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Anethole inhibits growth of recently emerged multidrug resistant toxigenic *Vibrio cholerae* O1 El Tor variant strains *in vitro*

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ABSTRACT. To search natural compounds having inhibitory effect on bacterial growth is important, particularly in view of growing multidrug resistant (MDR) strains of bacterial pathogens. Like other bacterial pathogens, MDR *Vibrio cholerae,* the causative agent of diarrheal disease cholera, is becoming a great concern. As an approach of searching new antimicrobial agents, here, we show that anethole, a well-studied natural component of sweet fennel and star anise seeds, could potentially inhibit the growth of MDR O1 El Tor biotype, the ongoing 7th cholera pandemic variant strains of toxigenic *V. cholerae*. The minimum inhibitory concentration (MIC) of anethole against diverse O1 El Tor biotype strains is evaluated as 200 μg/m*l*. Moreover, the effect of anethole is bactericidal and exerts rapid-killing action on *V. cholerae* cells. This study is the first report which demonstrates that anethole, purified from natural compound, is a potent inhibitor of growth of toxigenic *V. cholerae*. Our data suggest that anethole could be a potential antimicrobial drug candidate, particularly against MDR *V. cholerae* mediated infections.

KEY WORDS: anethole, El Tor variant, multidrug resistant, natural compounds, *Vibrio cholerae*

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Antimicrobial agents have been playing a pivotal role in controlling bacterial infections since their discovery. However, the recent situation of global emergence and spread of multidrug resistant (MDR) pathogenic bacteria demands the development of new antimicrobial agents [2]. The same scenario is continuing to combat toxigenic *Vibrio cholerae*, which is responsible for historical cholera pandemics. Multidrug resistance against nalidixic acid, furazolidone, trimethoprim, sulfamethoxazole and tetracycline in *V. cholerae* is spreading widely [13]. Moreover, azithromycin resistant *V. cholerae* is already reported, and resistance to ciprofloxacin is emerging, indicating a very few effective antimicrobial options for future treatment of cholera [18, 28].

The disease cholera is caused by the effect of cholera toxin (CT) encoded by the *ctxAB* genes and characterized by vomiting, profuse watery diarrhea and severe dehydration [10, 21]. The ability of production of CT is the hallmark of toxigenic *V. cholerae*. Although till date >200 'O' serogroups of this species have been reported, only O1 and O139 are responsible for cholera outbreaks [19]. Serogroups other than O1 and O139 (non-O1/-O139) caused sporadic cases of diarrhea and extra-intestinal infections [4]. *V. cholerae* strains belonging to O1 serogroup are further subdivided into 2 biotypes, El Tor and classical.

Cholera is an ancient disease, and so far, 7 pandemics have been recorded since the first pandemic began in 1817 [7]. Among *V. cholerae* O1 serogroup, the O1 El Tor biotype is responsible for the ongoing seventh pandemic of cholera, started in 1961, while the sixth and presumably the earlier pandemics were caused by the O1 classical biotype strains, which is now possibly extinct [23]. Recently emerged *V. cholerae* O1 El Tor hybrid strains (possess some attributes of classical biotype including *ctxB* gene allele) also categorized as El Tor variant strains produce more CT and cause more severe diarrhea than prototype El Tor [8, 9]. Analysis of the strains from recent devastating cholera outbreak in Haiti reveals that MDR O1 El Tor variant strains are now ruling the cholera world [24, 25]. As highly virulence epidemic *V. cholerae* strains are rapidly acquiring resistance towards the existing antimicrobial agents, it is time to develop or identify effective novel antimicrobial agents to fight them in near future.

It is generally expected that bioactive compounds as antimicrobial agents from plant origin are effective against infectious diseases and simultaneously subside many of the side effects often shown by the synthetic antimicrobials. Since ancient times, natural products from medicinal plants, such as spices, herbs, etc., have been used to treat enteric infections [15]. So, natural products of plant origin could be a reservoir to explore new antimicrobial agents against toxigenic MDR *V. cholerae* strains.

Much efforts and attention have already been paid to search effective antimicrobial agents from natural sources against *V. cholerae*. Previous reports have demonstrated that extracts from Japanese green tea (*Camellia sinensis*), 'neem' (*Azadirachta indica*), 'elephant garlic' and *Vitex negundo* leaf could effectively inhibit *V. cholerae* growth [12, 20, 26, 27]. We have also recently found that methanol extract of sweet fennel seed could potentially inhibit the

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Table 1. The relevant characteristics of *V. cholerae* strains used in this study

Strain ID	Serogroup/biotype	Antimicrobial resistance profile	Origin, Isolation year
NICED-1	O1 El Tor, *ctxB* genotype: El Tor	None	India, 1970
NICED-10	O1 El Tor, *ctxB* genotype: El Tor	None	India, 1970
NICED-3	O1 El Tor, *ctxB* genotype: El Tor	None	India, 1980
P130	O1 El Tor, *ctxB* genotype: El Tor	None	Peru, 1991
VC190	O1 El Tor, *ctxB* genotype: El Tor	None	India, 1993
CO533	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR	India, 1994
CRC27	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR	India, 2000
CRC41	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR	India, 2000
CRC87	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR	India, 2000
B33	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR	Mozambique, 2004
1'/2005	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA	India, 2005
2'/2005	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA	India, 2005
5′/2005	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA	India, 2005
2680713	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR	Bangladesh, 2006
2684269	O1 El Tor variant, *ctxB* genotype: Classical	SM, SXT, NA, FR, TE	Bangladesh, 2006

None: Not resistant to the tested antimicrobials, SM: Streptomycin, SXT: Sulfamethoxazole/trimethoprim, NA: Nalidixic acid, FR: Furazolidone, TE: Tetracycline.

growth of toxigenic *V. cholerae* (Chatterjee *et al.*, unpublished). Trans-anethole (*p*-methoxypropenyl benzene) is the major and active component (80–90%) of 'essential oil' extracted from sweet fennel and star anise seeds. Although antimicrobial activities of anethole (trans-anethole) against some bacteria, yeast and fungi are well established [6, 14], still there is no report regarding its effect on the growth of *V. cholerae*. In this study, we have examined the effect of purified anethole on the growth of toxigenic *V. cholerae* O1 El Tor strains.

MATERIALS AND METHODS

Test strains and ctxB genotyping: A total of 15 toxigenic *V. cholerae* O1 strains of different isolation origin and belonging to the El Tor biotype, as shown in Table 1, were selected randomly from the strain collection of the Laboratory of International Prevention of Epidemics, Osaka Prefecture University. To determine the *ctxB* gene alleles (classical or El Tor) of the tested strains, a mismatch amplification mutation PCR assay (MAMA-PCR) was carried out according to Morita *et al*. [16].

Antimicrobial susceptibility testing of the analyzed strains: Antimicrobial resistance pattern of the tested strains was determined by disc diffusion method according to guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) 2011 [5]. In brief, a single colony of *V. cholerae* from thiosulfate-citrate-bile salts-sucrose (TCBS: Eiken Chemical Co., Ltd., Tokyo, Japan) agar plate was grown in 3 m*l* of Mueller Hinton broth (Becton, Dickinson and Company, Franklin lakes, NJ, U.S.A.) at 37°C until the turbidity reached to 0.5 McFarland standards. By using sterile cotton swabs, an evenly distributed bacterial lawn was prepared on Mueller Hinton agar plates. Then, the antimicrobial discs were softly placed on each bacterial lawn. The inhibition zone of each antimicrobial agent was analyzed after overnight incubation at 37°C. The inhibition zone was determined as resistant or susceptible based on those of bacterial cells belonging to the family *Enterobacteriaceae* as guidelines of CLSI, 2011. *Escherichia coli* strain ATCC 25922 was used as an internal control. Antimicrobials (all from Becton, Dickinson and Company) used are as follows: sulfamethoxazole/trimethoprim (SXT) (1.25/23.75 μg), ampicillin (10 μg), chloramphenicol (30 μg), tetracycline (30 μg), norfloxacin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), kanamycin (30 μg), doxycycline (30 μg), azythromycin (15 μg), gentamycin (10 μg), furazolidone (50 μg) and ciprofloxacin (5 μg).

Media and growth conditions: The effect of trans-anethole (Nacalai Tesque, Kyoto, Japan; purity 98%) on the growth of *V. cholerae* O1 El Tor biotype strains was checked by co-culturing in AKI medium (0.5% NaCl, 0.4% Yeast extract and 1.5% Bactopeptone) supplemented with 0.3% $NaHCO_3$ (pH 7.4) at 37°C. A single colony of *V. cholerae* from TCBS agar plate was inoculated in AKI medium. After 12 hr of growth, optical density (OD) at 600 nm (OD_{600}) was adjusted to 1.0 and diluted 100-fold with fresh AKI medium [~10^7 colony-forming unit (CFU)/m*l*] and incubated both in the presence and absence of anethole. The culture condition was maintained according to Iwanaga *et al*. [11], with slight modifications. Briefly, cultures with or without anethole were kept under stationary condition for an initial 4 hr and then shifted to a shaking condition at 180 rpm for another 4 hr, unless otherwise needed. As anethole was dissolved and diluted in methanol, the final concentrations of methanol were always kept ≤0.5% in cultures. Appropriate dilutions of the culture samples were made with phosphate-buffered saline (PBS, pH 7.0) and spread on Luria–Bertani (LB) agar (Becton, Dickinson and Company) to see the bacterial viability as CFU/m*l*.

Determination of MIC and MBC of anethole: The minimum inhibitory concentration (MIC) of anethole against

Table 2. Effect of anethole on the growth of different *V. cholerae* strains

Strain ID	OD_{600} at different concentration of anethole (μg/m*l*)					
	0	50	100	150	200	300
NICED-1	1.83 ± 0.11	1.85 ± 0.13	1.73 ± 0.05	0.47 ± 0.09	0	0
NICED-10	1.80 ± 0.09	1.74 ± 0.05	1.57 ± 0.09	0.67 ± 0.14	0	0
NICED-3	1.38 ± 0.06	1.37 ± 0.06	1.30 ± 0.07	0.11 ± 0.02	0	0
P130	1.86 ± 0.11	1.76 ± 0.07	1.68 ± 0.05	0.48 ± 0.09	0	0
VC190	2.15 ± 0.05	1.94 ± 0.06	1.80 ± 0.10	0.38 ± 0.05	0	0
CO533	2.60 ± 0.05	2.50 ± 0.10	2.14 ± 0.06	0.52 ± 0.06	0	0
CRC27	2.46 ± 0.06	2.29 ± 0.06	2.18 ± 0.06	0.61 ± 0.07	0	0
CRC41	2.59 ± 0.08	2.47 ± 0.07	2.32 ± 0.11	0.75 ± 0.12	0	0
CRC87	2.12 ± 0.06	1.97 ± 0.07	1.70 ± 0.08	0.14 ± 0.07	0	0
B33	2.08 ± 0.06	1.87 ± 0.10	1.84 ± 0.07	0.62 ± 0.06	0	0
1’/2005	1.77 ± 0.06	1.66 ± 0.04	1.41 ± 0.08	0.48 ± 0.12	0	0
2’/2005	1.74 ± 0.07	1.64 ± 0.07	1.34 ± 0.07	0.19 ± 0.04	0	0
5′/2005	1.86 ± 0.11	1.85 ± 0.10	1.68 ± 0.06	0.46 ± 0.05	0	0
2680713	2.62 ± 0.09	2.53 ± 0.05	2.24 ± 0.14	0.57 ± 0.12	0	0
2684269	2.68 ± 0.09	2.55 ± 0.08	2.23 ± 0.06	0.72 ± 0.08	0	0

OD_{600}, Optical density at 600 nm; In all cases, values represent the mean (OD_{600}) ± SD of three independent bacterial cultures at respective anethole concentration.

tested *V. cholerae* strains was determined by broth macrodilution methods as described by previous authors [17] with some modifications. Briefly, cultures (~10^7 CFU/m*l*) without or with different concentrations of anethole were co-cultured in one m*l* of AKI medium according to our desired culture conditions. Then, the MIC was determined as the lowest concentration of anethole in which no growth of *V. cholerae* was observed at OD_{600} by using a spectrophotometer.

The minimum bactericidal concentration (MBC) of anethole was determined as the concentration of anethole which completely (~100%) killed the *V. cholerae* strains compared to that of untreated control. The MBC of anethole was confirmed by re-inoculating the broth cultures showing no visible bacterial growth onto the LB agar plates following overnight incubation at 37°C.

Time-kill studies: To examine the effect of anethole on the killing of *V. cholerae* strains in more detail, time-kill studies were performed. Aliquots of the cultures with (MBC) or without anethole were withdrawn at desired time-points, and bacterial viability was checked by spreading appropriate dilutions of samples onto LB-agar plates. Time–kill studies also demonstrate the least required time to exert bactericidal effect by anethole.

RESULTS

ctxB genotyping and antimicrobial resistance profile of the tested strains: Among 15 *V. cholerae* O1 El Tor strains, the *ctxB* gene alleles of 10 strains were confirmed as classical type by MAMA-PCR and categorized as El Tor variant. On the other hand, rest of the 5 strains, which carried El Tor type *ctxB* gene alleles, were confirmed as typical El Tor (Table 1). Antimicrobial resistance pattern of the tested strains is presented in Table 1. Surprisingly, all of the El Tor variant strains, which are the current epidemic strains, showed resistance to the multiple commonly used antimicrobial agents. On the other hand, prototype El Tor strains showed resistance to none of the antimicrobial agents, used in this study.

MIC and MBC of anethole: We tested the effect of anethole on the growth of 15 toxigenic *V. cholerae* O1 El Tor strains by co-culturing with different concentrations of anethole. Methanol (0.5%) alone was also added in a control assay as it had no detectable effect on *V. cholerae* growth (data not shown). As shown in Table 2, ≤100 μg/m*l* anethole did not show significant growth inhibitory effect on the analyzed *V. cholerae* strains compared to those of anethole untreated controls. But, 150 μg/m*l* anethole showed potent antibacterial activity against all of the tested strains. Moreover, no visible growth (determined at OD_{600}) was observed in the analyzed strains after co-cultured with ≥200 μg/m*l* anethole, even when those cultures were exposed to shaking growth phase for 4 hr.

We further investigated whether the effect of anethole on the growth of *V. cholerae* is bacteriostatic or bactericidal. To test this, we re-examined the cultures of each of the representative El Tor (P130) and El Tor variant strain (CRC41) both in the absence and presence of different concentrations of anethole onto the LB agar plate and compared the cell viability as CFU/m*l*. Due to some limitations in spreading bacterial cultures onto agar plate, we were unable to detect the viable cells below 10 CFU/m*l*. As shown in Fig. 1, a significant growth inhibition (P<0.01) of the tested strains was observed in presence of 150 μg/m*l* anethole compared to the anethole untreated controls. Moreover, no viable bacteria were detected after co-culturing with 200 μg/m*l* anethole in the case of both strains. We found that 200 μg/m*l* anethole is bactericidal at least in case of these 2 strains. So, we concluded that the effect of anethole on the growth of *V. cholerae* is bactericidal.

Anethole exerts rapid bactericidal effect: We tested the time-dependent killing effect of anethole on the strains

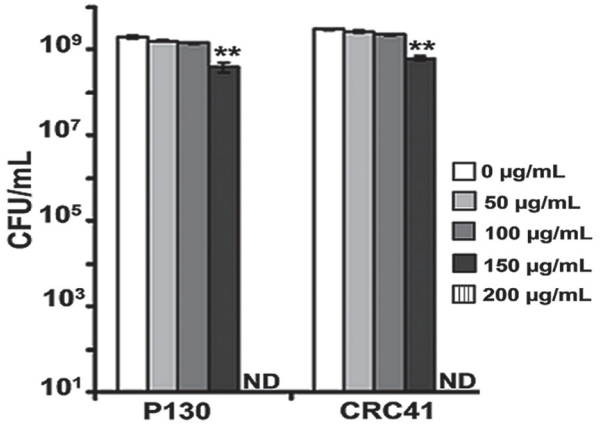

Fig. 1. Recovery of *V. cholerae* cells after incubating with different concentrations of anethole. *V. cholerae* cells were co-cultured with anethole in AKI medium, and bacterial viability was confirmed by inoculating those cultures onto the LB agar plates. *x*-axis indicates the strain ID. 'ND' indicates that no viable CFU was recovered after spreading of the 100 μl of bacterial cultures onto the agar plates followed by overnight incubation at 37°C. Values represent averages ± SD of three independent experiments. By using two-sample *t*-test, two asterisks (**) represent $P<0.01$ as compared with the anethole-free culture as a control.

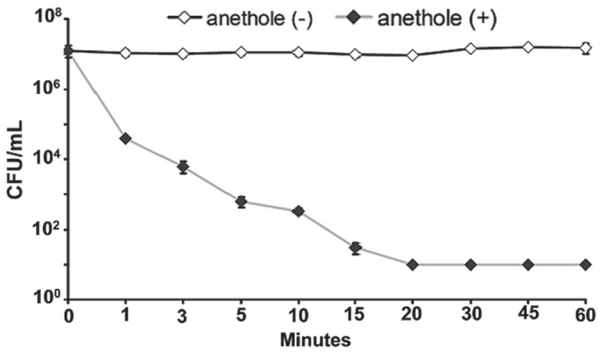

Fig. 2. Time-killing effect of anethole on *V. cholerae* O1 El Tor variant. *V. cholerae* O1 El Tor variant strain CRC41 was incubated with 200 μg/m*l* of anethole for 60 min. Viable bacteria were counted by inoculating cultures from each of the desired time points onto the LB agar plates. ≤10 CFU/m*l* indicates the below detection limit of the recovered bacteria. Data represented as the mean ± SD of three independent experiments.

P130 (typical El Tor) and CRC41 (El Tor variant) at our experimental conditions. Initially, we observed that 200 μg/m*l* anethole potentially killed the tested strains within one hr of incubation at 37°C (data not shown), as no CFU was detected following incubation of those cultures on the LB agar plates. Then, to monitor the anethole-mediated bactericidal effects in more detail, we shorten the incubation time (within 60 min) and analyzed the bactericidal effect of anethole on O1 El Tor variant strain CRC41. As shown in Fig. 2, anethole caused rapid killing of *V. cholerae* cells. Anethole killed 100% of the incubated *V. cholerae* cells within 20 min, as no CFU was detected following incubation of the cultures onto the LB agar plates.

DISCUSSION

During the second half of the 20th century, it was generally accepted that antimicrobial agents from medicinal plants have less side effects and thus are advantageous for therapeutic purposes than synthetic. Moreover, researchers are encouraged to investigate the antimicrobial activity of natural products as the traditional antimicrobial agents are losing their effectiveness against MDR pathogenic bacteria. As methanol extract of sweet fennel seeds showed potential growth inhibition in *V. cholerae* (Chatterjee *et al*, unpublished), it would be very useful if we could identify the active compounds exerting such effects. As an approach of searching active compounds in methanol extract of sweet fennel seeds, we targeted first anethole which accounts for 80% of the essential oil derived from sweet fennel seeds [22]. Besides having various medicinal properties, anethole and its natural reservoir sweet fennel seeds are generally used as a food additive or as a spice component. Although antimicrobial activities of anethole have already been well established against some bacteria, yeast and fungi [6, 14], effect on *V. cholerae* was not yet evaluated.

Recent cholera outbreaks caused by the O1 El Tor variant strains become more severe in terms of generating symptoms than past, might be due to the co-existence of MDR and higher cholera toxin production phenomena in them than prototype El Tor [9, 24, 25]. In this study, all of the O1 El Tor variant strains, which were confirmed by their *ctxB* genotyping, have recently been emerged as compared to the prototype El Tor strains (Table 1). Antimicrobial susceptibility testing also revealed that variant type strains are resistant to multiple drugs compared to prototype El Tor strains, which were found to be susceptible to all of the antimicrobial agents tested in this study. These observations also support the idea that recently emerged El Tor variant strains are replacing the prototype El Tor strains and simultaneously acquiring the MDR phenomena. If these trends continue, in near future, to treat infections mediated by MDR *V. cholerae* O1 El Tor variant strains will be very difficult unless novel antimicrobials are discovered.

We tested the effect of anethole on the growth of *V. cholerae* O1 El Tor strains in AKI medium supplemented with 0.3% $NaHCO_3$ (pH 7.4) which resembles the environment of human small intestine. Moreover, this medium is specially considered for maximum virulence induction by O1 El Tor strains when cultures were kept for an initial 4 hr stationary followed by a shaking growth phase [1, 11]. So, by considering the environment of host small intestine during early phase of *V. cholerae* infection, its closely mimic *in vitro* virulence inducing conditions in AKI medium was used to analyze the effect of anethole on the growth of *V. cholerae* O1 El Tor strains.

It is generally expected that purified compounds from medicinal plant could exert better antimicrobial effect

than whole extracts. In this study, MBC of anethole was evaluated as 200 μg/m*l* against all of the tested *V. cholerae* strains, also demonstrating the potentiality of the anethole as antimicrobial agent. Moreover, rapid-killing of *V. cholerae* cells by MBC of anethole (Fig. 2) demonstrates the efficacy of anethole as an antimicrobial drug. Although we did not study detail mechanisms of bactericidal effect of anethole, microscopic observations revealed morphological changes in anethole-treated cells compared to the curved-rod shape of untreated *V. cholerae* cells (data not shown).

To analyze whether the antimicrobial activity of anethole is *V. cholerae* specific or not, we tested the effect of anethole on the growth of two virulent *Vibrio parahaemolyticus* strains. Although detail data are not shown here, we found that $\geq$150 μg/m*l* anethole is bactericidal against them. Previously, the effect of essential oils of oregano, thyme and sassafras was evaluated as bactericidal against *V. parahaemolyticus* at a concentration of 100 μg/m*l* [3]. In this study, bactericidal effect of anethole against *V. parahaemolyticus* along with *V. cholerae* also supports the idea that anethole might have broad-spectrum antibacterial activity.

Because of having fewer side effects to hosts, anethole is confirmed as "GRAS" (Generally Recognized as Safe) by the FDA (Food and Drug Administration) and FEMA (Flavor Extract Manufactures Association) in the U.S.A. Correlating these with the findings in this study, it is strongly suggested that anethole could be used as future alternatives to control *V. cholerae* contamination in foods. Alternatively, daily intake of sweet fennel seeds containing anethole could be a cheap remedy for *V. cholerae*-mediated diarrhea and other extra-intestinal infections. Although further studies are necessary, we strongly believe that anethole could be a potential antimicrobial drug candidate against cholera, which remains a significant public health burden, especially in the developing world.

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