Synergistic Antimicrobial Effect of Ethanol, Sodium Chloride, Acetic Acid and Essential Oil Components

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Ethanol, NaCl, acetic acid and essential oil components were examined for their synergistic antimicrobial effects, using air-borne microorganisms and pure cultures of fungi. Antimicrobial assays were carried out at 27°C, using 2% glucose Sabouraud agar. In order to completely suppress the growth of all the contaminating air microorganisms for over 20 days, more than 8% (by volume) ethanol, 25% (wt/vol) NaCl, or 0.2% (by volume) acetic acid was required in the medium. All essential oil components examined, at a concentration of 1 or 2 mM, were only moderate or very weak in antimicrobial effect. However, when these substances were applied in pairs, in threes and in fours, they exhibited a potent antimicrobial effect at relatively or considerably lower concentrations.

The combination of 3% ethanol, 0.05% acetic acid and 1 mM perillaldehyde, and the combination of as low as 0.5% ethanol, 2% NaCl, 0.05% acetic acid and 0.5 mM perillaldehyde were sufficient to suppress completely the growth of all contaminating air microorganisms for over 20 days. The combination of 3% ethanol, 3% NaCl and 0.5 mM perillaldehyde or 0.05% acetic acid was also fairly potent in this respect. Other essential oil components, such as citral, citronellol, geraniol, cuminaldehyde and eugenol, were as effective as perillaldehyde; L-menthol, linalool and D-carvone were somewhat less effective; and hydrocarbons (α -limonene, α -pinene, β -pinene, camphene, β -myrcene and *p*-cymene), citronellal, 1,8-cineole, anethole and vanillin were very weak or almost completely ineffective in this respect. Similar synergistic antimicrobial effects of these substances were also observed when using pure cultures of fungi as inocula.

Our results strongly suggest that combinations of ethanol, NaCl, acetic acid and certain essential oil components, each at a considerably lower concentration, are applicable for effective preservation of foods without applying synthetic preservatives or other antimicrobial substances uncommon in usual foods.

It has long been well known that more than certain concentrations of some ordinary food components, such as sodium chloride (NaCl), acetic acid and ethanol, are effective in inhibiting the growth of various microorganisms; and there have been many reports on the antimicrobial activity of essential oils and their components.^{1~14)} However, from a practical point of view, a relatively high concentration of any one of these substances is required in foods or in culture media when singly applied to suppress completely or almost completely the growth of all contaminating microorganisms for a long period of time. This appears

to limit considerably the applicability of these substances to food preservation.

Recently we^{15,16)} found that 7 to 10% NaCl and 1 to 2 mM perillaldehyde or certain other essential oil components exhibited in combination a potent antimicrobial effect, while either of them alone, at the same concentration, respectively, was only moderate in this effect. Thereafter we¹⁷⁾ also found that relatively lower concentrations of NaCl ($\leq 5\%$), acetic acid ($\leq 0.1\%$), and certain essential oil components (≤ 0.5 mM), when added together to 2% glucose Sabouraud agar, exhibited a potent antimicrobial effect as compared with

any one of them alone.

In the present study, ethanol was examined for its antimicrobial effect in combination with NaCl, acetic acid and/or essential oil components.

It will be shown below that considerably lower concentrations of these substances, when applied together, are sufficient to exhibit a potent antimicrobial effect. Our results strongly suggest that combinations of these substances for the inhibition of microbial growth may have wide application in the field of food preservation.

MATERIALS AND METHODS

Chemicals. Ethanol, NaCl, acetic acid and essential oil components employed here were purchased from Wako Pure Chemical Industries, Co., Ltd., Tokyo, Japan, and used without further purification.

Assays of antimicrobial effect. Assays were carried out in the same manner as described elsewhere.¹⁷⁾ Briefly, the incubation of microorganisms was carried out at 27°C throughout. The culture medium employed for the assays was 2% glucose Sabouraud agar. When acetic acid was added to the medium (initial pH: 6.4) at concentrations of 0.05, 0.1, and 0.2%, the medium pH became 4.6, 4.25, and 4.0, respectively.

The growth-inhibitory effect on air-borne microorganisms was assayed by an agar plate technique. The agar plate medium was exposed to air for 30 min, and then the medium-containing petri dish was sealed, and the contaminating air microorganisms were incubated for more than 20 days. The inhibitory effect on pure cultured fungi was assayed on slants. The culture of each fungi which was used as an inoculum for control and test media was 9 to 11 day-old. The duration of inhibition of fungal growth on test media was taken as a relative measure of the antifungal effect. Whether fungal growth occurred or not was determined macroscopically.

RESULTS

Synergistic antifungal effect of ethanol, NaCl, acetic acid and essential oil components

In our preliminary studies, NaCl, at a concentration of as high as 10% (w/v), inhibited the growth of the fungi listed in Table I for only 1 to 3 days, and at 3% did not show the growth-inhibitory effect at all (Table I). Perillaldehyde, even at 1 mM, was approxi-

mately as weak as 10% NaCl in this respect. Acetic acid, at a concentration of 0.1% (by volume), was also considerably weak in this effect (Table I), but at 0.2% it exerted a fairly potent inhibitory effect on some of the fungi. As shown in Table I, ethanol, at a concentration of 3% (by volume), was very weak, and at 5% moderate in the antifungal effect. A concentration of 8% or more was required for ethanol to exhibit a potent growth-inhibitory effect.

However, 5% ethanol, when combined with 3% NaCl, exhibited a considerably potent antifungal effect. The combination of 0.1% acetic acid and 5% ethanol showed a potent growth-inhibitory effect on some of the fungi, but had no additive effect on *Paecilomyces lilacinus* (N). The combination of 5% ethanol and 0.5 mM perillaldehyde also exhibited a potent inhibitory effect on some of the fungi, but not on the others.

At an ethanol concentration of 3% or less, any of these combinations of two failed to exhibit a potent inhibitory effect on most of the fungi, and in some cases did not show even an additive effect.

As shown in Table II, any three out of ethanol, NaCl, acetic acid and perillaldehyde, when applied together, were considerably more potent than the combinations of two of them (Table I), and the combination of all these four substances was more potent than the combinations of three of them in the antifungal effect.

The duration of the growth inhibition caused by the combination of all the four substances was much longer for all of the fungi than the sum of the durations attributed to each substance alone (see also Table I).

Thus these substances together apparently exhibited a highly synergistic antifungal effect. Citronellol and citral, when applied with ethanol, NaCl and acetic acid in pairs, in threes and in fours, were individually also nearly as effective as perillaldehyde in this respect (data not shown). Table III shows the antifungal effects of other essential oil components in the presence of ethanol and NaCl.

TABLE I. SYNERGISTIC ANTIFUNGAL EFFECT OF ETHANOL WITH NaCl, ACETIC ACID AND PERILLALDEHYDE

Fungus	Duration of growth inhibition (days)															
	Additive															
	Ethanol (%)				NaCl (3%) +ethanol (%)				Acetic acid (0.1%) +ethanol (%)				Perillaldehyde (0.5 mm) +ethanol (%)			
	1	3	5	8	0	1	3	5	0	1	3	5	0	1	3	5
<i>Aspergillus oryzae</i> (IFO 4220)	0	0	2	>20	0	0	2	7	1	1	1	3	0	1	2	3
<i>Aspergillus niger</i> (43)	0	0	5	>20	0	0	2	4	1	1	2	4	0	2	3	17
<i>Penicillium citrinum</i> (FRI)	0	1	2	>20	0	0	1	6	1	1	1	5	0	1	2	6
<i>Penicillium viridicatum</i> (FRI)	0	1	7	>20	0	0	3	14	2	2	3	>20	0	2	6	19
<i>Fusarium graminearum</i> (W38)	0	0	2	>20	0	0	1	3	3	3	>20	>20	1	1	2	5
<i>Aureobasidium pullulans</i> (L-2)	0	1	7	>20	0	1	6	>20	2	1	2	>20	0	1	3	12
<i>Paecilomyces lilacinus</i> (N)	0	0	1	7	0	0	3	8	1	0	0	1	1	1	1	2

TABLE II. SYNERGISTIC ANTIFUNGAL EFFECT OF ETHANOL, NaCl, ACETIC ACID AND PERILLALDEHYDE

Fungus	Duration of growth inhibition (days)															
	Additive															
	Perillaldehyde (0.5 mm) + acetic acid (0.1%) + ethanol (%)				Acetic acid (0.1%) + NaCl (3%) + ethanol (%)				Perillaldehyde (0.5 mm) + NaCl (3%) + ethanol (%)				Perillaldehyde (0.5 mm) + acetic acid (0.1%) + NaCl (3%) + ethanol (%)			
	0	1	3	5	0	1	3	5	0	1	3	5	0	1	2	3
<i>Aspergillus oryzae</i> (IFO 4220)	2	2	2	3	2	2	8	>20	3	3	3	13	13	13	15	>20
<i>Aspergillus niger</i> (43)	2	2	2	15	1	2	3	8	7	5	10	>20	4	4	5	11
<i>Penicillium citrinum</i> (FRI)	2	3	4	13	1	1	3	7	2	2	5	9	4	4	11	>20
<i>Penicillium viridicatum</i> (FRI)	4	15	>20	>20	7	6	>20	>20	3	4	17	>20	>20	>20	>20	>20
<i>Fusarium graminearum</i> (W38)	13	>20	>20	>20	8	>20	>20	>20	3	5	6	>20	>20	>20	>20	>20
<i>Aureobasidium pullulans</i> (L-2)	4	5	7	>20	3	5	>20	>20	4	12	>20	>20	14	19	>20	>20
<i>Paecilomyces lilacinus</i> (N)	1	1	2	4	2	2	5	8	9	9	11	18	7	8	9	11

TABLE III. SYNERGISTIC ANTIFUNGAL EFFECT OF ESSENTIAL OIL COMPONENTS WITH ETHANOL AND NaCl

Additive (ethanol, 3% + NaCl, 3% +)		Duration of growth inhibition (days)						
		Fungus						
		<i>Aspergillus</i> <i>oryzae</i> (IFO 4220)	<i>Aspergillus</i> <i>niger</i> (43)	<i>Penicillium</i> <i>citrinum</i> (FRI)	<i>Penicillium</i> <i>viridicatum</i> (FRI)	<i>Fusarium</i> <i>graminearum</i> (W38)	<i>Aureobasidium</i> <i>pullulans</i> (L-2)	<i>Paecilomyces</i> <i>lilacinus</i> (N)
Nil		2	2	1	3	1	6	3
Cinnamaldehyde	0.5 mM	2	3	6	5	2	7	6
Citral	0.5 mM	2	6	5	10	3	17	9
Citronellal	1.0 mM	1	2	3	3	2	6	3
	2.0 mM	3	3	5	5	3	8	6
Cuminaldehyde	0.5 mM	3	4	4	9	3	16	3
Vanillin	2.0 mM	2	2	2	5	3	4	5
D-Carvone	1.0 mM	3	2	3	10	3	12	5
	2.0 mM	8	7	7	>20	12	>20	7
Citronellol	0.5 mM	4	5	7	13	5	>20	9
L-Menthol	0.5 mM	2	3	3	7	3	11	6
	1.0 mM	3	5	6	11	5	>20	9
Linalool	1.0 mM	2	3	3	7	3	10	4
	2.0 mM	3	7	6	13	5	14	5
Eugénol	0.5 mM	2	3	5	17	5	>20	6
1,8-Cineole	2.0 mM	1	2	2	3	1	5	2
D-Limonene	2.0 mM	1	1	1	3	1	5	2

Any one of the essential oil components alone, at a concentration of 1 or 2 mM, inhibited the growth of the fungi for only 0 to 3 days (data not shown). However, when combined with both 3% ethanol and 3% NaCl, eugenol and cuminaldehyde, at a concentration of 0.5 mM, and L-menthol, D-carvone and linalool, at 1 or 2 mM, individually exhibited a considerably potent antifungal effect. Citronellal, vanillin, 1,8-cineole and D-limonene, even at 2 mM, were only slightly effective or completely ineffective in this respect.

It should be noted that cinnamaldehyde, under the same experimental conditions, was less effective as compared with perillaldehyde, citronellol and eugenol, while cinnamaldehyde was more effective than these compounds when they were examined for the effect singly or in combination with NaCl.

Synergistic growth-inhibitory effect of ethanol, NaCl, acetic acid and essential oil components on air-borne microorganisms

The number and kinds of air-borne microorganisms in a region, and the degree of microbial contamination from air are considerably variable depending upon a number of factors, and thus results of antimicrobial assays using such microorganisms are more or less variable. However, the growth-inhibitory effect of substances on such microorganisms would, from a practical point of view, still be a reasonable measure for estimating their effectiveness in preserving foods. Thus various combinations of ethanol, NaCl, acetic acid and perillaldehyde or other essential oil components were examined for their growth-inhibitory effect on contaminating air microorganisms.

Ethanol, in a concentration range of 5 to 7%, and NaCl, at about 15%, individually exhibited a considerably potent growth-inhibitory effect on air-borne microorganisms. However, in order to completely suppress the growth of all the contaminating air microorganisms for over 20 days, an ethanol concentration of more than 8%, or a NaCl concentration of more than 25% was required in

the medium.

To obtain the same magnitude of the effect, an acetic acid concentration of 0.2% or more was required. Acetic acid, at 0.1%, was only moderate in this respect. (It should be kept in mind that the antimicrobial effect of acetic acid is potent only in the acidic region.)

Perillaldehyde, at a concentration of as high as 1 mM, was only moderate in this effect, and allowed considerable growth of various air microorganisms within several days after contamination.

When ethanol and NaCl were applied as a pair, the combination of 3% ethanol and 5% NaCl or the combination of 5% ethanol and 3% NaCl was sufficient to completely suppress the growth of all the contaminating air microorganisms for over 20 days.

The combination of 3% ethanol and 0.1% acetic acid or 1 mM perillaldehyde was considerably potent in this effect, but allowed a few of the contaminating air microorganisms to grow substantially within 20 days of incubation.

When any three of ethanol, NaCl, acetic acid and perillaldehyde were applied together, their antimicrobial effect became fairly more potent than the effects of the combinations of two of them. When 1% ethanol, 1 mM perillaldehyde and 0.05% acetic acid were applied together, the growth-inhibitory effect was con-

FIG. 1. Synergistic Growth-inhibitory Effect of Ethanol, Acetic Acid and Perillaldehyde on Air-borne Microorganisms.

Incubation was at 27°C for 20 days. (See text for details.)

FIG. 2. Synergistic Growth-inhibitory Effect of Ethanol, NaCl and Perillaldehyde on Air-borne Microorganisms. Incubation was at 27°C for 20 days. (See text for details.)

siderably potent, and the combination of 3% ethanol, 0.5 mM perillaldehyde and 0.05% acetic acid was also equally effective. Furthermore, when 3% ethanol, 1 mM perillaldehyde and 0.05% acetic acid were applied together, all the contaminating air microorganisms were completely inhibited from growing for more than 20 days (Fig. 1).

Figure 2 shows that 3% ethanol, 3% NaCl and 0.5 mM perillaldehyde exhibited together a fairly potent inhibitory effect on such microorganisms.

The combination of 3% ethanol, 3% NaCl and 0.05% acetic acid was also equally effective (data not shown).

When ethanol, NaCl, acetic acid and perillaldehyde were applied together, these substances exhibited a potent antimicrobial effect, each at a considerably lower concentration. Figure 3 shows that as low as 0.5% ethanol, 2% NaCl, 0.05% acetic acid and 0.5 mM perillaldehyde, when combined together, were sufficient to completely suppress the growth of all the contaminating air microorganisms for over 20 days.

Although results of this kind of experiment are inevitably somewhat variable, the results presented here clearly demonstrate that when ethanol, NaCl, acetic acid and perillaldehyde are applied in pairs, in threes and all together, the growth-inhibitory effect of these sub-

FIG. 3. Synergistic Growth-inhibitory Effect of Ethanol (0.5%), NaCl (2%), Acetic Acid (0.05%) and Perillaldehyde (0.5 mM) on Air-borne Microorganisms.

Incubation was at 27°C for 20 days. (A) ethanol + NaCl; (B) ethanol + acetic acid; (C) ethanol + NaCl + perillaldehyde; (D) ethanol + NaCl + acetic acid; (E) ethanol + acetic acid + perillaldehyde; (F) ethanol + NaCl + acetic acid + perillaldehyde.

stances on contaminating air microorganisms becomes increasingly potent.

Other essential oil components, such as citral, cinnamaldehyde, citronellol, geraniol, eugenol and cuminaldehyde, were approximately as effective as perillaldehyde in this respect. Hydrocarbons (*D*-limonene, α -pinene, β -pinene, *p*-cymene, camphene and β -myrcene), citronellal, vanillin, 1,8-cineole and anethole were very weak, and *L*-menthol, linalool and *D*-carvone were intermediate in this effect.

DISCUSSION

From a practical point of view, almost all of the known essential oil components are moderate or very weak in antimicrobial effect when applied singly. These compounds, at a concentration of as high as 1 or 2 mM, failed to effectively suppress the growth of a variety of air-borne microorganisms.

However, we¹⁶⁾ found previously that certain essential oil components, such as perillaldehyde, cinnamaldehyde, cuminaldehyde, citronellol and eugenol, when applied in combination with 7 to 10% NaCl, exhibited a

potent antimicrobial effect at a concentration of 1 mM or less. In relation to this, we¹⁶⁾ also showed that several spices (perilla, Japanese pepper, laurel and a kind of cherry), which are relatively high in essential oil content, were effective in preserving a Japanese food, "miso" (a bean paste), which contained about 10% NaCl. Thereafter we¹⁷⁾ found that as low as 3% NaCl, 0.1% acetic acid and 0.5 mM perillaldehyde or certain other essential oil components, when applied together, suppress completely the growth of all the contaminating air microorganisms for over 20 days, while any one of them alone was only moderate or very weak in this respect. Acetic acid and NaCl, when applied as a pair, were also considerably potent as compared with each alone.

In the present paper, various combinations, pairs, threes, and fours of ethanol, NaCl, acetic acid and certain essential oil components were shown to exhibit an additive or synergistic antimicrobial effect; above all, the combination of all these four substances was remarkably potent in this effect, that is, a combination of considerably lower concentrations of each substance was sufficient to completely suppress the growth of all the contaminating air microorganisms for a long period of time (Fig. 3).

These results, together with those previously reported, strongly suggest that combinations of ethanol, NaCl, acetic acid and essential oil components (all ordinary and familiar food components) for the inhibition of microbial growth may have wide application to effectively preserve certain foods without applying synthetic preservatives or antimicrobial substances uncommon in usual foods. The applicability of various combinations of these substances for food preservation is under investigation in our laboratories.

Recently Kurita (one of the authors) found that the magnitude of the antimicrobial effect of several neutral salts of alkali metals and chlorine or bromine (LiCl, LiBr, NaCl, NaBr and KCl) was well correlated with their effectiveness in reducing the activity of water. In addition, the antimicrobial effect of these salts

was independent of the concentrations of glucose (1~4%) and peptone (0.5~2%) in the medium, indicating that the effect of these salts is not directly due to their interactions with sugars and/or proteins (unpublished data).

These results appear to suggest that the antimicrobial effect of NaCl, as well as those of the other salts, is mainly through the interaction with water molecules in the medium and/or in the microbial cell.

In our previous studies^{14,18)} using various kinds of essential oil components and related compounds, each series of primary alcohols, phenolic compounds, aromatic aldehydes and normal organic acids was found to increase in antifungal activity as the hydrophobicity increased (the number of the carbon atoms of the compounds examined: ≤ 10 for the alcohols, aromatic aldehydes and organic acids; ≤ 11 for the phenolic compounds). This strongly suggests that the hydrophobic interaction of these compounds (including ethanol and acetic acid) with the fungus cell is involved in their antifungal actions.

However, other series of essential oil components, such as hydrocarbons, α,β -saturated aldehydes and some ether compounds, were all very low in this activity, while their hydrophobicity is sufficiently high.

Therefore it appears that a certain degree of hydrophobicity is necessary, but is not sufficient in itself for certain essential oil components to inhibit the fungal growth effectively.

Obviously the magnitude of the antifungal effect of these compounds is also related to the nature of their functional groups. Functional groups, such as hydroxyl, carboxyl, carbonyl and ether ones, are assumed to be different from each other in the mode of their interaction with water molecules, because these functional groups are not equal in the number of the proton-accepting sites and/or exchangeable protons.¹⁹⁾ In addition, as mentioned above, hydrocarbons, which have no functional group, are almost completely inactive in the antifungal action. Thus it is intriguing to speculate that the mode of the

interaction of their functional groups with water molecules (though not clear), as well as their hydrophobic interaction with the fungus cell, may be of crucial importance to their antifungal action.

As for α,β -unsaturated aliphatic aldehydes from essential oils (cinnamaldehyde, perillaldehyde and citral), Kurita *et al.*¹³⁾ suggested previously that these aldehydes, all good electron acceptors, may exhibit their antifungal effect through the formation of charge transfer complexes with certain electron donors, such as tryptophan, of the fungus cell.

To date, however, the precise mechanisms of individual antifungal (and also antimicrobial) actions of ethanol, NaCl, acetic acid and essential oil components have not yet been fully elucidated on the molecular basis; in addition, the mechanisms of the synergistic antimicrobial actions of these substances also remain to be clarified.

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