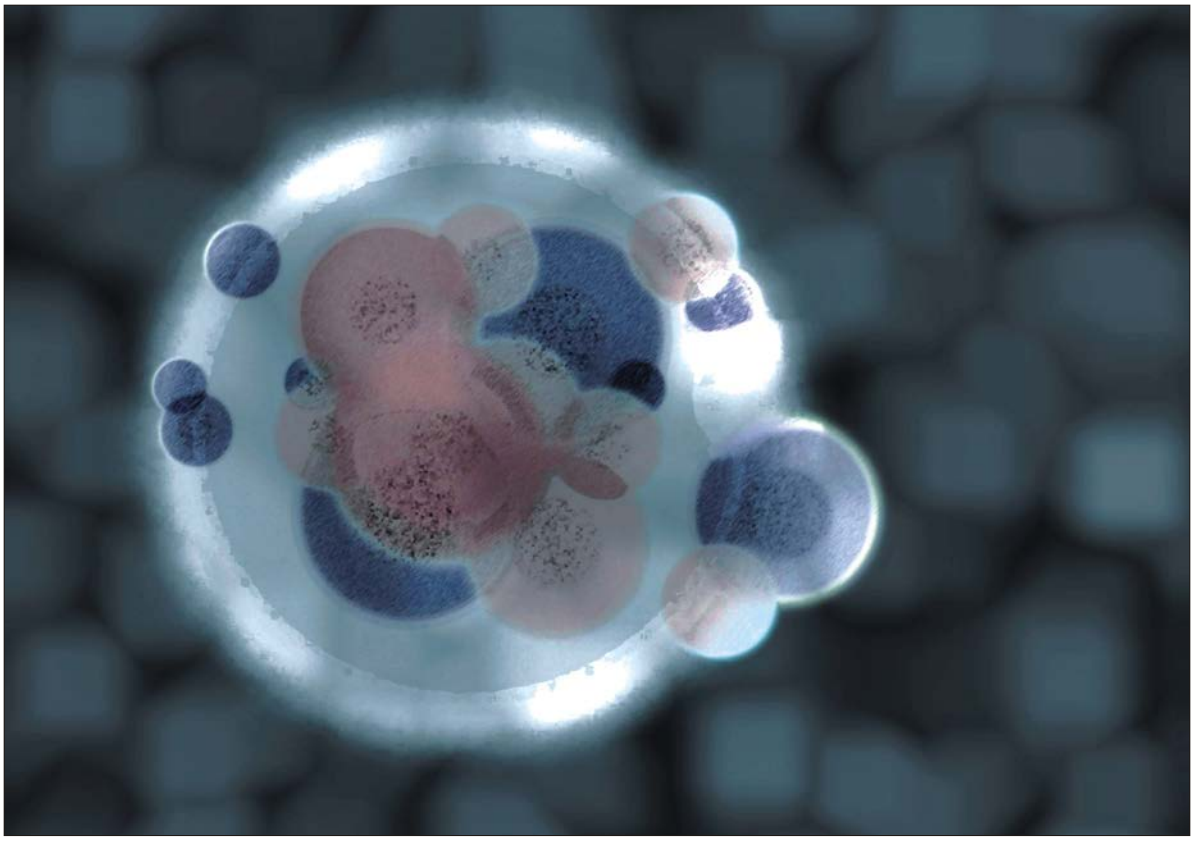

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 6 (1): 135-139, 2006
ISSN 1727-3048

Studies on Antimicrobial and Antioxidative Substance of Yuzu (*Citrus junos* hort. ex Tanaka) Seed

Yukiko Nakazono, Yuka Watanabe, Fumio Hashinaga and Kenjiro Tadera
Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan

Abstract: A yuzu (*Citrus junos* hort. ex Tanaka) seed extract with 80% methanol showed a potent antimicrobial activity against *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis*. The antimicrobial substance was purified by adsorption column chromatography and identified as *p*-methoxycinnamic acid by gas chromatography-mass spectrometry. Minimum inhibitory concentrations of *p*-methoxycinnamic acid against *M. luteus*, *S. aureus*, *E. coli* and *S. enteritidis* were 80, 60, 50 and 60 μg mL^{-1}, respectively. In addition, this structure suggested that *p*-methoxycinnamic acid had the antioxidative activity and we investigated also the antioxidative activity. The antioxidative activity of *p*-methoxycinnamic acid was about 70-80% of that of BHA, BHT and α-tocopherol.

Key words: Yuzu seed, *Citrus junos* hort. ex Tanaka, antimicrobial activity, antioxidative activity, *p*-methoxycinnamic acid

INTRODUCTION

Food deterioration, *viz.*, off- flavors, changes in color and texture and nutritional losses, which are caused by the spoilage and the oxidation, is an important problem in the food industry. The food poisoning is also caused by the spoilage. Oxidation is of major significance in human health as well as the food industry. Oxidative stress has been linked to diseases such as atherosclerosis, cancer and tissue damages in rheumatoid arthritis[1]. In order to avoid microbial and oxidative actions, synthetic preservatives such as sorbic, benzoic and propionic acids as are used in the food industry as antimicrobial substances and butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as antioxidative substances. However, the use of these synthetic substances has been restricted because of these carcinogenicity[2-4] and consumer's interests in the safety of food. Therefore, new effective and safe natural preservatives are demanded.

The yuzu fruit is a distinctive citrus fruit and recognized as a species, *Citrus junos* hort. ex Tanaka. The yuzu fruit is of the size of mandarin orange, with thick uneven skin and paler flesh containing many seeds[5]. The production of yuzu fruits in Japan is near 20,000 tons per year. The average percentage of fruits transformed into processed foods is from 50 to 70%. Since the seed of yuzu fruits is about 10% of the fruit weight[6], very large amounts of a by-product are formed every year. An increase in industrial wastes of yuzu seeds is one of the serious social problems.

It is also reported that extracts and essential oils from many plants have the antimicrobial and antioxidative activities. Seeds of fruits, *viz.*, grape (*Vitis vinifera*)[7] and citrus such as grapefruits (*Citrus paradisi* Macf.)[8] and Satsuma mandarin fruits (*Citrus unshiu* Marc.)[9], show a high growth-inhibiting activity against microorganisms. Seeds of fruits such as grape[10] and avocado (*Persea americana* Mill.)[11] have the antioxidative activity. It is thought that the seed contains the antimicrobial and antioxidative substances to protect itself.

In this study, for the purpose of utilization of yuzu seeds of the by-product, we surveyed the antimicrobial activity in yuzu seeds and identified an isolated antimicrobial substance. In addition, it was suggested from its structure that the isolated antimicrobial substance had the antioxidative activity. Therefore, we investigated also the antioxidative activity of the substance.

MATERIALS AND METHODS

Material: Yuzu seeds were obtained from a processing industry in Kagoshima City at October, 2001. After the seeds were dried at 60°C in an oven, they were stored at -20°C and ground to powder before use.

Corresponding Author: Yukiko Nakazono, Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan Tel/Fax: +81-99-285-8667

Isolation of antimicrobial substance

Extraction: Yuzu seeds (300 g) were extracted with three 600 mL portions of 80% methanol (MeOH) with a Soxhlet apparatus. The extracts were combined and concentrated to a small volume with a rotary evaporator.

Amberlite XAD-2 column chromatography: The 80% MeOH extract of yuzu seeds was applied to an Amberlite XAD-2 column (Organo, Co., Ltd., 5.0×40 cm). The elution was carried out at a flow rate of 0.25 mL min^{-1} with 300 mL portions of the water-MeOH mixtures in the various ratios.

Silica gel column chromatography: A silica gel column (Silica Gel C-300, Wako Pure Chemical Industries, Ltd., 2.0×25 cm) was developed at a flow rate of 0.3 mL min^{-1} with 100 mL portions of the hexane-ethylacetate (EtOAc) and EtOAc-MeOH mixtures in the various ratios.

TOYO PEARL HW-40 column chromatography: Antimicrobial compound was eluted from a TOYO PEARL HW-40 column (Tosoh, Co., Ltd. 2.0×42 cm) with MeOH at a flow rate of 0.3 mL min^{-1} and the eluate was collected by 10 mL aliquots.

Sephadex LH-20 column chromatography: A Sephadex LH-20 column (Pharmacia Fine Chemicals, Co., Ltd. 2.5×50 cm) was developed successively with 250 mL portions of 15, 50 and 70% MeOH at a flow rate of 0.3 mL min^{-1}. The effluent was collected by 5 mL aliquots and monitored at 280 nm.

Determination of the antimicrobial activity

Microorganisms and culture conditions: The following microorganisms were used for assaying the antimicrobial activity. Two strains of Gram-positive bacteria, *viz., Micrococcus luteus* (IFO-12708) and *Staphylococcus aureus* (IFO-14462) and two strains of Gram-negative bacteria, *viz., Escherichia coli* (IFO-3301) and *Salmonella enteritidis* (IFO-3313). A culture medium for bacteria consisted of 0.5% meat extract, 1.0% peptone and 0.25% sodium chloride, pH 6.5-6.6[12].

Assay procedure: Antimicrobial activity of the sample was assayed by the paper disc method[13].

The minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial that inhibited the growth of a particular microbe, was measured by the double fold-dilution method[14].

Determination of the antioxidative activity in the linoleic acid system : The antioxidative activity was assayed according to the thiocyanate method with AAPH reported by Shirasaka *et al.*[15], the absorbance at 500 nm showing the formation of lipid peroxides. All analyses were performed in triplicate and the data were averaged. Inhibition of lipid peroxidation was expressed as percentage of the control by using the following equation:

$$\text{Inhibition}(\%) = 100 - \frac{\text{Absorption at 500 nm of the sample}}{\text{Absorption at 500 nm of the control}} \times 100$$

Gas chromatography-Mass spectrometry (GC-MS): GC-MS spectrum was taken on a Polaris Q gas chromatograph-mass spectrometer (Thermo Electron, Co., Ltd.). Ionization voltage was 70 eV and the ion source temperature was kept at 200°C. The column (DB 17, J and W Scientific, Ltd.) temperature was programmed from 60 to 260°C at a rate of 20 °C min^{-1} after 2 min holding time. The split flow rate in the Right SSL method was 50 mL min^{-1}.

RESULTS AND DISCUSSION

The extraction efficiency of four solvents on the antimicrobial substance from yuzu seeds was investigated. The yuzu seed powder was successively extracted in a Soxhlet extractor with hexane, EtOAc, butanol (BuOH) and 80% MeOH. The extracts were separately concentrated under reduced pressure for removing the solvents. The yields were 19.8, 6.2, 9.0 and 14.5%, respectively. The antimicrobial activity of four extracts is shown in Table 1. None of the hexane and

Table 1: Antimicrobial activity of yuzu seed extracts with various solvents

	Solvents			
Microorganisms	Hexane	Ethylacetate	Butanol	80% Methanol
Gram-positive				
Micrococcus luteus	-	-	++	++++
Staphylococcus aureus	-	-	+	++++
Gram-negative				
Escherichia coli	-	-	+	+++
Salmonella enteritidis	-	-	+	+++

The diameter of the circle of inhibition was measured and antimicrobial activity was rated as follows : -, negative (0 mm); +, positive (1-3 mm); ++, moderately positive (4-6 mm); +++, strongly positive (7-10 mm); ++++, very strongly positive (>10 mm)

Table 2: Antimicrobial activity of the fractions obtained by Amberlite XAD-2 column chromatography

	Ratio	Microorganisms			
Fractions	Water : MeOH	*M. luteus*	*S. aureus*	*E. coli*	*S. enteritidis*
1	10 : 0	-	-	-	-
2-6	9 : 1-4 : 6	-	-	-	-
7	3 : 7	-	-	-	-
8	2 : 8	+	+	+	+
9	1 : 9	++	++	++	++
10	0 : 10	++++	++++	+++	++++

See the footnote of Table 1 regarding the symbols

Table 3: Antimicrobial activity of the fractions obtained by silica gel column chromatography

Fractions	Ratio					Microorganisms			
	Hexane	:	EtOAc	:	MeOH	*M. luteus*	*S. aureus*	*E. coli*	*S. enteritidis*
1	10	:	0			-	-	-	-
2-9	9-2	:	1-8			-	-	-	-
10	1	:	9			-	-	-	-
11			10	:	0	+	+	-	+
12			9	:	1	+	+	+	+
13			8	:	2	++++	++++	+++	++++
14			7	:	3	++++	++++	++++	++++
15			6	:	4	++++	++++	++++	++++
16			5	:	5	++++	++++	++++	++++
17			4	:	6	++	++	++	+++
18			3	:	7	+	+	+	+
19			2	:	8	-	-	-	-
20			1	:	9	-	-	-	-
21			0	:	10	-	-	-	-

See the footnote of Table 1 regarding the symbols

Table 4: Antimicrobial activity of fraction 7 obtained by Sephadex LH-20 column chromatography and some food preservatives (200 μg disc^{-1})

	Microorganisms			
	M. luteus	*S. aureus*	*E. coli*	*S. enteritidis*
Fraction 7	++++	++++	++++	++++
Sodium benzoate	++	++	+	++
Benzoic acid	+++	++	++	+
Gallic acid	+++	+++	+	+

See the footnote of Table 1 regarding the symbols

EtOAc extracts exhibited any antimicrobial activity against all bacteria. The BuOH and 80% MeOH extracts exhibited the antimicrobial effect against all the bacteria tested. The 80% MeOH extract showed the higher antimicrobial activity than that of the BuOH extract, indicating that the antimicrobial activity was more extracted with the higher polar solvents. Since the 80% MeOH extract showed the highest antimicrobial activity, it was used as an extraction solvent for the isolation of antimicrobial compound.

At all the isolation steps, the antimicrobial activity of the sample (200 μg) was assayed by the paper disc method[13] using four strains as the test bacteria. The 80% MeOH extract of yuzu seeds (yield : 43.5 g) was applied to an Amberlite XAD-2 column. Fractions 8, 9 and 10 with the antimicrobial activity (Table 2) were combined and purified by silica gel column chromatography. Fraction 11 to 18 showing the antimicrobial activity (Table 3) were combined and then the concentrate (yield : 1.8 g) was purified by TOYO PEARL HW-40 column chromatography. The fractions (yield : 1.2 g) with the antimicrobial activity were combined and applied to Sephadex LH-20 column chromatography. The effluent was pooled into eight fractions from absorption at 280 nm. Fraction 7 that was eluted with 70% MeOH (yield : 30 mg) only showed the antimicrobial activity. The antimicrobial activity of fraction 7 was considerably higher than that of food preservatives used in the food industry such as sodium benzoate, benzoic acid and gallic acid[16] (Table 4).

Fraction 7 was analyzed by GC-MS. Among the peaks on a gas chromatogram, the peak of the retention time 14.92 min was considered to show the antimicrobial activity. The mass spectrum of this peak compound was coincident with that of *p*-methoxycinnamic acid {m/z (relative intensity): 178 (100), 161 (30), 133 (16), 118 (6), 90 (6), 77 (11)}. The retention time on gas chromatography was confirmed to be identical with that of a specimen (Tokyo Kasei Kogyo, Co., Ltd.) of *p*-methoxycinnamic acid. Therefore, the compound was identified as *p*-methoxycinnamic acid. Minimum inhibitory concentrations of *p*-methoxycinnamic acid against *M. luteus*, *S. aureus*, *E. coli* and *S. enteritidis* were 80, 60, 50 and 60 μg mL^{-1}, respectively.

p-Methoxycinnamic acid showed a higher antimicrobial activity against Gram-negative *E. coli* than Gram- positive *S. aureus*. This result agreed to the finding of Balasubamanian *et al.*[17]. This is the first study to describe that *p*-methoxycinnamic acid had a good antimicrobial activity against *M. luteus* and *S. enteritidis*.

The structure of *p*-methoxycinnamic acid suggested that it had the antioxidative activity. We investigated the antioxidative activity of *p*-methoxycinnamic acid in the linoleic acid system. The antioxidative activity of *p*-methoxycinnamic acid was compared with that of BHA, BHT and α-tocopherol at the concentration of 40 μg mL^{-1}. The inhibition of *p*-methoxycinnamic acid, BHA, BHT and α-tocopherol against the lipid peroxidation was 48, 65, 69 and 60%, respectively.

Although cinnamic acid derivatives such as *p*-coumaric and caffeic acids widely distribute in plants, *p*-methoxycinnamic acid is seldom found in plants: the leaves of *Duranta repens* LINN[18], the bark of *Ailanthus integrifolia* LAMK (*Simaroubaceae*)[19], the stem of *Ficus septica*[20], brown rice [21] and the root of *Scrophalaria buergeriana*[22]. Its occurrence in plant seeds has not been reported.

p-Methoxycinnamic acid is reported to have the following functions: protecting primary cultures of rat hepatocytes from toxicity induced by carbon tetrachloride (CCl_4)[22]; attenuating glutamate-induced neurotoxicity in a dose-dependent manner when added to primary cultures of rat cortical cells[23]; anti-amnestic activity[24]; suppressive effect of the SOS-inducing activity of the mutagen, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (furylfuramide)[25]; α-glucosidase inhibitory activity[26].

p-Methoxycinnamic acid is probably applicable to a great variety of foods as a potential antimicrobial and antioxidative substance. Therefore, there is a possibility that yuzu seeds is utilized as a new functional material.

CONCLUSIONS

A search for components exhibiting antimicrobial activity in yuzu seeds was made. The antimicrobial substance in the 80% MeOH extract was purified by various adsorption column chromatographies and was identified as *p*-methoxycinnamic acid by GC-MS. MIC of *p*-methoxycinnamic acid against *M. luteus*, *S. aureus*, *E. coli* and *S. enteritidis* were 80, 60, 50 and 60 μg mL^{-1}, respectively. In addition, *p*-methoxycinnamic acid showed also the antioxidative activity. The results suggest that yuzu seeds is utilized as a new functional material.

REFERENCES

1. Halliwell, B., 1994. Free radicals and antioxidants. Nutr. Rev., 52 : 253-265.
2. Wattenburg, L.W., 1972. Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic antioxidants and ethoxyguin. J. Natl. Cancer Inst., 48: 1425-1430.
3. Wattenburg, L.W., 1978. Inhibition of chemical carcinogenesis. J. Natl. Cancer Inst., 60: 11-18.
4. Hocman, G., 1988. Chemoprovention of cancer: Phenolic antioxidants (BHT, BHA). Intl. J. Biochem., 20: 639-651.
5. Alan, D., 1991. Fruit. Mitchell Beazley Publishers, England, pp: 74.
6. Ito, S., 1991. Science of fruits. Asakura Syoten, Japan, pp: 142-143.
7. Jayaprakasha, G.K., T. Selvi and K.K. Sakariah, 2003. Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. Food Res. Intl., 36: 117-122.
8. Omi, K., 1999. The Japanese Specifications and Standards for Food Additives. Japan Food Additives Association Press, pp: 207.
9. Omi, K., 1999. The Japanese Specifications and Standards for Food Additives. Japan Food Additives Association Press, pp: 519.
10. Ariga, T., H. Hosoyama, S. Tokutake and J. Yamakoshi, 2000. Studies on functional properties of proanthocyanidins and their practical application. Nippon Nogei Kagaku Kaishi, 74: 1-6 (in Japanese).
11. Matsusaka, Y., J. Kawabata and T. Kasai, 2003. Antioxidative constituents in Avocado (Persea americana Mill.) seeds. Nippon Shokuhin Kagaku Kogaku Kaishi, 50: 550-552, (in Japanese).
12. Jeongmok, K., R.M. Maurice and I.W. Cheng, 1995. Antibacterial activity of some essential oil component against five food-borne pathogens. J. Agric. Food Chem., 43: 2834-2845.
13. Nguyen, V.C., T. Kurata, H. Kato and M. Fujimaki, 1982. Antimicrobial activity of kumazasa (*Sasa albo-marginate*). Agric. Biol. Chem., 46: 971-978.
14. Nishina, C., N. Enoki, S. Tawata, A. Mori, K. Kobayashi and M. Fukushima, 1987. Antibacterial activity of flavonoids against *Staphylococcus epidermidis*, a skin of bacterium. Agric. Biol. Chem., 51: 139-143.
15. Shirasaka, N., A. Kuramatsu, N. Kondo, S. Kondo, M. Iida, T. Hasegawa, T. Murakami and H. Yoshizumi, 1999, Isolation and characterization of antioxidative compounds from ume (*Prunus mume*) liqueur. Nippon Shokuhin Kagaku Kogaku Kaishi, 46: 792-798 (in Japanese).
16. Tesaki, S., S. Tanabe, M. Moriyama, E. Fukushi, J. Kuwabara and M. Watanabe, 1999. Isolation and identification of an antibacterial compound from grape and its application to foods. Nippon Nogei Kagaku Kaishi, 73: 125-128 (in Japanese).
17. Balasubramanian, N., B. Deepak, P. Devayani, M. Vishnukant and D. Avinash, 2004. Esters, amides and substituted derivatives of cinnamic acid: Synthesis, antimicrobial activity and QSAR investigations. Euro. J. Medic. Chem., 39: 827-834.
18. Kuo, Y.H., Z.S. Chen and Y.L. Lin, 1996. Chemical components of the leaves of *Duranta repens* LINN. Chem. Pharm., 44: 429-436.
19. Kosuge, K., K. Mitsunaga, K. Koike and T. Ohmoto, 1994. Studies on the constituents of *Ailanthus integrifolia*. Chem. Pharm., 42: 1669-1671
20. Ping, C.K., C.C. Chao, S.S. Li, Y.L. Chia, J.W. Shwu, A.G. Damu, L.W. Pei, S.K. Chang and S.W. Tian, 2002. Non-alkaloidal constituents from the stem of *Ficus septica*. J. Chin. Chem. Soc., 49: 113-116.
21. Hudson, E.A., P.A. Dinh, T. Kokubun, S. Monigue, J. Simmonds and G. Andreas, 2000. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and color cancer cells. Cancer Epidemiol., Biomarkers and Prevention, 9: 1163-1170.

22. Lee, E.J., S.R. Kim, J. Kim and Y.C. Kim, 2002. Hepatoprotective phenyl propanoids from *Scrophularia buergeriana* roots against CCl_4-induced toxicity: Action mechanism and structure activity relation ship. Planta Medica, 68: 407-411.
23. Kim, S.R. and Y.C. Kim, 2000. Neuroprotective phenylpropanoid esters of rhamnode isolated from roots of *Scrophularia buergeriana*. Phytochemistry, 54: 503-509.
24. Kim, S.R., S.Y. Kang, K.Y. Lee, S.H. Kim, G.J. Markelonis, T.H. Oh and Y.C. Kim, 2003. Anti-amunestic activity of E-*p*-methoxycinnamic acid from *Scrophularia buergeriana*. Cornitive Brain Res., 17: 454-461.
25. Miyazawa, M., Y. Okuno, S. Nakamura and H. Kameoka, 1998. Suppression of SOS-inducing activity of chemical mutagens by cinnamic acid derivatived from *Scrophulia ningpoensis* in the *Salmonella typhimurium* TA 1535/PSK1002 umu test. J. Agri. Food Chem., 46: 904-910.
26. Sirichai, A., S. Kasem, R. Sophon, P. Amorn, N. Nattaya, C. Warinthorn, D. Sujitra and Y. Sirintorn, 2004. Structure-activity relationships of *trans*-cinnamic acid derivatives on α-glucosidase inhibition. Bioorg. Medic. Chem. Lett., 14: 2893-2896.