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Determination of Antioxidant Activity and Characterization of Antioxidant Phenolics in the Plum Vinegar Extract of Cherry Blossom (*Prunus lannesiana*)

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Sakura-cha (salted cherry blossom tea) is a Japanese tea that is traditionally served at celebrations such as wedding ceremonies. The production of Sakura-cha includes the immersion of cherry blossom flowers in Japanese plum vinegar, and through this process, the byproduct (plum vinegar extract of cherry blossom) is obtained. In this study, the antioxidant activity of the plum vinegar extract of cherry blossom was examined. The plum vinegar extract of cherry blossom had a greater superoxide anion scavenging activity compared with red wine, which is a well-known strong antioxidant. Liquid chromatography–mass spectrometry analysis showed that cyanidin-3-glucoside, cyanidin-3-rutinoside, and caffeic acid were the major components in the phenolic extract prepared from plum vinegar extract of cherry blossom, and they possessed superoxide anion scavenging activity. Caffeic acid is mainly responsible for the scavenging activity of phenolic extract; the contributions of cyanidin-3-glucoside and cyanidin-3-rutinoside were minor.

KEYWORDS: Plum vinegar extract of cherry blossom; superoxide anion; antioxidant activity; anthocyanin; caffeic acid

INTRODUCTION

Recently, reactive oxygen species (ROS) have attracted a great deal of attention. During metabolic processes of aerobic organisms, molecular oxygen accepts electrons to generate ROS such as superoxide, hydrogen peroxide, and hydroxyl radical. ROS play important beneficial roles in living species, such as killing of bacteria and apoptosis of defective cells. On the other hand, ROS have been implicated in aging and a number of diseases, such as cancer, arteriosclerosis, and neurodegenerative disorders, because they can alter lipids, proteins, and DNA (*1*–*3*). Many living species have several antioxidative defense systems against oxidative stress induced by ROS. These systems include antioxidative enzymes such as catalase and superoxide dismutase (SOD). SOD has been identified to play an important role in life span determination (*4*). In every organism, a balance needs to be maintained between the generation of ROS and the elimination of ROS by the antioxidative defense systems. However, when the amount of ROS generation exceeds the limit of defense mechanisms, the imbalance leads to lifestyle-related diseases. Recently, some studies have shown that a high intake of antioxidant food may decrease the risk of incidence of such diseases (*5*–*7*). Therefore, much attention is currently focused on antioxidants obtained from natural food resources.

Cherry blossom (*Prunus* sp.) blooms with white or pink flowers in the spring and is one of the most popular flowers in Japan. The salted leaves and petals as well as the fruit of cherry blossom are used for food production. In the town of Yusuhara (Kochi Prefecture, Japan), there are various products from cherry blossom (*Prunus lannesiana*) such as Sakura-cha (infusion of salted cherry buds). In Japan, cherry blossom products embody spring or a celebration. Therefore, Sakura-cha is traditionally served for celebrations such as wedding ceremonies. The production processes of Sakura-cha are as follows. Harvested cherry buds are washed with water and then immersed in salt water to remove the insects in the cherry buds and subsequently in Japanese plum vinegar (byproduct obtained after the production of pickled plum) for storage. The byproduct, known as Sakura-cha Ekisu in Japanese (plum vinegar extract of cherry blossom), is obtained after the immersion processes. It is used as a coloring and flavoring agent in food because of its red-purple color and its cherry blossom-like aroma. Of the red-purple

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pigments in plant materials, anthocyanins are widely known. These compounds are polyphenols, and they have various biological properties such as antioxidant activity (*8, 9*), anti-inflammatory activity (*10, 11*), inhibition of platelet aggregation (*12*), antimicrobial activity (*13*), and antitumor activity (*14*). Of these activities, antioxidant activity has received the most attention recently. Therefore, several studies have been conducted in the past decade on the antioxidant activity of polyphenols. For example, the benefits of antioxidants in eggplant (*15*), bilberry (*16*), barley bran-fermented broth (*17*), red vinegar produced through fermentation with purple sweet potato (*18*), and extracts of cherry blossoms in Korea (*19, 20*) have been reported. Therefore, we expected that the extract derived from *P. lannesiana* in Japan would have the activity as well.

In the present study, we focused on the antioxidative activity of the plum vinegar extract of cherry blossom. With the aim of applying the extract to various food products, the antioxidant activity and antioxidants in the plum vinegar extract of cherry blossom were investigated.

MATERIALS AND METHODS

Reagents. Hypoxanthine was bought from Nacalai Tesque (Kyoto, Japan); xanthine oxidase from buttermilk was from Oriental Yeast (Tokyo, Japan); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid, and SOD from bovine erythrocytes were from Sigma-Aldrich Co. (St. Louis, MO); 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) was from Labotec (Tokyo, Japan); dimethyl sulfoxide (DMSO) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were from Wako Pure Chemical Industries (Osaka, Japan); and cyanidin-3-glucoside and cyanidin-3-ruthinoside were from Funakoshi (Tokyo, Japan). SOD Assay Kit-WST was bought from Dojindo Laboratories (Kumamoto, Japan). Red wines were purchased from a local supermarket. Other chemicals used were of the highest grade available and were used without further purification. Purified water from a Millipore Autopure WQ-501 (Yamato Scientific, Japan) was used in all experiments.

Preparation of Plum Vinegar Extract of Cherry Blossom. The plum vinegar extract of cherry blossom was prepared according to the following process. Cherry blossom flowers were collected from *P. lannesiana* (Ooshimazakura in Japanese) found near Yusuhara, Kochi Prefecture, Japan. These flowers were then washed with water and immersed in salt water. The flowers were dehydrated and then immersed in Japanese plum vinegar. After immersion, they are separated from the plum vinegar and can then be used as Sakuracha. The leftover plum vinegar can be used as plum vinegar extract of cherry blossom. The plum vinegar extract of cherry blossom prepared according to the above process was kindly donated by Japan Agricultural Cooperatives Tsunoyama (Kochi, Japan).

Preparation of Phenolic Extract. The plum vinegar extract of cherry blossom was diluted with an equivalent volume of 5% formic acid. This solution (80 mL) was adsorbed by 100 mL of Amberlite XAD-7HP (Organo Co., Tokyo, Japan), cation-exchange resin, and washed with 200 mL of water and then with 200 mL of 5% formic acid. The adsorbed fraction was eluted with 200 mL of 5% formic acid in ethanol and then evaporated under vacuum. The residue was dissolved in 3 mL of methanol containing 1% hydrochloric acid. This solution was mixed with 30 mL of diethyl ether and then precipitated by centrifugation at 1200*g* for 5 min. The precipitate was evaporated and then used as phenolic extract for subsequent HPLC or liquid chromatography–mass spectrometry (LC-MS) analysis.

Superoxide Anion Scavenging Activity Analysis. The superoxide anion scavenging activity of the sample was measured according to the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-1) method and the electron spin resonance (ESR) method. The WST-1 method was performed with the SOD Assay Kit-WST according to Ukeda et al. (*21*). Twenty microliters of sample, 200 μL of WST working solution, and 20 μL of enzyme working solution were mixed. The mixture was incubated for 10 min at room temperature. WST-1 formazan formed in the reaction of superoxide anion with WST-1 was spectrophotometrically measured at 440 nm using a microplate reader (Immuno Mini NJ-2300, Nalje Nunc, NY). The IC_{50} value was determined as the concentration of sample that inhibited the formation of WST-1 formazan by 50%. The SOD equivalent activity of the sample was calculated by comparing the IC_{50} value of the sample with that of the SOD preparation.

The ESR method was performed as described by Noda et al. (*22*). Fifty microliters of 4 mM hypoxanthine, 30 μL of DMSO, 50 μL of sample, 20 μL of 4.5 M DMPO, and 50 μL of xanthine oxidase (0.1 unit/mL) were mixed in a 96-well microplate. The mixture was then placed in a special flat cell. DMPO−superoxide spin adduct was analyzed with an ESR spectrometer (FR 30 free radical monitor; JEOL, Tokyo, Japan) under the following conditions: magnetic field, 335.6 ± 5 mT; power, 4 mW; sweep time, 2 min; modulation, 100 kHz, 0.1 mT; amplitude, 320; and time constant, 0.1 s. The intensity of the ESR signal was corrected against Mn^{2+}, internal standard.

DPPH Radical Scavenging Activity Analysis. DPPH radical scavenging activity analysis was performed according to the procedure reported by Yamaguchi et al. (*23*). Sample solution (0.2 mL) was mixed with 0.1 M Tris-HCl buffer (pH 7.4, 0.8 mL) and then added to 1 mL of 0.125 mM DPPH in ethanol. This solution was incubated at room temperature for 20 min in the dark, and the absorbance at 517 nm by DPPH was measured. The IC_{50} value was determined as the concentration of sample that scavenged the DPPH radical by 50%. The Trolox equivalent activity of sample was calculated by comparing the IC_{50} value of the sample with that of Trolox.

HPLC and LC-MS Analysis. HPLC and LC-MS analysis of the phenolic extract was carried out according to the method of Feria et al. (*24*). HPLC conditions were as follows: column, Cosmosil 5C_{18}-MS-II (4.6 mm i.d. × 250 mm; Nacalai Tesque, Kyoto, Japan); detector, UV-2070 plus (JASCO Corp., Tokyo, Japan); wavelengths, 310 and 520 nm; solvent A, H_2O/HCOOH (9:1); solvent B, H_2O/CH_3CN/HCOOH (6:3:1); gradient program, solvent A, 80–15% for 70 min; and flow rate, 0.8 mL/min.

The LC-MS conditions were as follows: column, Cosmosil 5C_{18}-MS-II (4.6 mm i.d. × 250 mm; Nacalai Tesque); MS detector, Finnigan LCQ Duo (Thermo Fisher Scientific K.K., Kanagawa, Japan); ionization interface, ESI; capillary voltage, 10 V; capillary temperature, 190 °C; solvent A, H_2O/HCOOH (9:1); solvent B, H_2O/CH_3CN/HCOOH (6:3:1); gradient program, solvent A, 80–15% for 140 min; and flow rate, 0.2 mL/min.

Table 1. Antioxidant Activity of Plum Vinegar Extract of Cherry Blossom and Commercial Red Wines

sample	WST-1 method, SOD equivalent activity (units of SOD equiv/mL)	DPPH method, Trolox equivalent activity (mmol of Trolox equiv/L)
red wine 1	348	11.84
red wine 2	392	6.48
red wine 3	199	12.04
plum vinegar extract of cherry blossom	1212	39.65

RESULTS

Antioxidant Activity of Plum Vinegar Extract of Cherry Blossom. Superoxide anion scavenging activity of plum vinegar extract of cherry blossom was determined using the WST-1 method. The plum vinegar extract of cherry blossom showed dose-dependent superoxide anion scavenging activity. As shown in **Table 1**, the SOD equivalent activity of plum vinegar extract of cherry blossom was calculated to be 1212 units/mL, whereas those of commercial red wines were 199–348 units/mL. The plum vinegar extract of cherry blossom also showed dose-dependent DPPH radical scavenging activity, and the Trolox equivalent activity of the extract was calculated to be 39.65 mM (**Table 1**). This activity was higher than the activities of commercial red wines (6.48–12.04 mM).

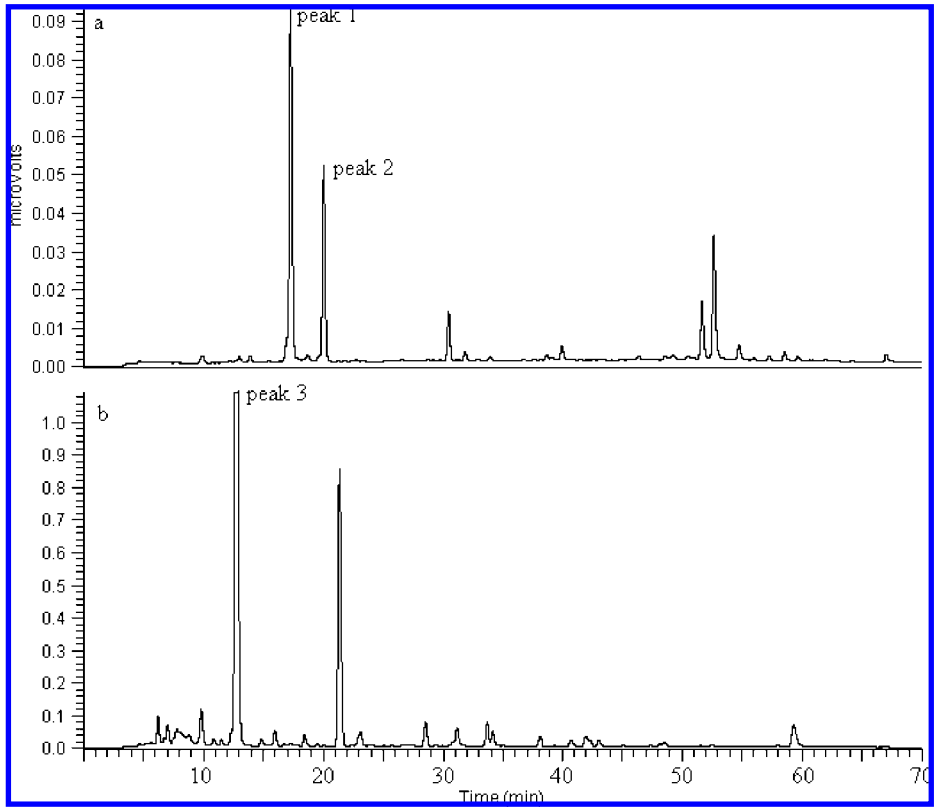

Figure 1. HPLC chromatogram of phenolic extract. Analysis was performed at 520 nm (**a**) and at 310 nm (**b**). HPLC analysis was carried out according to the conditions described under Materials and Methods.

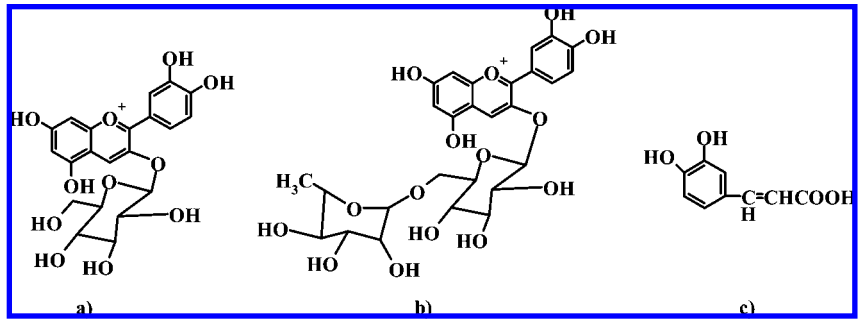

Figure 2. Antioxidant components in phenolic extract: (**a**) cyanidin-3-glucoside; (**b**) cyanidin-3-rutinoside; (**c**) caffeic acid.

Identification of the Major Components in the Phenolic Extract of Plum Vinegar Extract of Cherry Blossom. To obtain the antioxidant component from the plum vinegar extract of cherry blossom, the phenolic extract was prepared using Amberlite XAD-7HP polymeric resin. This procedure provided 1.4 mg of the phenolic extract from 1 mL of the plum vinegar extract of cherry blossom. The superoxide anion scavenging activity of the phenolic extract (0.1 mg/mL) was measured using the WST-1 and ESR methods. The phenolic extract scavenged the superoxide anion by 67.2% (ESR method) and 97.5% (WST-1 method).

It is known that anthocyanins, which have a catechol structure on the B ring of the aglycon, exhibit high antioxidant activity (*25*). Thus, it was presumed that the phenolic extract contained cyanidins or delphinidins as antioxidants. The analysis of anthocyanins was performed with HPLC. Two major peaks (17.28 and 20.05 min) were detected on the chromatogram at 520 nm (**Figure 1a**), and one main peak (12.89 min) was detected at 310 nm (**Figure 1b**). These major peaks were identified on the basis of their retention time, absorbance spectrum, MS fragment pattern, and cochromatography when compared with authentic standards.

The LC-MS analysis of peak 1 showed a positively charged molecular ion $[M + H]^+$ at *m*/*z* 449, and MS-MS analysis showed a fragment ion at *m*/*z* 287. Additionally, the authentic cyanidin-3-glucoside had the same retention time and absorbance spectrum (λ_{max}, 280 and 517 nm) as those at peak 1. Therefore, peak 1 was identified as cyanidin-3-glucoside (**Figure 2a**). Peak 2 had a $[M + H]^+$ ion at *m*/*z* 595 and MS-MS ions at *m*/*z* 449 and 287. The fragment ions at *m*/*z* 449 and 287 correspond to cyanidin-3-glucoside and cyanidin, respectively, which suggests the structure of cyanidin-3-rutinoside. Peak 2 was identified as cyanidin-3-rutinoside from the comparison of retention time and absorbance spectrum with the authentic compound (**Figure 2b**). Similarly, peak 3 was identified as caffeic acid because of the same molecular ion ($[M - H]^-$ at *m*/*z* 179), retention time, and absorbance spectrum of authentic caffeic acid (**Figure 2c**).

Table 2. Concentration of Major Components in Phenolic Extract

	concn (mg/g, dry wt of phenolic extract)
cyanidin-3-glucoside	5.0
cyanidin-3-rutinoside	2.7
caffeic acid	210.0

Table 3. Comparison of Authentic Caffeic Acid with Phenolic Extract on Superoxide Anion Scavenging Activity (ESR Method)

	inhibition (%)
phenolic extract	67.2 ±2.3
caffeic acid	79.5 ±1.8

Caffeic acid and phenolic extract were used at concentrations of 0.021 and 0.1 mg/mL in these analysis, respectively. Values are expressed as the means ± SD ($n = 3$).

Superoxide Anion Scavenging Activity of Antioxidant Components in the Phenolic Extract of Plum Vinegar Extract of Cherry Blossom. The contents and antioxidant activity of cyanidin-3-glucoside, cyanidin-3-rutinoside, and caffeic acid identified in phenolic extract were investigated. The amount of these components in the phenolic extract was calculated using peak area on the HPLC chromatogram. As shown in **Table 2**, cyanidin-3-glucoside, cyanidin-3-rutinoside, and caffeic acid were contained at levels of 5.0, 2.7, and 210 mg in 1.0 g of the phenolic extract. The antioxidant activity of the phenolic extract (0.1 mg/mL) was compared with those of authentic cyanidin-3-glucoside (0.5 μg/mL), cyanidin-3-rutinoside (0.27 μg/mL), and caffeic acid (0.021 mg/mL). As described above, the phenolic extract scavenged superoxide anion by 67.2% (ESR method) and 97.5% (WST-1 method), whereas cyanidin-3-glucoside and cyanidin-3-rutinoside scavenged the superoxide anion by only <1.0% with both methods. As shown in **Table 3**, caffeic acid scavenged the superoxide anion by 79.5% (ESR method) and 99.1% (WST-1 method), which were as much as those of the phenolic extract. These results indicated that caffeic acid was the main component, responsible for antioxidant activity in the phenolic extract. Caffeic acid was not detected in Japanese plum vinegar by HPLC analysis. This indicated that it was not the constituents of the plum vinegar but rather those of the cherry blossom.

DISCUSSION

The plum vinegar extract of cherry blossom showed dose-dependent superoxide anion and DPPH radical scavenging activity. Red wine is well-known as a functional beverage with antioxidant activity. The three commercial red wines used in our experiment exhibited radical scavenging activities of 6.48–12.04 mmol of Trolox equiv/L, and the activities were equal to those of popular red wines (*23, 26*). The plum vinegar extract of cherry blossom had a radical scavenging activity of 39.65 mmol of Trolox equiv/L, which was higher than the activities of red wine. In addition to the DPPH radical scavenging activity, the superoxide anion scavenging activity of the extract was also higher than the activities of red wines. We considered the plum vinegar extract of cherry blossom to be a useful and functional beverage, because it had a higher scavenging activity than did red wine for a hydrophilic superoxide anion and a lipophilic DPPH radical.

To elucidate the antioxidant in the plum vinegar extract of cherry blossom, the phenolic extract was prepared. Then, the antioxidant activity of the phenolic extract was compared with that of plum vinegar extract of cherry blossom. The dried phenolic extract was dissolved in water at the level of 0.1 mg/mL. Because 1.4 mg of phenolic extract was obtained from 1 mL of plum vinegar extract of cherry blossom, plum vinegar extract of cherry blossom was diluted 14-fold with water (corresponding to 0.1 mg/mL of phenolic extract). The 0.1 mg/mL of phenolic extract scavenged superoxide anion by 67.2% with the ESR method, and the 14-fold diluted plum vinegar extract of cherry blossom showed 90% inhibition. Therefore, it was considered that the main antioxidants in the plum vinegar extract of cherry blossom were contained in the phenolic extract. The antioxidant activity of the phenolic extract measured using the WST-1 method was higher than that with the ESR method. Moriyama et al. (*27*) showed that acylated anthocyanins with caffeic acid have the same tendency and suggested that this was due to the pH dependency of the sample because the reaction pH values in the WST-1 and ESR methods were 10.2 and 7.4, respectively.

The main components in the phenolic extract were isolated by reversed-phase HPLC. Cyanidin-3-glucoside and cyanidin-3-rutinoside were identified in the phenolic extract by LC-MS. These anthocyanins had high antioxidant activity, but they contributed little to the antioxidant activity of the phenolic extract because few cyanidins were contained in the phenolic extract.

Additional LC-MS and antioxidant analysis identified caffeic acid as the main antioxidant in the phenolic extract. Various studies have shown the antioxidant activity of phenolic acids such as caffeic acid derived from plant foods (*28, 29*). According to Mattila et al., caffeic acid dominated in plum and cherry (*30*). Our findings with the plum vinegar extract of cherry blossom agreed with findings in the literature. Jung et al. reported different antioxidants (quercetin and its glucoside) in the methanol extract of cherry blossom (*Prunus serrulata* var. *spontanea*) (*19*). Japanese plum vinegar used as extraction solvent in the preparation of plum vinegar extract of cherry blossom is the salted water containing some plum constituent. Therefore, it was thought that Japanese plum vinegar could not extract the lipophilic antioxidant quercetin. Caffeic acid is usually present as a chlorogenic acid, which is the ester of caffeic acid with quinic acid, and not as a free form. However, chlorogenic acid was not detected on the HPLC chromatogram of the phenolic extract, which indicated that caffeic acid in the extract is present as a free acid. The caffeic acid content calculated was 210 mg/g of the phenolic extract and 0.29 mg/mL of the plum vinegar extract of cherry blossom, which indicated that the plum vinegar extract of cherry blossom has a higher caffeic acid content than does prune juice (*29*), a beverage containing a high concentration of hydroxicinnamic acids such as caffeic acid.

After ingestion, food components are metabolized via various mechanisms. To understand the implication of the plum vinegar extract of cherry blossom in human health, it is important to determine its bioavailability in humans. The main antioxidant in the extract, caffeic acid, is known to have good bioavailability. For example, Takenaka et al. (*31*) showed that caffeic acid is stable in artificial digestive juice consisting of NaCl, pepsin, and HCl. Also, it has been reported that caffeic acid is present in the blood after oral administration in the rat or rabbit (*32, 33*). Olthof et al. (*34*) showed that almost all of the authentic caffeic acid was absorbed in the small intestine of humans. Mateos et al. (*35*)

concluded that caffeic acid could be taken up by the liver using the human hepatoma HepG2 cell as a hepatic model system. Caffeic acid that is taken up by the human body faces neutral pH in the intestinal tract, blood, and cells. At this pH, caffeic acid has shown strong antioxidant activity because the ionization of catecholic hydroxyl groups in caffeic acid has resulted in the formation of phenolate anion, which has a high antioxidant activity (*36*). Because caffeic acid can be taken up by the body without modification and exhibits antioxidant activity under physiological conditions, it is expected to scavenge superoxide anion and free radical in vivo.

Until now, the plum vinegar extract of cherry blossom has been used only as a natural source for coloring or flavoring food. However, our experiment indicates the superior antioxidant activity of plum vinegar extract of cherry blossom. As described above, superoxide anion is a precursor of hydroxyl radical with high reactivity against biological molecules. Consequently, it is thought that the superoxide anion scavenging activity of the plum vinegar extract of cherry blossom could provide important beneficial effects on health. Furthermore, the main antioxindant in the extract (caffeic acid) has good bioavailability in humans. Hence, it is expected that the plum vinegar extract of cherry blossom could be used as functional food product.

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