Studies on Natural Antioxidants in Citrus Species. I. Determination of Antioxidative Activities of Citrus Fruits

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The antioxidative activities of twenty types of citrus fruits were investigated with a screening method which is based on rat liver microsomal lipid peroxidation induced by dihydronicotinamide adenine dinucleotide phosphate (NADPH) and adenosine diphosphate (ADP). The activities of the exocarp were greater than those of the sarcocarp and the activities from immature fruits (collected in July—August) were greater than those from mature fruits. The strongest antioxidative activity was found in ponkan (*Citrus reticulata* BLANCO) collected in July.

Keywords antioxidative activity; lipid peroxidation; rat liver microsome; citrus; ponkan; immature fruit; exocarp; fruits thinning

In recent years, farmers who cultivate citrus fruits in Japan have been discarding some of the immature fruits as a means of fruit thinning. The quantities discarded are quite considerable, so to make use of them is highly desirable. We have already reported the antioxidative activities of methanol (MeOH) extracts of about 130 types of crude drug,[1] and have identified several antioxidative components, such as *l*-epicatechin in Theae Folium,[2] curcumin in Curcumae Rhizoma[3] and geniposidic acid in Plantaginis Semen.[4] Recently, besides their use as food additives, antioxidants have been proposed as possible therapeutic drugs for geriatric diseases, as lipid peroxidation in a whole live organism has been suggested to have some correlation with geriatric diseases,[5] such as arteriosclerosis,[6] diabetes,[7] cancer[8] and senile dementia.[9] Therefore, it is highly possible that antioxidants may be developed into potential drugs which affect the course of these diseases, as well as having use as essential food additives, which protect oils and fats against peroxidation.

In this study, the antioxidative activities of various citrus fruits collected at different times were investigated with a screening method based on rat liver microsomal lipid peroxidation induced by dihydronicotinamide adenine dinucleotide phosphate (NADPH) and adenosine diphosphate (ADP).

Materials and Methods

Citrus Fruits Tested and Crude Drugs The twenty types of citrus fruits tested are shown in Table II and were all collected from the farm of the Shizuoka Prefectural Citrus Experiment Station (Shimizu, Japan). Each citrus fruit tested was peeled as soon after collection as possible and the exocarp was air-dried at room temperature. Each dried exocarp (20 g) was extracted with 100 ml MeOH under reflux for 30 min, and each freeze-dried sarcocarp (20 g) was extracted with 200 ml MeOH under reflux for 30 min. Each extract and 30 ml of MeOH solution used for the washing of the residue, were mixed and concentrated to dryness under reduced pressure and, when required for use, dissolved or suspended in 0.5 M Tris–HCl buffer (pH 7.5) with a small amount of Tween 80. All crude drugs originating from citrus species were purchased from Niiya Co., Ltd. (Shimizu, Japan).

Chemicals NADPH and ADP were purchased from Sigma Chemical Co. (U.S.A.), and thiobarbituric acid (TBA) was purchased from Nacalai Tesque Inc. (Tokyo, Japan). DL-α-Tocopherol, used as a reference compounds, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals used were of the highest grade commercially available.

Antioxidative Test Preparation of Microsomes: Male Wistar rats, which weighed 330—370 g, were purchased from the Shizuoka Laboratory Animal Centers, Hamamatsu, Japan, and were deprived of food but allowed free access to water 18 h prior to the experiment. They were anesthetized with ether, exsanguinated from the abdominal artery and the liver was perfused with ice-cold saline *via* the portal vein and removed quickly. The liver tissues were cut into small pieces and then homogenized with 9 times their volume of 1.15% (w/v) KCl, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), at 4 °C with a Potter–Elvehjem type teflon homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C, the resultant supernatant was centrifuged at 10000 *g* for 30 min at 4 °C, and then this supernatant was centrifuged at 78000 *g* for 90 min at 4 °C to obtain the pellet of the microsomal fraction. The resultant microsomal pellet was suspended in 0.5 M Tris–HCl buffer (pH 7.5) and the microsomal protein concentration was determined by the method of Lowry *et al.*[10] with bovine serum albumin as the standard.

Lipid Peroxidation Test[11] A mixture of microsomes (2.5 mg protein/ml) and 0.5 M Tris–HCl buffer (pH 7.5), which contained 2 mM (final concentration) ADP and the required MeOH extract test solution was pre-incubated at 37 °C for 3 min, after which the reaction was started by adding 0.2 mM (final concentration) NADPH solution; the final volume of the reaction mixture was 1 ml. The reaction was terminated after 30 min by cooling the mixture to 4 °C rapidly, and the lipid peroxides in the mixture were determined by our TBA method[12] with malondialdehyde (MDA) as the standard. The control preparations were prepared in an identical manner but no test solution was added and the lipid peroxide level in the controls increased about 100 times (from 0.18—0.20 mM to 17.4—19.2 mM, MDA) compared with that in the tube to which no ADP and NADPH solution was added. Therefore, antioxidative activity of each citrus sample was expressed as the percentage inhibition of the lipid peroxide level compared with the level in the control preparation.

Results and Discussion

Antioxidative Activities of Crude Drugs Originating from Citrus Species Prior to our experiments with immature citrus fruits, we assayed the antioxidative activities of MeOH extracts of crude drugs, which originated from citrus species, such as *Citri leiocarpae exocarpium* (Seihi, Chinpi, Kippi in Japanese) and *Aurantii pericarpium* (Touhi), *Aurantii fructus immaturus* (Kijitu). Their activities, expressed as the concentrations which produced 50% inhibition compared with the control samples (IC_{50}, μg/ml), are shown in Table I. Seihi, which is prepared from the exocarp of immature *Citrus unsiu* fruits, was found to possess the strongest antioxidative activity. These results suggested, therefore, that the exocarp of immature citrus fruits was likely to be sources of antioxidants.

Antioxidative Activities of MeOH Extracts of the Citrus Fruit Tested i) Antioxidative Activities: The citrus fruits tested ware collected in 3 different seasons, in July,

September (Sept.) and December (Dec.), and the concentration of each MeOH extract studied was 50 μg/ml. The results are shown in Table II.

First, the antioxidative activities of the exocarp and sarcocarp of ponkan (*C. reticulata* BLANCO) and unshu-mikan (*C. unsiu*) were compared, and the antioxidative activities of the extracts of their exocarp were greater than those of their sarcocarp. Therefore, in subsequent experiments, we used the exocarp of the citrus fruits only as source material for the MeOH extracts.

The MeOH extracts which showed intensive antioxidative activity, (at a concentration of 50 μg/ml) were as follows; ponkan in July > ponkan in Sept. > daidai (*C. aurantium*) in July > ohbeni-mikan (*C. tangerina*) in July > kishu-mikan (*C. kinokuni*) in July > navel-orange (*C. sinensis*) in July.

Next, we determined the IC_{50} values of some MeOH extracts, which demonstrated an inhibition of 60% or over, compared with control samples, at 50 μg/ml. The results are shown in Table III. Of the samples tested, the IC_{50} of ponkan in July was the lowest, *i.e.*, this species possessed the strongest antioxidative activity. The IC_{50} value of the extract of ponkan (3.86 μg/ml) indicated it was about 9.8 times stronger as an antioxidant than the crude drug Seihi. The IC_{50} of DL-α-tocopherol, which was used here as a reference compound, was 1.07 μg/ml. These results suggested that a rather effective antioxidative component may exist in the exocarp of ponkan collected in July.

ii) Change of Antioxidative Activity with Collection Time: Table II also shows the changes of activity observed at different collection times. Particular attention was paid to certain citrus species, such as ponkan, daidai, navel-orange, lemon (*C. limon*), Iyokan (*C. iyo*), and related types, all of which demonstrated high antioxidative activity and could be collected in July, Sept., and Dec. The antioxidative activity of all these types decreased as the collection time became later. Therefore it is obvious that the antioxidative activities of citrus fruits decline as they mature.

We selected the exocarp of ponkan collected in July as the experimental material for searching for the antioxidative constituents, because it showed the strongest activity and longest duration of activity in our antioxidative test. We are now preparing to report on the chemical structures of the antioxidative constituents we have isolated from ponkan.

TABLE I. List of IC_{50} Values of MeOH Extracts of Crude Drugs Originating from Citrus Species against Rat Microsomal Lipid Peroxidation Induced by NADPH–ADP

Crude drugs	Source plants	Part	Stage at collection time	IC_{50} (μg/ml)
Seihi *Citri leiocarpae exocarpium*	*C. unsiu* and related plants	Peel	Immature	38.0
Chinpi *Citri leiocarpae exocarpium*	*C. unsiu* and related plants	Peel	Mature	227.5
Touhi *Aurantii pericarpium*	*C. daidai* or *C. aurantium*	Peel	Mature	296.8
Kijitu *Aurantii fructus immaturus*	*C. unsiu*, *C. daidai* and related plants	Fruit	Immature	329.8
Kippi *Citri leiocarpae exocarpium*	*C. grandis* and related plants	Peel	Mature	359.6

TABLE II. Inhibitory Effects of MeOH Extracts of Citrus Fruit against Rat Liver Microsomal Lipid Peroxidation Induced by NADPH–ADP

Sample name Exocarp	Citrus type and related species	Latin name	MeOH extract yield (g)[a]			Inhibition (%)		
			July	Sept.	Dec.	July	Sept.	Dec.
	Citrus							
Daidai	Archicitrus	*Citrus aurantium* LINN. form. KABUSU	4.42	2.89	4.81	81.7	54.7	46.4
Natsu-daidai		*C. natudaidai* HAYATA	2.71	2.53	4.49	60.0	34.9	27.0
Iyokan		*C. iyo* HORT. *ex* TANAKA	1.96	2.48	5.63	70.0	37.9	26.7
Navel Orange		*C. sinensis* OSBECK	1.76	1.77	4.45	75.1	47.2	24.4
Hyuga-natsu		*C. tamurana* HORT. *ex* TANAKA	2.55	2.75	5.40	47.4	38.5	28.7
Hassaku		*C. hassaku* HORT. *ex* TANAKA	4.23	2.24	5.40	51.6	35.3	25.9
Buntan		*C. grandis* OSBECK	5.62	2.92	5.22	44.5	35.1	32.5
Lemon		*C. limon* BURMANN form. *Lisbon*	2.11	2.73	4.15	71.8	47.4	45.7
Kimikan		*C. flaviculpus* HORT. *ex* TANAKA	2.79	2.29	5.12	61.8	54.0	43.1
Busshukan		*C. Medica* LINN. var. *sarcodactylis* SWINGLE	1.71	4.10	N.D.	48.2	32.8	N.D.
Unshu-mikan	Metacitrus	*C. unsiu* MARC.	1.22	2.35	5.54	65.3	38.7	22.4
Ponkan		*C. reticulata* BLANCO	1.59	3.09	5.82	100.0	82.5	36.7
Kishu-mikan		*C. kinokuni* HORT. *ex* TANAKA	N.D.	1.23	4.66	N.D.	76.7	42.0
Kouji		*C. leiocarpa* HORT. *ex* TANAKA	N.D.	1.75	3.74	N.D.	56.6	55.8
Oobenimikan		*C. tangerina* HORT. *ex* TANAKA	N.D.	1.26	2.65	N.D.	77.4	56.3
Kobenimikan		*C. erythrosa* HORT. *ex* TANAKA	N.D.	2.41	N.D.	N.D.	52.3	N.D.
Yuzu		*C. junos* SIEB. *ex* TANAKA	2.07	2.18	5.24	62.0	52.7	40.5
Ichan-limon		*C. Shiangyuan* HORT. *ex* TANAKA	3.11	2.48	5.12	48.1	37.4	38.2
Kinkan	*Fortunella*	*Fortunella*	N.D.	2.70	8.95	N.D.	27.4	30.8
Karatachi	*Poncirus*	*Poncirus trifoliata* RAFINESQUE	3.62	4.34	N.D.	50.7	40.2	N.D.
Sarcocarp								
Unshu-mikan		*C. unsiu* MARC.	2.24	6.32	8.80	41.8	45.3	36.2
Ponkan		*C. reticulata* BLANCO	2.15	5.24	7.10	46.5	47.4	29.8

a) MeOH extract yield (g): yield (g) of MeOH extract, obtained from extracting each dried peel (20 g) with 100 ml MeOH and each freeze-dried flesh (20 g) with 200 ml MeOH, under reflux for 30 min. N.D., not determined due to immaturity or maturity of fruit.

TABLE III. List of IC_{50} (μg/ml) Values of MeOH Extracts of the Main Citrus Species Tested against Microsomal Lipid Peroxidation Induced by NADPH–ADP

Citrus	IC_{50} (μg/ml)		Citrus	IC_{50} (μg/ml)	
	July	Sept.		July	Sept.
Daidai	26.8	>60	Yuzu	49.5	>60
Natsu-daidai	40.8	>60	Kishu-mikan	>60	26.3
Iyokan	51.4	>60	Oobenimikan	>60	18.0
Navel orange	36.4	>60	Kimikan	43.1	>60
Limon	40.3	>60	Reference compound		
Unshu-mikan	45.8	>60	DL-α-Tocopherol		1.07
Ponkan	3.86	18.4			

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