *V. cholerae*: Recently emerged *V. cholerae* O1 El Tor variants are documented to cause more severe diarrhea (4,5). There-

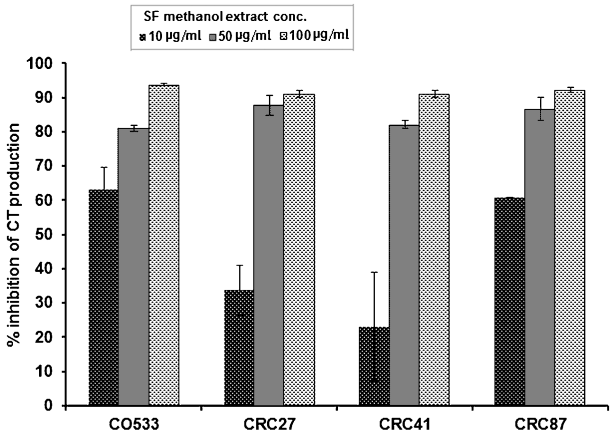

Fig. 1. Dose-dependent inhibitory effects of methanol extract of sweet fennel (SF) seeds on CT production in some *Vibrio cholerae* O1 El Tor variant strains. The inhibitory effects of methanol extract of SF seeds at various concentrations on CT production comparing with those of control cultures without the extract (0.2% methanol only) are presented as ''% inhibition of CT production'' in the y-axis. SF methanol extracts at various concentrations (0, 10, 50, and 100 μg/ml) were applied to O1 El Tor variant strains (CO533, CRC27, CRC41, and CRC87), and CT production was measured by bead-ELISA. The data is represented by mean ± SD.

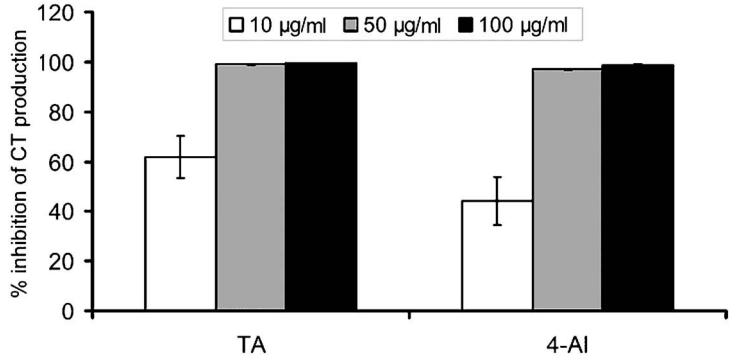

Fig. 2. Dose-dependent inhibitory effects of essential oil constituents of sweet fennel seeds on CT production in *Vibrio cholerae* O1 El Tor variant strain CO533. The inhibitory effects of *trans*-anethole (TA) and 4-allylanisole (4-AI) at various concentrations on CT production comparing with that of control cultures without the TA/4-AI (0.2% methanol only) are presented as ''% inhibition of CT production'' in the y-axis. Various concentrations (0, 10, 50, and 100 μg/ml) of TA/4-Al were applied to O1 El Tor variant strain CO533, and CT production was measured by bead-ELISA. The data is represented by mean ± SD.

fore, 4 O1 El Tor variant strains (CO533, CRC27, CRC41, and CRC87) having high CT production capabilities were selected for CT quantification with different doses of sweet fennel methanol extract (Table 1). We observed significant inhibition of CT production in all 4 O1 El Tor variant strains using different sub-bactericidal concentrations of sweet fennel seed methanol extract compared to the without-extract controls (Fig. 1). In the presence of different doses of sweet fennel methanol extract (10, 50, and 100 μg/ml), the amount of detectable CT in the culture supernatants were drastically reduced in a dose-dependent manner; 63–94% inhibition in CO533, 34–91% in CRC27, 23–91% in CRC41, and 61–92% in CRC87.

Essential oil components of sweet fennel seeds repress CT production in *V. cholerae*: It has been reported that *trans*-anethole, 4-allylanisole, fenchone, and limonene are the major components of sweet fennel seeds (21). In this study, commercially available *trans*-anethole and 4-allylanisole were used to analyze whether these are the active components responsible for repression of CT production. Among the *V. cholerae* strains analyzed in this study, a high CT-producing O1 El Tor variant CO533 was selected to analyze the effects of active components of sweet fennel seed extract. In this study, different doses (10, 50, and 100 μg/ml) of pure compounds were used, and no significant effect on the growth of O1 El Tor variant CO533 strain was observed (data not shown). Higher concentrations (50 and 100 μg/ml) of *trans*-anethole and 4-allylanisole were able to inhibit CT production drastically (92–93%; Fig. 2). Surprisingly, comparatively lower concentrations of these compounds (10 μg/ml) were also able to inhibit ~50% of CT production in the CO533 strain. Moreover, both of the compounds inhibited CT production in *V. cholerae* in a dose-dependent manner (Fig. 2).

DISCUSSION

Spices are popular, not only for their flavor and fragrance, but also for their medicinal values. Moreover, in tropical and subtropical countries, where diarrheal diseases like cholera are recurrent, peoples usually use an abundance of spices and herbs in their daily life. Different spices including sweet fennel are already well known for their disease healing capacity. Fennel seeds are recommended to reduce or cure many ailments such as abdominal pains, colic in children, constipation, diarrhea, flatulence, gastritis, irritable colon, and stomachache (12,22). In this study, we observed that sub-bactericidal concentrations of sweet fennel seed methanol extract and its major components could drastically inhibit CT production in different serogroups of *V. cholerae*.

The increase in antimicrobial resistance among enteric pathogens is an issue of great concern and reinforces the need for novel strategies to control epidemic diseases like cholera (23). However, any kind of antimicrobial agent targeting bacterial viability can be expected to impose selective pressure on the development of antimicrobial resistance. In contrast, the use of natural compounds to repress virulence factors without affecting bacterial growth has advantages such as preserving the host's indigenous micro-flora and can be expected to impose less selective pressure on the development of antimicrobial resistance (9). Based on this hypothesis, in the present study, the methanol extract of sweet fennel seed, which is a popular natural product, was selected and examined to investigate whether it can effectively inhibit CT production in *V. cholerae* without affecting growth. Different serogroups of *V. cholerae*, especially O1 El Tor variant strains, were selected because they are the predominant cause of current cholera pandemics in many developing countries (6,24).

As CT is the major virulence factor in toxigenic *V. cholerae*, inhibition of CT production with natural compounds like spices could be a unique strategy to reduce the severity of the disease. Even though many spices are known to possess antibacterial properties (8,13,25,26), very few extensive studies have evaluated their potential to inhibit toxin production in pathogenic bacteria (14,15). The present study has revealed that a methanol extract of sweet fennel seeds significantly inhibited CT production in different serogroups of *V. cholerae*, without affecting growth (Table 1 and Fig. 1). Although the degree of inhibition of CT production in *V. cholerae* strains varied (even within the same serogroup or biotype), a methanol extract of sweet fen-

nel seeds inhibited CT production irrespective of serogroups and biotypes. Thus, the inhibitory effect on toxin production of the analyzed spice appears to be a general phenomenon, but not serogroup or biotype specific. However, CT production inhibition by sweet fennel seed extract was relatively low in strains of *V. cholerae* that naturally produce low amount of CT (Table 1). In a recent study, we have shown that trans-anethole, a major component of sweet fennel seed extract, could suppress CT production in O1 El Tor variant strain CRC41 by affecting a virulence regulatory cascade (27). Considering these facts, we speculate that mutation of any of the genes in the virulence regulatory cascade could be the reason for the lower degree of CT inhibition by sweet fennel seed extract in those particular strains. However, further studies are needed to validate this hypothesis.

Recently, we have also reported the mechanism behind the repression of virulence factors expression in *V. cholerae* with the red chili component, capsaicin (14). Another report has shown similar result on the prevention of CT production in the presence of a small synthetic molecule 4-[N-(1,8-naphthalimide)]-n-butyric acid (28). However, very few natural compounds have been established as repressing CT production. Zhong et al. (29) has shown that red bayberry fruit extract could also inhibit CT production in *V. cholerae* at sub-bactericidal concentrations, but the mode of action behind this effect has not yet been established. Therefore, natural compounds, which are essential food ingredients and produce fewer side effects, could be effective to combat cholera. Further, two main essential oil components of fennel seeds, *trans*-anethole and 4-allylanisole, were tested against CT production to find out the agents responsible for CT production inhibition by the methanol extract of sweet fennel seeds (Fig. 2). Our data suggested that *trans*-anethole and 4-allylanisole could be probable agents in the methanol extract of sweet fennel seeds that exert CT inhibitory effects in O1 El Tor variant strains. In a recent study, *trans*-anethole, the most prevalent constituent of sweet fennel seed, was reported to be a probable antimicrobial agent against MDR *V. cholerae* strains (8); however, at sub-bactericidal concentrations of *trans*-anethole and 4-allylanisole, bacterial growth was not affected. Both components are generally recognized as safe by the 'Expert Panel of the Flavor' and 'Extract Manufacturer's Association' (30), and are approved by the US Food and Drug Administration (FDA) for food use.

In conclusion, the methanol extract of sweet fennel seeds and its essential oil components, *trans*-anethole and 4-allylanisole, can inhibit the CT production in various serogroups of *V. cholerae* without affecting growth. Further studies are needed to understand the inhibitory mechanism against CT production by sweet fennel seed methanol extract, *trans*-anethole, and 4-allylanisole. Thus, daily intake of sweet fennel seeds containing *trans*-anethole and 4-allylanisole could be an inexpensive and alternative approach to prevent cholera.

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Conflict of interest None to declare.

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