Potent Antioxidant and Radical-Scavenging Activity of Chenpi - Compensatory and Cooperative Actions of Ascorbic Acid and Citric Acid

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Abstract: Dried peels of Satsuma mandarin (Citrus unshiu Marcov.) have been used as traditional Chinese and Japanese medicine, which are called ‘Chenpi’. In our present study, cold and hot water extracts of Chenpi exhibited a strong inhibitory activity against linoleic acid peroxidation and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity compared with 0-methanol extract. When these extracts were separated into ethanol-soluble(ES) and ethanol-precipitable fractions, the dominant antioxidant and radical-scavenging activities were detected in the ES fractions, which suggests that these antioxidant and radical-scavenging activities are responsible for water-soluble and low-molecular-weight substances. As possible active principles for antioxidant activities in the water extracts, the contents of ascorbic acid and citric acid in these extracts were measured, and the antioxidant and radical-scavenging activities of these substances were assayed at various concentrations. The experimental results indicate that the antioxidant activity against lipid peroxidation in the water extracts is dominantly associated with citric acid, and the DPPH radical-scavenging activity of the water extracts is majorly responsible for ascorbic acid, suggesting a compensatory action of ascorbic acid and citric acid in expression of the antioxidant and radical-scavenging activities of Chenpi.

Key words: Chenpi, antioxidant and radical-scavenging activities, ascorbic acid, citric acid.

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Introduction

Free radical-mediated damage to body cells is associated with the causation and progression of chronic diseases such as atherosclerosis, diabetes and cancer [1,2]. On the other hand, various vegetables and fruits have been considered as preventive factors against these chronic diseases, and antioxidant components in these foods are important because they have antioxidant and radical-scavenging activities against the free radical attack on proteins, nucleic acids and lipids in body cells [3,4].

Among these edible plants, citrus fruits have been traditionally used as healthy foods. Pliny, an ancient Roman naturalist, first used the word 'Citrus' which labeled the fruit a medicine. Among citrus fruits, the dried peels of citrus fruits, called Chenpi, have been traditionally used as a medicine for improvement of bronchial and asthmatic conditions or cardiac and blood circulation in China and Japan [5].

However, the details of the antioxidant activity of citrus peels has not been analyzed except in several studies. For example, the methanol extracts of citrus peels including several phenolic substances showed relatively weaker antioxidant activity than the methanol extracts of citrus seeds [6]. A flavanone glycoside, hesperidin, is contained abundantly in citrus peels [7] and is considered as one of main antioxidant substances in Chenpi. Furthermore, other researchers studied the contents of flavonoids and phenolic compounds and antioxidant activity in hot water extracts of citrus peels [8,9]. We then compared the effects of the extracts treated with various solvents which were prepared from fresh and dried peels of Satsuma mandarin (Citrus unshiu Marcov.) on lipid peroxidation, and found that the hot water extract of dried peels showed the strongest antioxidant activity among various extracts [10]. In this study, the content of ascorbic acid in the hot water extract of Chenpi could not explain its remarkable antioxidant activity, which suggested the existence of other unknown factors responsible for the strong antioxidant activity.

In our present study, cold- and hot-water extracts of Chenpi showed very strong antioxidant and radical-scavenging activities, and we elucidated the compensatory and cooperative actions of ascorbic acid and citric acid as active principles in these extracts.
Materials and Methods

Chemical reagents
Potassium iodide, aluminium chloride, soluble starch, 2,6-dichlorophenol-indophenol (DCIP), ethylenediamine tetraacetic acid (EDTA), metaphosphoric acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Japan Co. (Tokyo, Japan). o-phenanthroline and linoleic acid were purchased from Kanto Chemical Co. (Tokyo, Japan). Ascorbic acid and citric acid were purchased from Wako Pure Chemical Co. (Osaka, Japan). Potassium iodide solution was prepared by 2 g of KI in 100 ml of ethanol, and aluminium chloride solution was prepared from 2 g of AlCl₃ and 20 µg of o-phenanthroline in 100 ml of ethanol. A starch solution contained 1 g of soluble starch and 20 g of NaCl in 10 ml of distilled water. Other chemical reagents used in the present study were purchased from Sigma-Aldrich Japan, except for specific reagents which are described in the text.

Preparation of the extracts from the peels of Satsuma mandarin (Chenpi)
The peels were separated from the fresh Satsuma mandarin (Citrus unshiu Marcov.) supplied from the Shizuoka branch of the Japanese Agriculture Association (Shizuoka, Japan). The fresh peels were dried in a silica gel desicator at 4°C, and the weight of dried peels became approximately 25% of the total weight of the fresh peels. The dried peels were minced extensively by an electric mill, combined with 10 volumes of cold water (4°C), hot water (90°C) or methanol and stood for 3 days at 4°C. The extract was centrifuged at 1500 × g for 15 min and the supernatant was used as the test sample.

Separation of ethanol-soluble (ES) and ethanol-precipitable (EP) fractions from Chenpi extracts
After each extract (10 ml) of Chenpi was combined and mixed with three volumes of cold ethanol in the presence of 30 mM NaCl, it was kept in a deep freezer for 3 hr at −40°C. The mixture was centrifuged at 1500 × g for 15 min and the supernatant was recovered and concentrated with a rotary evaporator at 4°C as ES fraction (10 ml). The precipitate was dissolved in 10 ml of cold distilled water or methanol as EP fraction. The ES and EP fractions were stored in a deep freezer at −40°C.

Measurement of ascorbic acid content
Ascorbic acid content was measured by a modification of the previous method [11]. One ml of the test samples including ascorbic acid was mixed with 1 ml of ice-cold 10% metaphosphoric acid and 2 mM EDTA for deproteinization and stabilization of ascorbic acid. After 600 µl of 50 mM citrate/acetate buffer (pH 3.5) was added to the mixture, 300 µl of DCIP solution (0.1 mg/ml) was added and mixed. One min later, the absorbance of the mix-
ture was measured at 520 nm. Standard ascorbic acids were dissolved in 5% metaphosphoric acid and the standard curve of ascorbic acid was made by using the assay method described above.

Measurement of citric acid content

The citric acid content was measured by a modification of the previous method [12]. 0.1 M NaOH solution in water (W/V) was exactly prepared by the quantitation with 0.05 M oxalic acid. 20 μl of 1% phenolphthalein solution in ethanol (W/V) was added and mixed in 10 ml of Chenpi extract and then 10 μl of NaOH solution was added and mixed repeatedly until the pinkish color of the Chenpi extract disappeared. The citric acid content in the Chenpi extract (mg/ml) was calculated by the following equation: 0.64 × (V/1000) / (10.02 + V/1000) (V: Volume of 0.1 M NaOH solution (μl))

Assay for the antioxidant activity against lipid peroxidation

Hydroperoxide generation from oxidized linoleic acid was assayed by the modified method of Asakawa and Matsushita [13]. One ml of each extract or test solution was mixed with 1 ml of linoleic acid solution and 2 ml of 50 mM phosphate buffer (pH 7.5) in a glass test tube and stood for two weeks at 33°C. As a negative control experiment, 1 ml of each solvent was used in place of 1 ml of the extract or test solution. The test solution was prepared, including oxidized linoleic acid solution (200 μl), 250 μl of KI solution, 250 μl of aluminium chloride solution and 1 ml of hexane were added to a test tube, mixed and incubated for 5 min at 37°C under dark condition. Starch solution (0.25 ml) and 7.5 ml of 10 mM HCl were added, mixed and shaken vigorously. The solution was centrifuged at 1,500 × g for 5 min, and the absorbance of the lower layer was measured at 560 nm using a Shimadzu UN-1600 spectrophotometer.

Assay for radical-scavenging activity

The radical-scavenging activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the modified method of Brand-Williams [14]. An aliquat of water or methanol (200 μl) containing different test substances was mixed with 3.8 ml of 0.1 mM DPPH in methanol prepared daily. The extract or test solution was diluted successively with water or methanol, and a control experiment was performed with water or methanol alone and compared with that of the test solutions. The decrease of absorbance at 520 nm was measured at 15 min intervals for 3 hrs using a Shimadzu UV-265 spectrophotometer.

Statistical analysis

The experimental result was expressed as the mean and standard error of triplicate assays. Relative activity(%) was expressed by the following equation: (the mean value of sample-treated experiment/ the mean value of the control experiment) × 100%. The statistical com-
Comparison between the control and sample-treated experimental groups was carried out using Student's t test. A value less than 0.05 was considered to be significantly different.

Results

Antioxidant activity of Chenpi extracts against lipid peroxidation

The dried peels of Satsuma mandarin (Chenpi) were milled, and their extracts were prepared by cold water, hot water and methanol, and the effects of these extracts on the hydroperoxide generation from oxidized linoleic acid were compared. As shown in Fig. 1, strong antioxidant activities were observed in cold and hot water extracts, which showed 72 and 77.1% inhibition against hydroperoxide generation, respectively (P<0.001). The methanol extract exhibited a relatively weak antioxidant activity (30.1% inhibition, P<0.05). Then, these extracts were treated with ethanol and separated into ethanol-soluble (ES) and ethanol-precipitable (EP) fractions and the antioxidant activities of both fractions were assayed. As indicated in Fig. 2, the ES fractions of cold and hot water extracts showed relatively strong activities (51.4 and 58.1% inhibition, P<0.001) compared with that of methanol extract (20% inhibition, P<0.025). However, the EP fractions of all extracts did not exhibit any significant antioxidant activities. This result indicates that the major antioxidant activities against lipid peroxidation in Chenpi are associated with water- and ethanol-soluble low-molecular-weight substances.

As possible active substances responsible for the antioxidant activity against lipid peroxidation in the water extracts of Chenpi, we assumed ascorbic acid and citric acid, according to previous studies [3,4]. Then we measured the contents of these substances in cold- and hot-

![Fig. 1](image_url)

Fig. 1. Antioxidant effect of Chenpi extracts on hydroperoxide generation in lipid peroxidation. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student's t test. P values of the extract-treated experiments are shown as follows. SD: standard deviation, *: P < 0.05, **: P < 0.001.
Fig. 2. Antioxidant effect of ES and EP fractions of Chenpi extracts on hydroperoxide generation in lipid peroxidation. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student’s $t$ test. $P$ values of the extract-treated experiments are shown as follows. ES : ethanol-soluble, EP : ethanol-precipitable, SD : standard deviation, *: $P<0.025$, **: $P<0.001$.

Fig. 3. Antioxidant effect of ascorbic acid on hydroperoxide generation in lipid peroxidation. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student’s $t$ test. $P$ values of the extract-treated experiments are shown as follows. SD : standard deviation, *: $P<0.05$.

The ascorbic acid contents in cold- and hot-water extracts were estimated to be 250 and 220 $\mu$g/ml, respectively. As shown in Fig. 3, the ascorbic acid concentrations of 50–250 $\mu$g/ml caused apparent weak antioxidant effects without a dose-dependency (12.7–19.9% inhibition, $P<0.05$). This result indicates that the antioxidant activity of ascorbic acid itself is too insufficient to explain the strong antioxidant activity of water extracts of Chenpi, as shown in Fig. 1.
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![Graph showing antioxidant effect of citric acid on hydroperoxide generation in lipid peroxidation](image)

**Fig. 4.** Antioxidant effect of citric acid on hydroperoxide generation in lipid peroxidation. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student's t test. P values of the extract-treated experiments are shown as follows. SD: standard deviation, *: \( P < 0.05 \), **: \( P < 0.001 \).

![Graph showing scavenging activity of Chenpi extracts against DPPH radical](image)

**Fig. 5.** Scavenging activity of Chenpi extracts against DPPH radical. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student’s t test. P values of the extract-treated experiments are shown as follows. DPPH: 1, 1-diphenyl-2-picrylhydrazyl, SD: standard deviation, *: \( P < 0.001 \), **: \( P < 0.0001 \).

Next, the citric acid contents in the cold and hot water extracts were estimated to be 1.0 and 1.3 mg/ml, respectively. As shown in Fig. 4, when different concentrations of citric acid (250–1000 µg/ml) were added to the lipid peroxidation system, they showed dose-dependent inhibitory activities. Especially, the high concentration of citric acid (1 mg/ml) equivalent to that of cold water extract showed about 80% inhibition against lipid peroxidation (\( P < 0.001 \)), which was roughly equal to the activities in the cold and hot water extracts of Chenpi.
These results suggest that the dominant antioxidant activity against lipid peroxidation in the water extracts of Chenpi is associated with citric acid, and ascorbic acid plays a minor role in expressing antioxidant activity in water extracts.

**DPPH radical-scavenging activity of Chenpi extracts**

Next, we analyzed radical-scavenging activities of cold and hot water and methanol extracts of Chenpi. As shown in Fig. 5, the cold and hot water extracts showed remarkable radical-scavenging activities (98.3 and 95.3% inhibition, \( P < 0.0001 \)), and methanol extract also expressed a relatively strong activity (about 70% inhibition, \( P < 0.001 \)). These extracts were then separated into ES and EP fractions by ethanol treatment. As indicated in Fig. 6, the dominant radical-scavenging activities of these extracts were observed in the ES fractions of all the extracts, and the ES fractions of the water extracts exhibited much stronger activity than that of the methanol extract. However, the radical-scavenging activities in the EP fractions of all the extracts were relatively low (Fig. 6). This result suggests that the major radical-scavenging activities in Chenpi are associated with water- and ethanol-soluble and low-molecular-weight substances. We also assumed ascorbic acid and citric acid as possible active principles responsible for DPPH radical-scavenging activities in the water extracts. As shown in Fig. 7, ascorbic acid showed strong radical-scavenging activities at 100, 250 and 500 \( \mu g/ml \) in a dose-dependent fashion (82.2, 95.3 and 98.3% inhibition). Especially, the concentration equivalent to that in the cold water extract (250 \( \mu g/ml \)) showed a remarkable radical-scavenging activity. However, citric acid exhibited relatively weak radical-scavenging activities at 100–1000 \( \mu g/ml \) (17.8–22% inhibition) and no dose-dependency was observed (Fig. 8). These results suggest that DPPH radical-scavenging activity in the water extracts of Chenpi is mainly due to ascorbic acid.

![Fig. 6. Scavenging activity of ES and EP fractions of Chenpi extracts against DPPH radical. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student’s t test. \( P \) values of the extract-treated experiments are shown as follows. ES: ethanol-soluble, EP: ethanol-precipitable, DPPH: 1,1-diphenyl-2-picrylhydrazyl, *: \( P < 0.05 \), **: \( P < 0.001 \).](image-url)
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**Fig. 7.** Scavenging activity of ascorbic acid against DPPH radical. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student's *t* test. *P* values of the extract-treated experiments are shown as follows. DPPH: 1, 1-diphenyl-2-picrylhydrazyl, SD: standard deviation, *:* *P* < 0.01, **:* *P* < 0.001, ***:* *P* < 0.0001.

**Fig. 8.** Scavenging activity of citric acid against DPPH radical. The column and bar represent the mean and SD of triplicate assays. The statistical comparison between the control and sample-treated experiment was carried out using Student's *t* test. *P* values of the extract-treated experiments are shown as follows. DPPH: 1, 1-diphenyl-2-picrylhydrazyl, SD: standard deviation, *:* *P* < 0.05.

extracts of Chenpi is majorly responsible for ascorbic acid and a minor radical-scavenging activity is derived from citric acid.

**Discussion**

A part of our present study confirms our previous result that the water extracts of dried
peels of Satsuma mandarin (Chenpi) show much stronger antioxidant activities than the methanol extract against lipid peroxidation [10]. We then focussed on the antioxidant and radical-scavenging activities of cold and hot water extracts of Chenpi and further analyzed the active principles in the extracts responsible for these activities. The dominant activities of these extracts were observed in ES fractions prepared by ethanol treatment (Fig. 2 and 6), which indicates that the antioxidant and radical-scavenging activities of Chenpi extracts are associated with water-soluble and low-molecular-weight substances. We assumed ascorbic acid and citric acid as possible principles responsible for the antioxidant and radical-scavenging activities of Chenpi extracts. Interestingly, both substances showed qualitatively different antioxidant and radical-scavenging activities.

Citric acid showed the strong antioxidant activity against lipid peroxidation (Fig. 4), but exhibited very weak radical-scavenging activity against DPPH (Fig. 7). As a possible mechanism for the antioxidant activity of citric acid against lipid peroxidation, we considered a chelating ability of citric acid for transition metal ions such as ferrous and copper ions. Namely, lipid peroxidation is accelarated by metal ion-dependent reaction, which promotes hydroperoxide generation [15]. Although the radical-scavenging activity of citric acid itself is not so strong (Fig. 7), its chelating activity might cause the strong suppression against hydroperoxide generation in lipid peroxidation (Fig. 4).

In contrast, ascorbic acid exhibited a strong scavenging activity against DPPH radical (Fig. 6), but showed very weak antioxidant activities against lipid peroxidation (Fig. 3). This result can be explained by previous studies by other researchers. The reductive form of ascorbic acid has a strong radical-scavenging activity, but reacts with a trace amount of ferrous or copper ion and converts to its oxidized form. During these reactions, highly reactive radicals such as superoxide anion, hydrogen peroxide and hydroxyl radical are generated [16,17]. Possibly, this property of ascorbic acid induces a prooxidant effect on lipid peroxidation. On the other hand, a chelating activity of citric acid might cause a protective effect against lipid peroxidation induced by metal ion and ascorbic acid. To confirm the above mentioned notion, we carried out the following experiment. As shown in Fig. 9, the combined supplement with 200 μg/ml ascorbic acid and 2 μM FeCl₃ showed a prooxidant effect on lipid peroxidation compared with the negative control experiment. Then the addition of citric acid (250–1000 μg/ml) caused a dose-dependent inhibition against lipid peroxidation induced by ferrous ion and ascorbic acid. This result indicates a possibility that the strong antioxidant activities of the cold- and hot-water extracts of Chenpi might be responsible for the cooperative action of ascorbic acid and citric acid in the extracts.

Furthermore, in our present study, the methanol extract of Chenpi also showed significant antioxidant and radical-scavenging activities in spite of its relatively weak activities compared with the water extracts (Fig. 1 and 5). As possible antioxidants in the methanol extract of Chenpi, the flavonoid group including flavones, flavanones and phenolic acids can be considered according to previous studies [6–9].
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**Fig. 9.** Effect of citric acid against lipid peroxidation induced by ascorbic acid and ferrous ion. The column and bar represent the mean and SD of triplicate assays. As a positive control experiment, 200 μg/ml ascorbic acid and 2 μM FeCl₃ were added to the lipid oxidation system. The statistical comparison between the negative and positive control experiments was carried out using Student’s t test. (*: \( P < 0.01 \)). Furthermore, different concentrations (250, 500 and 1000 μg/ml) of citric acid were applied to the lipid oxidation system induced by ascorbic acid and ferrous ion. The statistical comparison between the positive control and citric acid-treated experiments was carried out using Student’s t test. \( P \) values are shown as follows. **: \( P < 0.001 \), ***: \( P < 0.0005 \).

Although a detailed pharmacological analysis of flavonoids in Chenpi has not been performed, some flavonoids might be responsible for its chemopreventive effect on chronic diseases. For example, citrus-derived flavonoids such as tangeretin and nobiletin caused hypolipidemic effects on diet-induced hypercholesterolemia in hamsters [18], which may be associated with their antioxidant or radical-scavenging activities.

As another interesting problem, the consumption of citrus peels, but not the consumption of citrus juices, is associated with a reduced risk of human skin cancer [19]. Generally, the antioxidant substances in citrus peel extracts are more abundant than those in citrus juices. For example, ascorbic acid, flavonoids and phenolic acids in citrus peels showed higher concentrations than those of the citrus juices [7, 20–21].

Possibly, abundant amounts of ascorbic acid, citric acid, flavonoids and other antioxidant substances in citrus peels seem to show additive or synergistic effects on expressing the antioxidant or radical-scavenging activities, possibly associated with the preventive effects on various chronic diseases such as cardiovascular diseases and cancer.
References

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陳皮の抗酸化・ラジカル消去活性におけるアスコルピン酸とクエン酸の補完・協同作用について

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要旨: 中国や日本では古来より乾燥した温州みかんの皮を「陳皮」と称して循環器系疾患や呼吸器系疾患の予防や改善の目的で漢方薬・生薬・薬膳料理などに利用してきた。本研究ではこれらの陳皮の薬理効果の作用メカニズムのひとつとして抗酸化・ラジカル消去活性に注目して分析した結果、陳皮の冷水・熱水抽出液にきわめて強い抗酸化・ラジカル消去活性が認められた。そこで水抽出液をエタノール可溶性画分と沈澱画分に分けたところ、主要な活性はエタノール可溶性画分に認められた。そこでその活性本体に対応する水溶性・低分子性物質としてアスコルピン酸とクエン酸を仮定してそれらの陳皮水抽出液中の濃度を測定し、その濃度に対応する両者の抗酸化・ラジカル消去活性を分析したところアスコルピン酸は強い1,1-diphenyl-2-picrylhydrazyl (DPPH) ラジカル消去活性を示したが、リノール酸の過酸化脂質の生成に対する作用は弱かった。ところが、クエン酸は逆に DPPH ラジカル消去活性は弱かったが、リノール酸の過酸化脂質の生成に対して強い抑制作用を示した。さらにクエン酸は微量金属とアスコルピン酸によるリノール酸の過酸化脂質の生成の促進を消去・抑制することを見出した。以上の実験結果により陳皮の水抽出液の示す強い抗酸化・ラジカル消去活性はアスコルピン酸とクエン酸がお互いにそれぞれの作用を補完するとともに一方の物質の酸化促進作用を他の物質が消去・抑制するためであることが示唆された。

キーワード: 陈皮, 抗酸化, ラジカル消去, アスコルピン酸, クエン酸。

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