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The Antibacterial Activities of Peppermint Oil and Green Tea Polyphenols, Alone and in Combination, against Enterohemorrhagic *Escherichia coli*

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The antibacterial activities of peppermint oil and its 53 constituents against *Escherichia coli* were examined in a preliminary screening test. Peppermint oil and 15 of its constituents had significant bactericidal activity against non-pathogenic *E. coli* and enterohemorrhagic *E. coli* O157:H7. Polyphenols from green tea and four catechins from green tea also had antibacterial activity aganist *E. coli* O157:H7 in liquid culture medium. Peppermint oil and three of its constituents, namely, menthol, menthone and neomenthol, killed the bacteria within one hour at concentrations above 400 μg/ml in phosphate-buffered saline. Polyphenols from green tea showed bacteriostatic activity in the culture medium, and bactericidal activity in PBS. This latter effect only became evident after several hours. A synergistic effect was observed for peppermint oil and for menthol when combined with green-tea polyphenols. These results suggest that the antibacterial activities of peppermint oil and green-tea polyphenols might involve different mechanisms and combinations of peppermint oil or the active constituents of peppermint oil with green-tea polyphenols might be useful as natural antibacterial agents against *E. coli* O157:H7.

Key words : Enterohemorrhagic *E. coli* O157:H7/Antibacterial properties/Combined effect/Peppermint oil/Green-tea polyphenols.

INTRODUCTION

Peppermint oil, one of the most popular flavorings worldwide, is used extensively in the production of foodstuff and confectioneries. Peppermint oil includes many constituents such as menthol, menthon and neomenthol. Peppermint oil and its constituents have been reported to have various biological activities including those which are antibacterial (Dikshit and Husain, 1984; Maiti et al., 1985; Shapiro et al., 1994; Sivropoulou et al., 1995), insecticidal (Mansour et al., 1986), antiallergic (Arakawa et al., 1992) and antimutagenic (Samejima et al., 1995). In particular, the antibacterial activity of peppermint oil and its constituents against non-pathogenic *Escherichia coli* has been reported, but not against enterophathogenic *E. coli* O157:H7 (Okamura, 1974; Sivropoulou et al., 1995).

The polyphenols of green tea, such as various catechins, have also been reported to have bactericidal activity against strains of enterohemorrhagic *E. coli* O157:H7 (Toda and Shimamura, 1996).

In this study, we examined the antibacterial activities of peppermint oil and its constituents, as well as the effects of combinations of peppermint oil or its constituents with polyphenols from green tea, aganist *E. coli* O157:H7.

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MATERIALS AND METHODS

Bacterial strains

A non-pathogenic strain of *E. coli*, IFO 3301, was used in preliminary screening experiments and was

provided by the Institution for Fermentation, Osaka, Japan. Enterohemorrhagic *E. coli* O157:H7 strain no. 9664 was used in subsequent tests. Strain no. 9664 was derived from a clinical isolate and was provided by the Tokyo Metropolitan Research Laboratory of Public Health.

Chemicals

The essential oil of peppermint (*Mentha piperita* L.), produced in the Midwest of the U. S. A. in 1996, was used. Menthol, menthone, neomenthol, menthofuran, (+)-limonene, piperiton, *cis*-jasmone, (−)-myrtenol and carvacrol were obtained from Extrasynthese S. A. (Genay, France). (−)-Epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg) and (−)-epigallocatechin gallate (EGCg) were obtained from Kurita Industry Co., Ltd. (Tokyo). Eugenol and 2-ethylfuran were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo). Ocimene was obtained from Fluka Chemie AG (Buchs, Switzerland). (−)-Menthyl acetate, terpinen-4-ol, 1,8-cineole, terpineol, caryophyllene, (−)-carvone, linalool, 3-octanol, 1-octen-3-ol, 2-methylbutyl *iso*-valerate, *iso*-valeraldehyde, *iso*-amyl *iso*-valerate, *iso*-pulegol, neomenthyl acetate, *iso*-amyl alcohol, mint lactone, *cis*-3-hexenol, *cis*-2-pentenol, piperitol, 3-heptanol, hexyl aldehyde, menthyl valerate, 3-methyl cyclohexanone, *trans*-2-hexenol, phenylethyl *n*-valerate, 1-octen-3-yl acetate, *iso*-valeric acid, hexyl alcohol, pulegone, β-ionone, 3-octyl acetate, *cis*-3-hexenyl acetate, geranyl acetone, citronellyl acetate, α-pinene, β-pinene, myrcene, α-terpinene, γ-terpinene, ρ-cymene and terpinolene were gifts from Takasago International Co. (Tokyo).

Preparation of polyphenols from green tea and green tea in beverage form

Polyphenols were prepared from green tea as described previously (Horita, 1989). Leaves of green tea (*Camellia sinensis*), *Sen-cha*, pulverized to fragments of less than 2 mm (100 g) were extracted with two liters of 80% (v/v) ethanol at room temperature for two hours. The extract after evaporation (20 g) was fractionated by gel filtration (TOYOPEARL HW-40; Tosoh Co. Ltd., Tokyo) and, finally, a dry powder (11.7 g) was obtained that was used as green-tea polyphenols. The purity of the polyphenols was 98.7% as polyphenols, as estimated by the Folin-Denis method (Nakabayashi, 1968). Levels of ECg, EGC and EGCg were 6.6%, 44.7% and 47.3% (w/w), respectively, as determined by high-performance liquid chromatography (HPLC).

Green tea, in beverage form, was prepared as follows. Leaves of green tea (50 g), *Sen-cha*, were extracted with two liters of distilled water at 95℃ for 5 min. An extract (14.8 g) was obtained by lyophilization and dissolved at 7.4 mg/ml in culture medium to give a concentration equivalent to that in the common beverage. The polyphenol content of the extract was 33.2% (w/w), as determined by the Folin-Denis method. Levels of EC, ECg, EGC and EGCg were 2.9%, 2.1%, 12.8% and 14.8% (w/w), as determined by HPLC, respectively.

Preliminary screening of the antibacterial activities of peppermint oil and its constituents

The bactericidal activities of peppermint oil and its constituents against *E. coli* strain IFO3301 were determined as follows. Each test material was dissolved in methanol and each solution was diluted with phosphate-buffered saline (PBS; Nissui Pharmaceutical Co., Ltd., Tokyo) to give a concentration of 500 μg/ml. The test bacteria were inoculated in the solutions of each test material in PBS at 10^6 cfu/ml and suspensions were incubated at room temperature. Samples were removed after 5 h and 24 h from the each suspension. Each sample was diluted appropriately and spread on a Mueller-Hinton agar (MHA; Difco Laboratories Detroit, MI) plate for determination of the number of viable cells. The concentration of methanol in each test solution was always less than 2% (v/v). At this level, menthol did not affect the growth of the test bacteria.

Effects on the growth of *E. coli* O157:H7

The effects of the test materials on the growth of enterohemorrhagic *E. coli* were determined as follows. Mueller-Hinton broth (MHB; Difco Laboratories) was used in this experiment. Test materials were dissolved in methanol, and 200 μl of each solution were added to 10-ml aliquots of MHB to give final concentrations of 50 to 800 μg/ml in culture tubes of a biophotorecorder (TN-2612; ADVANTEC, Tokyo; Akimoto et al., 1998; Arai et al., 1996). Each culture tube was inoculated with an overnight culture of the test strain. The concentration of methanol in each culture was always less than 2% (v/v), and did not affect the growth of the bacteria. Each test tube was sealed with a butyl-rubber stopper after inoculation and incubated at 37℃, with shaking at 40 rpm. The growth of bacteria in each tube was monitored in terms of optical density (O. D.) at 660 nm.

The minimum inhibitory concentrations (MICs) of the test materials against the test strain were determined from the results obtained with the biophotorecorder. The MIC was the lowest concentration of the test material in the test tubes at which no increase in turbidity was recorded within 18 h. After

the MIC had been determined, the minimum bactericidal concentration (MBC) of each test material was determined by spreading each culture from the culture tubes on MHA plates and incubating the plates overnight at 37℃.

Bactericidal activities in PBS against *E. coli* O157:H7

The bactericidal activities of the test materials in PBS against enterohemorrhagic *E. coli* O157:H7 were determined as follows. Test materials were dissolved in methanol, and 200-μl aliquots of the solutions were added to 10-ml aliquots of PBS in culture tubes of the biophotorecorder. Then the test bacteria were inoculated into the culture tubes at 10^5 cfu/ml. Each tube was tightly sealed with a butyl-rubber stopper and incubated at 37℃ with shaking at 40 rpm. Samples were removed at 0, 1, 3, 5 and 24 h, and numbers of viable cells were determined by spreading samples on MHA plates by the spiral plating method (Autoplate Model 3000; Spiral System Instruments, Inc., Bethesda, MD, U. S. A.).

Effects in combination

The effects of peppermint oil, menthol, menthone and neomenthol in combination with green-tea polyphenols were determined in terms of the fractional inhibitory concentration (FIC) index by the checkerboard method (Ellion et al., 1954). Values of FIC between 1.0 and 0.5 indicated the additive effects and values of 0.5 or less indicated synergistic effects (Matsumoto et al., 1998). This experiment was repeated at least twice for each combination.

RESULTS

Preliminary screening of antibacterial activities of peppermint oil, its constituents and green-tea polyphenols

The antibacterial activities of peppermint oil, its 53 constituents and polyphenols from green tea were determined first with a non-pathogenic strain of *E. coli* (Table 1). Peppermint oil and 15 of its constituents, namely, menthol, menthone, neomenthol, menthofuran, (+)-limonene, piperiton, 3-octanol, *cis*-jasmone, mint lactone, (−)-myrtenol, piperitol, eugenol, carvacrol, 2-ethylfuran and ocimene, had strong antibacterial activity that was evident within 24 h. The polyphenols from green tea also exhibited significant antibacterial activity.

Effects on the growth of *E. coli* O157:H7

The growth of *E. coli* O157:H7 in the presence of various concentrations of peppermint oil, polyphenols

TABLE 1. Antibacterial activities of peppermint oil and its constituents aganist *E. coli* IFO3301.

Test materials	Duration of incubation (h)		Test materials	Duration of incubation (h)	
	5	24		5	24
Peppermint oil	++[a]	+++	Piperitol	++	+++
Menthol	++	+++	3-Heptanol	+	++
Menthone	++	+++	Hexyl aldehyde	+	++
(−)-Menthyl acetate	+	+	Menthyl valerate	−	−
Terpinen-4-ol	+	++	Eugenol	++	+++
1,8-Cineole	−	++	3-Methyl cyclohexanone	−	−
Neomenthol	++	+++	Phenylethyl *n*-valerate	+	+
Terpineol	+	++	*trans*-2-Hexenol	+	+
Menthofuran	++	+++	1-Octen-3-yl acetate	++	++
Caryophyllene	−	+	*iso*-Valeric acid	−	−
(+)-Limonene	++	+++	Hexyl alcohol	−	+
Piperiton	++	+++	Pulegone	++	++
(−)-Carvone	+	++	β-Ionone	+	++
Linalool	+	++	3-Octyl acetate	+	−
3-Octanol	++	+++	Carvacrol	++	+++
1-Octen-3-ol	+	++	*cis*-3-Hexenyl acetate	−	+
2-Methylbuthyl *iso*-valerate	−	−	Geranyl acetone	−	+
cis-Jasmone	++	+++	Citronellyl acetate	−	+
iso-Valero aldehyde	++	++	2-Ethylfuran	++	+++
iso-Amyl *iso*-valerate	+	++	α-Pinene	++	++
iso-Pulegol	+	++	β-Pinene	++	++
Neomenthyl acetate	+	++	Myrcene	−	+
iso-Amyl alcohol	−	+	α-Terpinene	+	+
Mint lactone	++	+++	γ-Terpinene	−	−
cis-3-Hexenol	−	+	Ocimene	+	+++
cis-2-Pentenol	−	+	ρ-Cymene	++	++
(−)-Myrtenol	++	+++	Terpinolene	+	+
			Green tea polyphenols	++	+++

[a] +++, $<10^2$ cfu/ml; ++, $10^2 - <10^4$ cfu/ml; +, $10^4 - <10^6$ cfu/ml; −, $\geq 10^6$ cfu/ml.

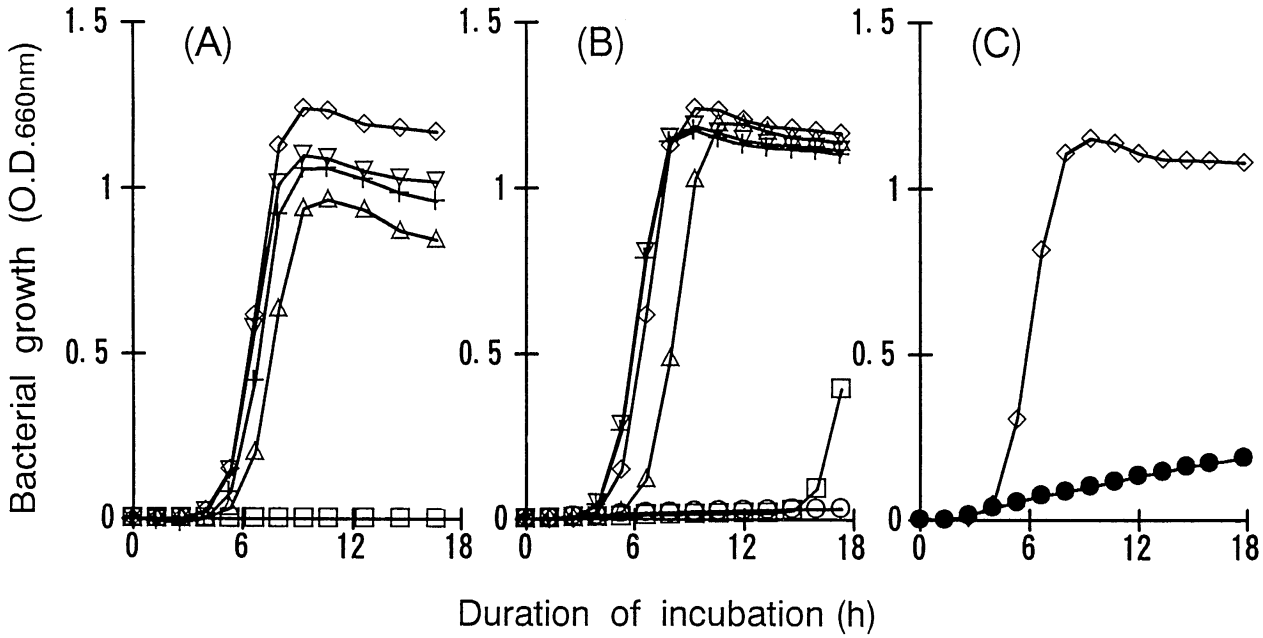

FIG. 1. Effects of peppermint oil, green-tea polyphenols and green tea, prepared as a beverage, on the growth of *E. coli* O157:H7. A, Peppermint oil; B, green-tea polyphenols; C, green tea, prepared as a beverage. The concentrations of test materials in MHB were 50 μg/ml (▽), 100 μg/ml (+), 200 μg/ml (△), 400 μg/ml (□) and 800 μg/ml (○). Effects of green tea, prepared as a beverage (●) and the control, namely MHB without additions (◇), are also indicated.

TABLE 2. The MICs and the MBCs of test materials with significant antibacterial activity against *E. coli* O157:H7.

Test material	MIC (μg/ml)	MBC (μg/ml)
Peppermint oil	400	400
Menthol	400	400
Menthone	400	400
Neomenthol	200	200
Menthofuran	>800	>800
(+)-Limonene	>800	>800
Piperitone	>800	>800
3-Octanol	800	800
cis-Jasmone	800	800
Mint lactone	800	800
(−)-Myrtenol	800	800
Piperitol	800	800
Eugenol	800	800
Carvacrol	200	200
2-Ethylfuran	800	>800
Ocimene	>800	>800
Green-tea polyphenols	800	>800
EC	>800	>800
EGC	800	>800
ECg	>800	>800
EGCg	400	>800

from green tea and tea prepared as a beverage is illustrated in Fig. 1. Peppermint oil inhibited growth at concentrations above 400 μg/ml, and dose-dependent inhibition was apparent from 50 to 200 μg/ml. Polyphenols from green tea inhibited growth at concentrations above 800 μg/ml, but viable cells were detected in the cultures. Green tea, prepared as a beverage, inhibited cell growth. However, the turbidity of cultures increased slowly but steadily, and viable cells remained in the culture.

The MICs and the MBCs of test materials that exhibited significant antibacterial activity in the preliminary screening are shown in Table 2. The MICs and the MBCs of peppermint oil and its constituents were similar except in the case of 2-ethylfuran (MIC, 800 μg/ml; MBC, >800 μg/ml). However, the MBCs of green-tea polyphenols and of two catechins, EGC and EGCg, were higher than the MICs, as indicated by the presence of viable cells in the cultures.

Bactericidal activities in PBS against *E. coli* O157:H7

The time-dependent changes in numbers of viable cells in solutions of PBS that contained peppermint oil, menthol, menthone and neomenthol were determined (Table 3). Menthol and menthone were present in peppermint oil at levels of about 41.5% and 22.4% (w/w), respectively. Neomenthol is a stereoisomer of menthol, and it was the most effective inhibitor of cell growth. When bacteria were exposed to peppermint oil, menthol and menthone at concentrations above 400 μg/ml, they died within one hour. Neomenthol, which killed the bacteria at concentrations above 200 μg/ml within 1h, was the most effective agent. The time-dependent changes in numbers

TABLE 3. Bactericidial activities of peppermint oil, green-tea polyphenols and its constituents in PBS aganist *E. coli* O157:H7.

Test material	Duration of incubation (h)	Concn (μg/ml)				
		50	100	200	400	800
Peppermint oil	1	−[a]	−	+	+++	+++
	3	−	−	+	+++	+++
	5	−	−	+	+++	+++
	24	−	−	+++	+++	+++
Menthol	1	−	−	−	+++	+++
	3	−	−	+	+++	+++
	5	−	−	+++	+++	+++
	24	−	−	+++	+++	+++
Menthone	1	−	−	−	+++	+++
	3	−	−	+	+++	+++
	5	−	−	+	+++	+++
	24	−	+	+++	+++	+++
Neomenthol	1	−	−	+++	+++	+++
	3	−	−	+++	+++	+++
	5	−	+	+++	+++	+++
	24	+	++	+++	+++	+++
Green-tea polyphenols	1	−	−	−	−	−
	3	−	−	−	−	+
	5	−	−	−	+	+++
	24	+	+++	+++	+++	+++
EGC	1	−	−	−	−	−
	3	−	−	−	+	+++
	5	−	−	−	+++	+++
	24	+++	+++	+++	+++	+++
EGCg	1	−	−	−	−	−
	3	−	−	−	−	−
	5	−	−	−	++	+++
	24	+	+++	+++	+++	+++
Green tea prepared[b]	1	−[b]				
	3	+++				
	5	+++				
	24	+++				

[a] +++, <10 cfu/ml; ++, 10^1 − <10^3cfu/ml; +, 10^3−<10^4 cfu/ml; −, ≧10^4 cfu/ml. [b] PBS plus 7.4 mg/ml green-tea extract.

TABLE 4. The FIC indices of the combination of peppermint oil, menthol, menthone and neomenthol with green-tea polyphenols with regard to *E. coli* O157: H7 .

Combination			FIC index[a]	Effect
Peppermint oil	+	green-tea polyphenols	0.50	Synergistic
Menthol	+	green-tea polyphenols	0.28	Synergistic
Menthone	+	green-tea polyphenols	0.75	Additive
Neomenthol	+	green-tea polyphenols	0.56	Additive

[a] FIC index: Values of FIC between 1.0 and 0.5 indicate the additive effects and values of 0.5 or less indicate synergistic effects.

of viable cells in solutions of green-tea polyphenols, EGC, EGCg and green tea, prepared as a beverage, in PBS are also shown in Table 3. Green-tea polyphenols at 800 μg/ml killed bacteria within 5 h. EGC and EGCg at 400 and 800 μg/ml, respectively, also killed the bacteria within 5 h. Green tea, prepared as a beverage, killed the bacteria within 3 h. The green-tea polyphenols EGC and EGCg at 100 μg/ml killed the bacteria within 24 h , but peppermint oil and its constituents at 100 μg/ml failed to kill the bacteria within 24 h.

Effects in combination

Combined effects on enterohemorrhagic *E. coli* O157:H7 were examined by the checkerboard method. Peppermint oil, menthol, menthone and neomenthol were separately combined with green-tea polyphenols. The results are shown in Table 4. A synergistic effect (FIC index ≦0.5) was observed for peppermint oil and for menthol with green-tea polyphenols. Menthone and neomenthol each had an additive effect on the effect of green-tea polyphenols (0.5< FIC index ≦1.0).

DISCUSSION

In preliminary screening experiments, we found that peppermint oil, 15 of its constituents and green-tea polyphenols had significant antibaterial activity (Table 1). Catechins, the polyphenolic constituents of green tea, have previously been examined for their antimicrobial activities (Hamilton-Miller, 1995; Sakanaka et al., 1989; Toda and Shimamura, 1996). In our experiments, we compared the antibacterial effects of peppermint oil and 15 of its constituents with the effects of green-tea polyphenols on *E. coli* O157:H7.

Peppermint oil and green-tea polyphenols inhibited the growth of *E. coli* O157:H7 at concentrations of 400 and 800 μg/ml, respectively, and the effects were evident within 18 h (Fig. 1). However, viable cells remained in cultures that contained green-tea polyphenols at 800 μg/ml. These results showed that peppermint oil and green-tea polyphenols inhibited cell growth in culture medium that contained proteins, with the former having a bactericidal effect and the latter a bacteriostatic effect. Green tea, prepared as a beverage, also inhibited cell growth within 18 h, but the turbidity of the culture increased slowly, perhaps because of the browning of some components of green tea by oxidation or because of the binding of tea components with proteins in the medium. Green tea, prepared as a beverage, contained catechins at about 2,300 μg/ml but failed to kill the bacteria. The potency of the catechins might have been reduced by browning reactions or by being bound to components in the medium. It was clear, however, that green-tea polyphenols and green tea itself had bacteriostatic effects in culture medium.

The respective MICs and MBCs of peppermint oil and its constituents were the same. The MBCs of green-tea polyphenols; of green tea, prepared as a beverage; and of two catechins in green tea were greater than the MICs, and viable cells remained in the cultures (Table 2). Toda and Shimamura (1996) reported that the antibacterial activity of EGCg was due to its destructive effect on bacterial membranes and that it made the bacteria adhere to each other. Tannins, which are polyphenols in green tea, are well known to bind to proteins. The bactericidal effects of catechins seemed to be weakened by the presence of proteins in the culture medium. The results described above confirmed that peppermint oil and its constituents have bactericidal activity and green-tea polyphenols have bacteriostatic activity against *E. coli* O157:H7 in liquid culture medium.

The time-dependent changes in numbers of viable cells in solutions of PBS that contained peppermint oil, three of its constituents, green-tea polyphenols, two green-tea polyphenols, and green tea, prepared as a beverage, are shown in Table 3. Peppermint oil at more than 400 μg/ml killed the bacteria within 1 h. Neomenthol was the most effective of the three constituents of peppermint oil tested. Green-tea polyphenols at 800 μg/ml killed cells within 5 h. Green-tea polyphenols at more than 100 μg/ml killed the bacteria with 24 h, but peppermint oil did not kill the bacteria at the same concentration. Green-tea polyphenols had bacteriostatic activity in liquid culture medium and bactericidal activity in PBS, but the effects were apparent after a longer time than those of peppermint oil. The mechanisms of bactericidal activity of peppermint oil and green-tea polyphenols may be different.

We observed synergistic effects (FIC index, $\leqq 0.5$) when we combined peppermint oil or menthol with green-tea polyphenols. Menthone and neomenthol had additive effects when combined with green-tea polyphenols ($0.5 <$ FIC index $\leqq 1.0$). The combination of peppermint oil and green-tea polyphenols, with their apparently different effects on *E. coli* O157:H7, seemed to be most effective. Peppermint oil is often used in the production of foods and confectionery and green tea is a common luxury article in Asia. From the point of biological safety, the application of these materials is not regulated. Our results suggest that peppermint oil and the combinations of peppermint oil with green-tea polyphenols might be useful as natural antibacterial agents against *E. coli* O157:H7.

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