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Characterization of novel antimicrobial compounds from mango (*Mangifera indica* L.) kernel seeds

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Abstract

The antimicrobial properties of mango seed kernel ethanol extract (MKE) were investigated. Minimum inhibitory concentrations (MICs) of the MKE against 18 species, of 43 strains, containing food-borne pathogenic bacteria were determined using the agar dilution method. The MKE had a broad antimicrobial spectrum, and was more active against gram-positive than gram-negative bacteria with a few exceptions. The antimicrobial activity of the MKE was stable against heat (121°C, 15 min), freezing (−20°C, 16 h) and pH treatment (pH 3–9) normally used in food processing. Chemical analysis showed that the MKE was composed of 79.5% polyphenol and 21.7% carbohydrate. The MKE was separated by reverse-phase HPLC with 10–30% acetonitrile linear gradient to characterize antimicrobial compounds. There were two fractions having antimicrobial activity; both peaks had maximum absorbance at 275 nm. These results indicated that the active component of the MKE was a type of polyphenol.

1. Introduction

In recent years, there has been a dramatic increase in number of reported poisoning outbreaks caused by food-borne pathogenic bacteria. In 1996, one of the serious outbreaks caused by *Escherichia coli* O157:H7 occurred in Sakai, Japan (National Institute of Infections Diseases and Infectious, Diseases Control Division, Ministry of Health and Welfare of Japan, 1997). Consequently, there has been considerable interest in preventing food contamination by food-borne pathogens. Traditionally, various methods, such as heating, reducing water activity, smoking, fermentation, and adding antimicrobial agents, have been used to prevent spoilage of foods. The addition of antimicrobial agents has been a particularly effective method for controlling microbial contamination.

Unlike chemically synthesized antimicrobial agents, those from natural sources are acceptable to consumers. Thus, one area of research is the development of the new agents from natural sources. Antimicrobial agents from spices and their essential oils (Beuchat, 1994), food plants (Walker, 1994), vegetable and fish oils (Dallyn, 1994), and bacteriocin, antimicrobial peptides produced by bacteria (Hill, 1995) have been reported.

Mango (*Mangifera indica* L.), which belongs to the family *Anacardiaceae*, order *Rutales*, is grown naturally or cultivated mainly in tropical and subtropical regions, and is one of the most popular edible fruits in the world. Mango seed kernel is promising as a source of fat, cocoa butter substitutes, and other food substitutes (Lakshminarayana, Chandrasekhara Rao & Ramalingaswamy, 1983). Parmar and Sharma (1986) reported that mango seed kernel enhanced oxidative stability of fresh-type cheese, ghee.

Here, we report the antimicrobial activity of an ethanol extract of mango seed kernel (MKE) with antimicrobial properties against food-borne pathogenic bacteria and the chemical characteristics of MKE.

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2. Materials and methods

2.1. Strains and culture conditions

The strains used in this study are listed in Tables 1 and 2. All strains without culture collection names are stock cultures of the laboratory of Hygiene, Kagawa Nutrition University.

The running cultures for the assay were propagated in nutrient broth(NB) (Difco laboratories, Detroit, USA) at 37°C for 16 h. In the case of *Vibrio parahaemolyticus*, NB containing 8% (w/v) sodium chloride was used for propagation. For lactic acid bacteria (LAB), MRS broth (Difco laboratories, Detroit, USA) was used for propagation.

Table 1
Antimicrobial activity and MICs of MKE

No.[c]	Indicator strain[a,b]	MIC (ppm)
1	Enteropathogenic *E. coli* O127:H21	2500
2	Enteropathogenic *E. coli* O124:HNM	2500
3	Enteropathogenic *E. coli* St&LT	2500
4	Enteropathogenic *E. coli* O157:H7(VT1&VT2)	2500
5	Enteropathogenic *E. coli* O157:H7(VT2)	1000
6	Enteropathogenic *E. coli* O157:H7(nonVT)	2500
7	Enteropathogenic *E. coli* O111:HNM(VT1&VT2)	2500
8	Enteropathogenic *E. coli* O26:HNM(VT1&VT2)	2500
9	*E. Coli* IFO 3301	2500
10	*Salmonella* Enteritidis	2500
11	*Salmonella* Typhimurium	2500
12	*Klebsiella aerogenes*	2500
13	*Aeromonas hydrophila* O1, ATCC 7966	2500
14	*Aeromonas hydrophila* O11	1000
15	*Aeromonas hydrophila* O21	2500
16	*Campylobacter jejuni*	100
17	*Vibrio parahaemolyticus*	500
18	*Yersinia enterocoliticia*	100
19	*Staphylococcus aureus* A type	1000
20	*S. aureus B* type	50
21	*S. aureus C* type	50
22	*S. aureus D* type	50
23	*S. aureus E* type	50
24	*S. aureus* TSST-1	50
25	*S. aureus* (MRSA)	50
26	*Bacillus cereus* (Emetic type)	500
27	*B. cereus* (Diarrheal type)	500
28	*B. cereus* IFO 13484	500
29	*B. subtilis*	500
30	*B. licheniformis*	500
31	*B. lichenformis*	500
32	*B. pumilus*	500
33	*Clostridium botulinum*	< 50
34	*Cl. perfringens*	< 50
35	*Listeria monocytogenes*	100

[a] Abbreviations: IFO,Institute for fermentation Osaka; ATTC, American Type Culture Collection.

[b] The strains without number were culture collections of Kagawa Nutrition University.

[c] From 1 to 18, gram-negative bacteria; 19–35, gram-positive bacteria.

2.2. Preparation of mango seed kernel extract (MKE)

We purchased mango at the retail shop in Kawagoe, Japan. Mango seed kernels were obtained by removing shells, and then ground using a homogenizer. Ethanol (99.5%) was added to the mango seed kernel triturate to 2:1 (v/w) and kept overnight in a dark place with gentle shaking. After removing insoluble materials by filtration (Advantec, No.2, Tokyo, Japan), the solvent was evaporated with a rotary evaporator. The residue, suspended in sterilized Milli Q water, was used as the MKE for the following experiments.

2.3. Antimicrobial assay

Antimicrobial assay was performed in the agar dilution method. Triptic Soy (TS) agar (Difco laboratories, Detroit, USA) and MRS agar (Difco laboratories, Detroit, USA) was used. Sodium chloride 8% (w/v) was added to TS agar using *Vibrio parahaemolyticus* as an indicator strain. An MRS plate was used for the assay against LAB.

The MKE was diluted with sterilized water for the assay. The MKE was added to the agar medium retained at 50°C, and then poured into a sterile dish (diameter 9 cm, Iwaki glass Co. Ltd., Tokyo, Japan). The overnight culture of indicator strains was diluted to approximately 10^6 cfu/ml with sterilized phosphate-buffered saline (pH 6.8) and then struck on the plates using a sterilized loop. The plates were incubated at 37°C for 24 h. The plates for LAB were incubated under anaerobic conditions using BBL Gas pack system (BBL, Maryland, USA). The MICs were defined as the lowest concentrations of the MKE capable of preventing growth of the indicator strains. For the fractions separated by HPLC, an antimicrobial titre was defined as

Table 2
Antimicrobial activity and MICs of MKE against lactic acid bacteria

	Indicator strain[a,b]	MIC (ppm)
36	*L. brevis* IFO 3345	1000
37	*L. casei* ATTC 7469	> 2500
38	*L. plantarum* IAM 1041	> 2500
39	*Leuconostoc mesenteroides*	1000
40	*Pediococcus acidilactici*	2500
41	*E. faecalis* IAM 10067	1000
42	*E. fecium M*	2500
43	*S. thermophilus* NRIC 1747	2500

[a] Abbreviations: IFO, Institute for fermentation Osaka; ATCC, American Type Culture Collection; IAM, Institute of Applied Microbiology, the University of Tokyo; NRIC, NODAI Institute-Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan.

[b] The strains without number were culture collections of Kagawa Nutrition University.

the reciprocal of the highest dilution showing a distinct inhibition of the indicator, and expressed in terms of arbitrary units (AU) per ml. All antimicrobial experiments were performed in duplicate using independently-prepared plates and a larger titre was used as the MICs.

2.4. Chemical analysis

Total nitrogen content was determined by the Kjeldahl method according to AOAC methods 955.04 (Association of Official Analytical Chemists [AOAC] 1995). Total polyphenol content was determined by the Folin–Ciocalteu method (Peterson, 1979) with garlic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a standard for calibration. Total sugar content was measured by the phenol-H_2SO_4 method with glucose as a standard (Dubois, Gillers, Hamilton, Redbers & Smith, 1956). Fatty acid and ash contents were determined by the method of Southgate (1971) and AOAC methods 942.05 (AOAC, 1995), respectively. The coloured qualitative reaction with an alkaline solution of ferric chloride, and the precipitate reaction with lead acetate were performed to examine the existence of polyphenols in the MKE (Lee & Widmer, 1996). All quantitive analyses were performed in duplicate and the value was calculated as a mean. Qualitative analysis was performed to examine the existence of flavones by Mg–HCl reaction (Lee & Widmer, 1996).

2.5. Effect of pH and temperature on stability of the MKE

The pH stability was measured with three kinds of buffers: a 50 mM sodium acetate buffer (pH 3.0–5.0), 50 mM sodium phosphate buffer (pH 6.0–7.0) and 50 mM tris–HCl buffer (pH 8.0–9.0). The MKE was exposed to the buffers for half an hour. After treatment, the MKE was diluted appropriately with PBS(-) and antimicrobial activity was measured by the agar dilution method as described previously, using *Escherichia coli* IFO 3301 as an indicator strain. The heat stability was measured following corresponding treatments for half an hour.

2.6. Reverse-phase high performance liquid chromatography (HPLC) analysis

The MKE was dissolved in 10% (v/v) acetonitrile and separated by an HPLC (HPLC system, model 1050, Hewlett Packard, Waldbronn, Germany) attached to a reversed-phase ODS column (TSK-gel ODS, 4.5×250 mm, Tosoh, Tokyo, Japan). Linear gradient elution was performed starting with 10% (v/v) acetonitrile in Milli Q water, and ending with 30% (v/v) acetonitrile at a flow rate of 1 ml/min at room temperature. Eluates were detected at 280 nm and fractionated. They were dried with a centrifugal evaporizer (model CVE-200D, EYELA, Co. Ltd., Tokyo, Japan). Each fraction was dissolved in sterilized Mill Q water to analyze the antimicrobial activity. The antimicrobial activities were assayed as described above.

3. Results

3.1. Antimicrobial activity

The antimicrobial activity of the MKE against 43 strains (18 species) determined by the agar dilution method is shown in Tables 1 and 2.

The MKE showed an antimicrobial activity against both gram-positive and -negative bacteria except for LAB at 2500 ppm. MICs against gram-negative bacteria were varied among species. MICs of the MKE against *E. coli* (Nos. 1–9), *Salmonella* sp. (Nos. 10 and 11), *Klebsiella aerogenes* (No. 12), and *Aeromonas hydrophila* (Nos. 13–15) were higher than 1000 ppm. MKE showed the same level of MIC (2500 ppm) against *E. coli*, which had different serotype, toxin producing ability and infection types, with an exception (No. 5. 1000 ppm). On the other hand, MICs of the MKE, against both *Campylobacter jejuni* (No. 16) and *Yersinia enterocolitica* (No.18), were 100 ppm.

The MKE showed remarkable antimicrobial activity against gram-positive strains (Tables 1 and 2); the MICs of the MKE against *Staphylococcus aureus* (Nos. 20 to 25), *Bacillus* sp. (Nos. 26–32), *Clostridium* sp. (Nos. 33 and 34) and *Listeria monocytogenes* (No. 35) varied in the range between 50 and 500 ppm. The MICs of the MKE, against 8 species of LAB (Table 2, Nos. 38–43) and *Staphylococcus aureus* (No. 19), were higher than 1000 ppm in MRS agar and TS agar, respectively.

3.2. pH and temperature stability

Table 3 shows heat and pH stability of the MKE. The antimicrobial activity of the MKE was stable at pH 3.0–9.0.

Table 3
pH and heat-stability of the MKE

Treatment	Activity recovered (%)
pH3	100
pH4	100
pH5	100
pH6	100
pH7	100
pH8	100
pH9	100
121°C, 15 min	100
121°C, 60 min	25
25°C, 16 h	100
4°C, 16 h	100
−20°C, 16 h	100
No treatment	100

The MKE was stable after heating at 121°C for 15 min, but the activity was reduced to about 1/4 when heated at 121°C for 60 min. The MKE was also stable against freezing.

3.3. Chemical analysis of the MKE

The MKE formed yellow and brown precipitation by qualitative analysis with alkaline solution and ferric chloride, respectively, which indicated the MKE contained polyphenols (Lee & Widmer, 1996). The MKE formed two layers precipitation by the lead acetate precipitation method; the white upper layer indicated tannin, while the yellow bottom layer suggested flavones. The MKE produced hydrogen gas, characteristic of flavones reacted with $MgCl_2$.

The chemical composition of the MKE is shown in Table 4. Total polyphenol content of the MKE was 79.5%, while total carbohydrate, nitrogen, ash and fat content were 21.7, 3.1, 1.6 and 0.5%, respectively.

3.4. HPLC analysis for the MKE

Fractionation of the MKE was performed by reverse-phase HPLC to identify active compounds. The HPLC profile and an antimicrobial activity of each peak are shown in Fig. 1. Two fractions eluted from 31 to 32 min and from 34 to 35 min had an antimicrobial activity, with 10 and 20 AU, respectively. The recovery of the activity was 19%. The maximum absorbances for both peaks were found at 275 nm.

4. Discussion

Characteristics of a novel antimicrobial compound in ethanol extract of mango kernel seed were studied. The qualitative analysis of the MKE showed that polyphenols are the major component of the MKE. From the HPLC analysis, there were two fractions with antimicrobial activity; both peaks had maximum absorbance at 275 nm, which also indicated the existence of polyphenols.

It is known that various polyphenols have an antimicrobial activity (Hara & Ishigami, 1989; Herald & Davidson, 1983; Sakanaka, Okubo, Akachi, Mabe & Matsumoto, 1997; Zhao, Chung, Milow, Kang & Stevens, 1997). The antimicrobial spectra of the MKE and other sources of polyphenols, crude catechins in green tea and crude theaflavins in brown tea, are shown in Table 5. The MICs of the crude catechins and crude theaflavins are lower against gram-positive bacteria than against gram-negative bacteria. The antimicrobial-spectrum of the MKE has the same tendency. However, there are a few differences in the antimicrobial spectra between the MKE and crude cathechins or theaflavins. The MICs of the MKE against *Campylobacter jejuni* and *Yersinia enterocolitica,* are 100 and 50 ppm, while those of the crude catechins and theaflavins are greater than 1000 ppm. The difference suggests that the molecular structures of the polyphenols, as active components in the MKE, differ from those in green or brown tea.

The antimicrobial spectrum of the MKE is more effective against gram-positive bacteria than gram-negative bacteria, although the antimicrobial activity against lactic acid bacteria is ineffective (Tables 1 and 2). The structures of cell envelope, including cytoplasmic membrane and cell wall component, are different between gram-positive and -negative bacteria (Hugo & Russell, 1987). Because antimicrobial agents generally make contact with the cell envelope first, the structural difference may play a key role in the susceptibility. Numerous studies have been carried out to study the mode of action of antimicrobial agents containing antibiotics (Franklin & Snow, 1989).

The antimicrobial peptide, nisin, produced by *Lactococci lactis,* has a similar antimicrobial spectrum to the MKE. Nisin generally has a bacteriostatic effect against only gram-positive bacteria. The primary site of the action of nisin against vegetative cell is considered to be the cytoplasmic membrane (Henning, Mets & Hammes, 1986; Jung, 1991; Ruhr & Sahl, 1985), because nisin interacts with the phospholipid of cytoplasmic membranes and forms a nisin-phospholipid complex. To argue the difference of susceptibility of the MKE against gram-positive and gram-negative bacteria, the mode of action must be studied in detail. More detailed characterization and biochemical studies are being carried out in our laboratory.

Antimicrobial substrates are potentially useful as food additives to extend the shelf-life of foods, in particular unheated products such as salads. Because the MKE has heat and pH stability (Table 3), it could be applied in a variety of foods. The disadvantage of the MKE is its low antimicrobial activity against gram-negative bacteria. Therefore, the MKE should be used, together with other antimicrobial components, effective against gram-negative bacteria, such as organic acids.

Safety as a food additive is of most importance for industrial application of the MKE. There are already some papers that report the safety of mango seed kernels as food additives (Arogba, 1997; Rukmini, 1987; Rukmini &

Table 4
Chemical components of the MKE

Component	Weight (mg/100 mg dry wet)
Carbohydrate	21.7
Total nitrogen	3.1
Ash	1.6
Fat	0.5
Polyphenol	79.5

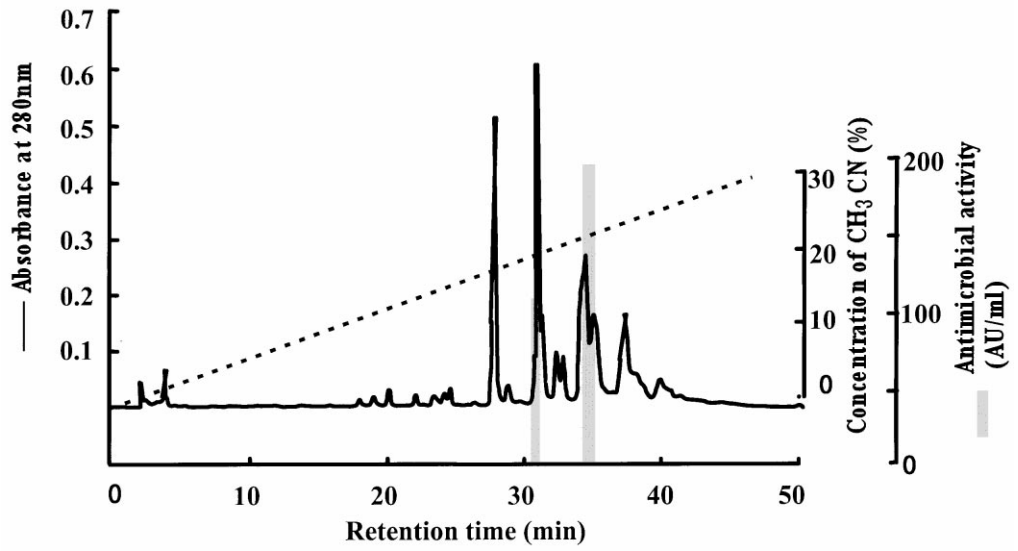

Fig. 1. HPLC elution profile of the MKE using an ODS column and an antimicrobial activities. Absorbance at 280 nm is shown as a solid line and gradient during HPLC elution is shown as a dotted line. Antimicrobial activities (AU) of corresponding fractions are shown as bars.

Table 5
Comparison of the antimicrobial activity (MIC) of the MKE and polyphenols

Indicator strain	MKE (ppm)	Crude catechins from green tea[a] (ppm)	Crude theaflavins from tea[a,b] (ppm)
Escherichia coli	> 1000	> 1000	> 1000
Salmonella sp.	2500	> 1000	> 1000
Aeromonas sp.	> 1000	> 400	300–700
Campylobacter jejuni	100	> 1000	> 1000
Vibrio sp.	500	200–500	300–400
Yersina enterocolitica	50	> 1000	> 1000
Stabylococcus aureus	50–1000	450	500
Bacillius sp.	500	600	500
Clostridium sp.	< 50	400	200

[a] MICs were determined in Nutrition agar (Hara & Ishigami, 1989). Crude catechins were obtained by ethyl acetate extract from green tea.
[b] Crude theaflavins were obtained by methylisobutylketone extract from brown tea.

Vijayaraghavan, 1984). Although more precise toxicity studies are needed, the MKE seems promising as a food additive for extending the shelf- life of a variety of food products.

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