Journal of Medicinal Plants Research Vol. 4(12), pp. 1156-1160, 18 June, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR09.463
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Antioxidant activity of Chinese mei (*Prunus mume*) and its active phytochemicals

Daozong Xia[1, 2], Jiayi Shi[1], Jinyan Gong[1], Xiaoqin Wu[1], Qing Yang[2] and Ying Zhang[1]*

[1]Department of Food Science and Nutrition, College of Biosystem Engineering and Food Science, Zhejiang University, Hangzhou, 310029, China.
[2]College of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou 310053, China.

Accepted 3 June, 2010

Prunus mume **fruits have been used as a healthy food and traditional drug in China. The present study investigated the phenolic compounds and antioxidant activity of ethyl acetate extract from fruits of *P. mume* in China. Total phenol content was determined as gallic acid equivalents by the Folin-Ciocalteu method. The antioxidant activity was measured by DPPH and $ABTS^{+}$ radicals scavenging and lipid peroxidation inhibition activities. Three chlorogenic acid isomers, namely, chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid, were isolated and purified by preparative HPLC from the extract and identified by ultra-violet (UV), MS and NMR and the latter two compounds were identified in *P. mume* fruits for the first time. The contents of these isolated compounds were quantified by HPLC. Results showed that chlorogenic acid was of the highest level in these three isomers. The ethyl acetate extracts demonstrated activity to some degree in all the antioxidant assays. In all tested assays, all of the isolated chlorogenic acid isomers exhibited strong antioxidant activities, which were almost the same. The results showed that chlorogenic acid isomers are the key phenolic compounds which are responsible for antioxidant activity of the ethyl acetate extract from *P. mume* fruits in China.**

Key words: *Prunus mume*, fruits, antioxidant activity, chlorogenic acid isomers, free radicals scavenging.

INTRODUCTION

Chinese mei, *Prunus mume* Sieb. *et* Zucc. (Rosaceae) has been widely cultivated as an ornamental plant and its fruit is consumed as a garnish foodstuff and drink in China (Choi et al., 2002; Ng et al., 2005). In Chinese traditional medicine, various parts of this plant (example an immature fruit, leaf, stem bark, flower and seed) are used as herbal medicines for alleviating fever, cough and intestinal disorders (Matsuda et al., 2003). The fruit-juice concentrate of *P. mume* could markedly improve the fluidity of human blood (Chuda et al., 2009) and showed strong anti-tumor effects on human pancreatic cancer and dog fibrosarcoma (Adachi et al., 2007). Jo has reported the methanolic extract from the fruits of *P. mume* might significantly decrease the 2-thiobarbituric acid reactive substances (TBARS) value and hexanal content of chicken breast meat (Jo et al., 2006). The condensed juice of *P. mume* showed no toxic effect on normal human blood cells (Adachi et al., 2007).

It is well known that oxidative damage of biological molecules in human body is involved in obesity, aging, lipid metabolism disorders, diabetes, coronary heart disease and cancer. Recently, phenolic compounds have received growing attention, because they have been reported to have antioxidant, hypolipidemia, anti-mutagenic and anti-carcinogenic activities (Gursoy and Tepe, 2009; Shi et al., 2009). Concerning the phenolic compounds, chlorogenic acid was detected from the ethanolic extract of *P. mume* fruits in Japan (Ina et al., 2004), but there have been no studies on fruits of *P. mume* cultivated in China. The profile of phenolic compounds may vary with country of origin.

In a preliminary study, we found the ethyl acetate extract of *P. mume* fruits could improve lipid profile by lowering serum total cholesterol, triacylglycerol, low-density lipoprotein cholesterol concentrations. In order to supply

*Corresponding author. E-mail: zhang_ying61@vip.163.com.Tel (Fax): +86 571 86049803.

more scientific evidences for the research and development of the fruits of *P. mume* in China in the field of pharmaceutical and functional foods, this study mainly investigated the antioxidant activity of the ethyl acetate extract from fruits of *P. mume* in China and identified their phenolic compounds for the first time. Furthermore, the contents and the antioxidant activities of the identified phenolic compounds were also compared and evaluated.

MATERIALS AND METHODS

General

DPPH (2, 2-Diphenyl-1-picryl-hydrazyl), ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid), TBA (thiobarbituric acid reactive substances), Folin-Ciocalteu's phenol reagent, Ascorbic acid and chlorogenic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neochlorogenic acid and cryptochlorogenic acid were purchased from Chengdu Purification Technology Development Co. Ltd (Chengdu, China). ^{1}H NMR and ^{13}C NMR spectrometry was recorded on an Avance DMX-500 (500 MHz) NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). UV data were obtained from HPLC with DAD analysis (Waters, Milford, MA, USA). Mass spectral data were obtained with an APEX III Fourier-transform ion cyclotron resonance mass spectrometry (Bruker Daltonics Inc., Billerica, MA, USA). Ultrapure water made by the Milli-Q system (Millipore, Bedford, USA) was used during both preparative and analytical HPLC analysis. All solvents used for chromatography were of HPLC grade. All other chemicals were of analytical reagent grade.

Plant material and extraction preparation

The fruits of *P. mume* were obtained from a farm in the province of Zhejiang (China), between May and June, 2008 and identified by Prof. K. R. Chen, College of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou, China. Voucher samples were prepared and deposited at the herbarium of Zhejiang University. The fruits was air-dried, ground and extracted three times with ethyl acetate at 65°C for 3 h each, then extract were filtered through Whatman No. 1 filter paper, The solvent from extract was removed under vacuum and the residues were lyophilized (Detianyou FD-18, Beijing). The dry extract was stored in glass vials at -70°C until tested and analyzed.

Determination of total phenolic compounds concentration

The amount of phenolic compounds in *P. mume* extract was determined by the modified Folin-Ciocalteu method (Jimoh et al., 2007). Samples (0.5 mL) were mixed with 0.9 mL of tenfold diluted Folin-Ciocalteu reagent and 3.6 mL sodium carbonate solution (75 g/L). After standing for 30 min at room temperature, the absorbance was measured at 765 nm. The content of total phenolics was expressed as milligrams of gallic acid equivalents (GAE) per g of dry extract.

Isolation and purification of phenolic compounds

The extract was dissolved in methanol and filtered through a 0.45 µm membrane filter. This solution was fractionated by preparative reverse-phase HPLC. The chromatographic separation was performed on a Waters DeltaPrep 600 preparative chromatography system equipped with Waters Prep LC Controller and Waters 2996 Photodiode Array Detector (Waters, Milford, MA, USA). The preparative reverse-phase HPLC was performed on a Hedera® ODS-2 preparative column (10 µm, 300 × 30 mm I.D., Hanbon Sci. and Tech, Jiangsu, China). The mobile phases included glacial acetic acid/water (2:98, v/v) (solvent A) and methanol (solvent B). A gradient elution program was used for preparative separation as follows: 88:12(A/B) to 55:45(A/B) in 50 min, 0:100 (A/B) in a further 40 min. The flow rate was 10 mL/min while the monitored wavelength was 328 nm. The eluate was collected in three fractions. These fractions were further isolated separately by preparative reverse-phase HPLC [Luna C_{18} column (10 µm, 250 × 10 mm I.D., Torrance, CA, USA), acetonitrile: glacial acetic acid/water (2:98, v/v) = 15:85 (v/v), flow rate: 3 mL/min, the monitored wavelength: 328 nm] to yield three pure compounds from the 5 g extract as follows: compound 1 (21.3 mg), compound 2 (57.2 mg) and compound 3 (27.8 mg).

HPLC analysis

The *P. mume* extract and each purified compound from the preparative HPLC separation were analyzed by analytical HPLC, which was performed on a Waters 2695 HPLC chromatograph (Waters, Milford, MA, USA) with a Luna C_{18} column (5 µm, 250 × 4.6 mm I.D.) purchased from Phenomenex (Torrance, CA, USA). A gradient elution programme was used with the mobile phase, combining solvent A [glacial acetic acid/water (2:98, v/v)] and solvent B (acetonitrile) as follows: 100:0 to 90:10(A/B) in 10 min, 90:10 to 75:25 (A/B) in 20 min. The flow rate was 0.7 mL/min. The column temperature was maintained at 40°C. Signal was monitored at 328 nm with the diode array detector (DAD).

Standard solutions of compounds 1 - 3 were prepared at 1 mg/mL in methanol/water (50:50, v/v). They were diluted to make seven concentrations (5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100 µg/mL) calibration curves. The extract was dissolved in methanol/water (50:50, v/v) (1 mg/mL). The solutions were filtered through a 0.45 µm membrane filter and aliquots of the filtrate (15 µL) were injected. All of the above experiments were replicated three times. These tested compounds in the ethyl acetate extract were quantified from the calibration curve (Table 1).

Determination of DPPH radical scavenging activity

The capacity of *P. mume* extract and isolated phenolic compounds to remove DPPH• were determined by a slight modification of a previous described procedure (Shirwaikar et al., 2006). Briefly, 2 ml of various concentrations of samples and 2 ml of freshly prepared 0.2 mM DPPH solution were thoroughly mixed and kept in the dark for 30 min. An equal amount of methanol and DPPH served as control. The absorbance of the reaction mixture at 517 nm was measured with a spectrophotometer (PE lambda 17, USA).

Determination of ABTS radical scavenging activity

The radical scavenging activity of *P. mume* extract and isolated phenolic compounds for $ABTS^{\bullet+}$ were determined by a previous described procedure (Pari and Suresh, 2008). Shortly, $ABTS^{\bullet+}$ was generated by the interaction of 5.0 ml ABTS solution (1.8 mM) mixed with 1.25 ml Potassium persulfate (2.0 mM) and kept in dark at room temperature for 2 h then dilute five times with phosphate buffer pH 7.0 (0.02 mM). Then, 0.4 ml various concentrations of samples in methanol, mixed with 3.6 ml ABTS solution, and kept in the dark for

10 min. The absorbance was measured at 734 nm.

Table 1. Calibration curves[a] and LOD[b] for three standard compounds under the proposed HPLC method.

Name of the compounds	Slope, *a*	Intercept, *b*	R^2	LOD (µg/mL)
Neochlorogenic acid	66,701 (± 288)	-138,015 (± 26,248)	0.9994	0.21
Chlorogenic acid	60,900 (± 221)	37,052 (± 4350)	0.9996	0.16
Cryptochlorogenic acid	51,372 (± 123)	-27,128 (± 3176)	0.9999	0.29

[a]For each calibration curve, the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (µg/mL), a is the slope, b is the intercept and R^2 is the correlation coefficient. SD values are given in parenthesis. [b]LOD: the limits of detection correspond to concentrations giving a signal-to-noise ratio of 3.

Lipid peroxidation inhibition

Healthy Sprague-Dawley rat's brains were quickly removed and washed with ice-cold normal saline. The brain tissue was homogenized in 9 volumes of ice-cold 5 mM Potassium phosphate buffer (pH 7.4) using a glass homogenizer. The inhibition of lipid peroxidation activity was determined by the method reported previously (Pari and Suresh, 2008). The reaction mixture was composed of tissue homogenate 0.6 ml, phosphate buffer (50 mM, pH 7.4) 0.4 and 0.3 ml $FeSO_4$ (1 mM), 0.3 ml H_2O_2 (10 mM), and 0.3 ml of samples. The reaction mixtures were incubated at 37°C for 30 min and the reaction was terminated on ice by adding 1 ml of 15% (v/v) trichloroacetic acid to the mixture, which was then centrifuged at 3,000 rpm for 10 min. About 1.5 ml supernatant was incubated with 1 ml of 1% thiobarbituric acid at 100°C for 15 min. After a cooling period, TBARS generated were spectrophotometrically determined at 532 nm.

RESULTS AND DISCUSSION

Extraction

The extract yield was 27.3 ± 2.1 mg ethyl acetate extract/g dry weight of fruits of *P. mume*. Total phenolic content estimated as 316.4 ± 23.2 mg gallic acid equivalents/g dry weight of the extract.

Structure identification of isolated compounds

Three compounds were isolated and purified from the ethyl acetate extract by preparative reverse-phase HPLC as described previously. UV, MS, ^1H and ^{13}C NMR spectral data of the three isolated compounds agree well with the data reported (Nakatani et al., 2000; Bilia et al., 2001). From these data, Compounds 1, 2 and 3 were thus identified as three chlorogenic acid isomers, namely, neochlorogenic acid, chlorogenic acid and cryptochlorogenic acid. The structures of compounds 1 - 3 are shown in Figure 1.

HPLC assay for measuring phenolic compounds in *P. mume* extract

HPLC chromatogram is shown in Figure 2. The results of quantitative analyses showed the content of neochlorogenic acid, chlorogenic acid and cryptochlorogenic acid was 20.33 ± 0.12, 55.09 ± 0.26, 26.72 ± 0.15 mg/g (dry weight of the ethyl acetate extract of *P. mume* futits), respectively. According to the chromatogram and quantitative analyses, chlorogenic acid was identified as the principal phenolic compounds of the ethyl acetate extract from *P. mume* fruits, followed by cryptochlorogenic acid and neochlorogenic acid and the latter two compounds were identified in *P. mume* fruits for the first time. The content ratio of neochlorogenic acid to chlorogenic acid to cryptochlorogenic acid is 20:54:26.

DPPH radical scavenging activity

This assay provided information on the reactivity of the samples with a stable free radical. Because of the odd electron, DPPH shows a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. Ascorbic acid exhibited the strongest scavenging activity with IC_{50} value of 4.83 µg/mL (Table 2). Three isolated chlorogenic acid isomers, the major phenolic compounds of *P. mume* fruits, displayed almost equivalent scavenging activity on DPPH• radical ($P > 0.05$). The DPPH• scavenging ability of the ethyl acetate extract is lower than that of ascorbic acid and chlorogenic acid isomers, but it may be enough to remove the DPPH• (the inhibition percentage of DPPH• was 76.3% at a dosage of 250 µg/mL), which may answer for its medicine use.

ABTS radical scavenging activity

ABTS is also a relatively stable free radical. The $ABTS^{\bullet+}$ model can be assessed the scavenging activity for both the polar and non-polar samples and the spectral interference is lessened as the absorption maximum often used is a wavelength not normally encountered by natural products. Therefore, it was considered necessary to further assess the extract and isolated phenolic

compounds against the synthetic $ABTS^{\bullet+}$ (Shi et al., 2009).

Ascorbic acid, as a standard, exhibited the strongest scavenging activity of $ABTS^{\bullet+}$ with IC_{50} value of

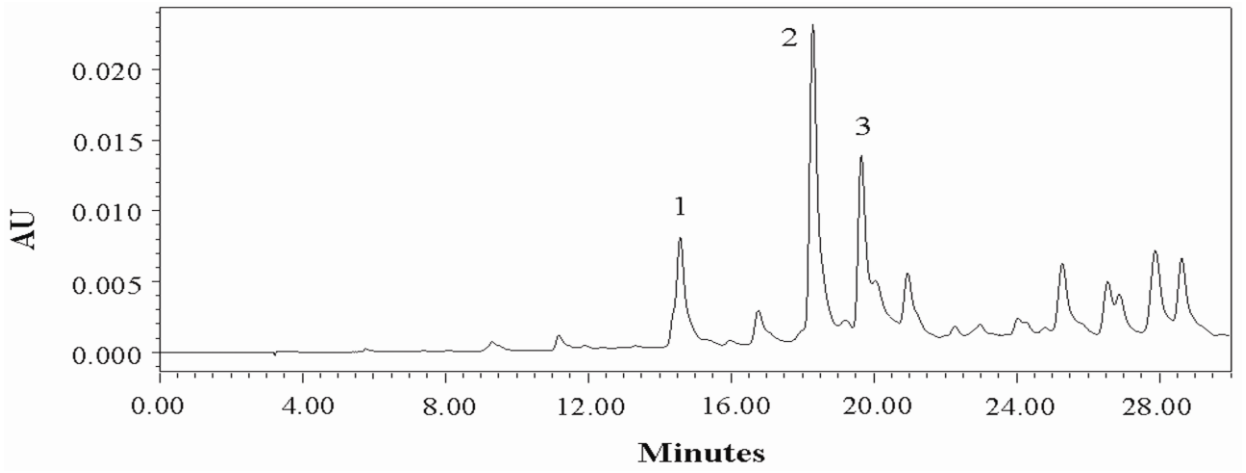

Figure 2. HPLC chromatogram of ethyl acetate extract of *P. mume* fruits. Neochlorogenic acid (1), chlorogenic acid (2) and cryptochlorogenic acid (3) were detected at 328 nm.

Table 2. Scavenging activities of ethyl acetate extract and isolated phenolic compounds on DPPH•, $ABTS^{\bullet+}$ and lipid peroxidation inhibition.

Samples	IC_{50} (μg/mL) for scavenging DPPH•	IC_{50} (μg/mL) for scavenging $ABTS^{\bullet+}$	IC_{50} (μg/mL) for lipid peroxidation inhibition
Ethyl acetate extract	30.04 ± 2.08^{a}	78.72 ± 2.96^{a}	89.23 ± 3.68^{a}
Neochlorogenic acid	8.97 ± 0.70^{b}	35.01 ± 1.94^{b}	49.65 ± 1.92^{b}
Chlorogenic acid	8.78 ± 0.68^{b}	33.37 ± 1.97^{b}	48.06 ± 1.68^{b}
Cryptochlorogenic acid	9.56 ± 0.74^{b}	36.53 ± 2.16^{b}	51.37 ± 1.83^{b}
Ascorbic acid	4.83 ± 0.38^{c}	17.86 ± 1.04^{c}	21.64 ± 0.70^{c}

The data are expressed as mean value ± SD (n = 3), values in the same column followed by different letters ard significantly different ($P < 0.05$).

17.86 μg/mL. The ethyl acetate extract and isolated phenolic compounds were also able to scavenge $ABTS^{\bullet+}$ radicals with a concentration dependent manner (Table 2). The ethyl acetate extract from *P. mume* fruits has a mild scavenging activity on $ABTS^{\bullet+}$ radical (IC_{50} = 78.72 μg/mL) and the inhibition percentage was 67.7% at a dosage of 250 μg/mL. In conclusion, the $ABTS^{\bullet+}$ scavenging capacity of samples exhibited the descending order: ascorbic acid > chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid > the ethyl acetate extract.

Inhibition of lipid peroxidation

The inhibitory effects of antioxidants on lipid peroxidation were due to the scavenging of free radicals (Mahakunakorn et al., 2004). For as much as the ethyl acetate extract and isolated phenolic compounds exhibited moderate free radicals scavenging activities, so we further studied their lipid peroxidation inhibition activity. As shown in Table 2, ascorbic acid exhibited the strongest inhibition of lipid peroxidation with IC_{50} value of 21.64 μg/mL. *P. mume* extract and isolated phenolic compounds also significantly inhibited the formation of TBARS in brain homogenates in a concentration-dependent manner. The inhibition percentage of *P. mume* extract was 66.4% at a dosage of 250 μg/mL. Based on the IC_{50} values, the hierarchy of lipid peroxidation inhibition activity was in agreement with that of DPPH and $ABTS^{+}$ radicals scavenging capacity. In brief, lipid peroxidation inhibition capacity of samples exhibited the descending order: ascorbic acid > chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid > the ethyl acetate extract. The ethyl acetate extract and isolated phenolic compounds were believed to intercept the free-radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable free radicals, which do not initiate or propagate further oxidation of lipids.

Conclusions

The ethyl acetate extract of *P. mume* fruits in China exhibited the antioxidant potent to some degree in *in vitro* assays and contains a high concentration of biologically

active chlorogenic acids, namely, chlorogenic acid, neochlorogenic acid and cryptochlorogenic acid. Squalene synthase plays an important role in the cholesterol biosynthetic pathway, chlorogenic acid could inhibit the squalene synthase of pig liver with an IC_{50} level of 100 nM (Choi et al., 2007), which partly explained the anti-obesity and hypolipidemia effects of the ethyl acetate extract of *P. mume* fruits in China. Accordingly, the ethyl acetate extract of *P. mume* fruits can be utilized as sources of these chlorogenic acids. Further studies are needed to investigate the pharmacological mechanisms of *P. mume* fruits extract in anti-obesity and improving lipid profile, which is of potential research and development value in the field of pharmaceutical and functional foods.

ACKNOWLEDGEMENTS

This study was supported by the Key Technologies R and D Program for Agriculture and Forestry, Hangzhou Science and Technology Bureau, China (20062413B27), the Zhejiang Natural Science Fund, Science and Technology Department of Zhejiang Province, China (Y207783), and the Key Research Fund of Zhejiang Chinese Medical University (Z08002).

REFERENCES

Adachi M, Suzuki Y, Mizuta T, Osawa T, Adachi T, Osaka K, Suzuki K, Shiojima K, Arai Y, Masuda K, Uchiyama M, Oyamada T, Clerici M (2007). The "*Prunus mume* Sieb. et Zucc" (ume) is a rich natural source of novel anti-cancer substance, Int. J. Food. Prop. 10: 375-384.

Bilia AR, Bergonzi MC, Mazzi G, Vincieri FF (2001). Analysis of plant complex matrices by use of nuclear magnetic resonance spectroscopy: St. John's wort extract, J. Agr. Food Chem. 49: 2115-2124.

Choi H, Eo H, Park K, Jin M, Park EJ, Kim SH, Park JE, Kim S (2007). A water-soluble extract from *Cucurbita moschata* shows anti-obesity effects by controlling lipid metabolism in a high fat diet-induced obesity mouse model. Biochem. Biophys. Res. Commun. 359: 419-425.

Choi SY, Chung MJ, Sung NJ (2002). Volatile *N*-nitrosamine inhibition after intake Korean green tea and Maesil (*Prunus mume* Sieb. *et* Zucc.) extract with an amine-rich diet in subjects ingesting nitrate. Food. Chem. Toxicol. 40: 949-957.

Chuda Y, Ono H, Ohnishi-Kameyama M, Matsumoto K, Nagata T, Kikuchi Y (1999). Mumefural, citric acid derivative improving blood fluidity from fruit-juice concentrate of Japanese apricot (*Prunus mume* Sieb. et Zucc), J. Agr. Food. Chem. 47: 828-831.

Gursoy N, Tepe B (2009). Determination of the antibacterial and antioxidative properties and total phenolics of two "endemic" Lamiaceae species from Turkey: *Ballota rotundifolia* L. and *Teucrium chamaedrys,* C. Koch. Plant. Food. Hum. Nutr. 64: 135-140.

Ina H, Yamada K, Matsumoto K, Miyazaki T (2004). Effects of benzyl glucoside and chlorogenic acid from *Prunus mume* on adrenocorticotropic hormone (acth) and catecholamine levels in plasma of experimental menopausal model rats. Biol. Pharm. Bull. 27: 136-137.

Jimoh FO, Sofidiya MO, Afolayan AJ (2007). Antioxidant properties of the methanol extracts from the leaves of Paullinia pinnata. J. Med. Food. 10: 707-711.

Jo SC, Nam KC, Min BR, Ahn DU, Cho SH, Park WP, Lee SC (2006). Antioxidant activity of *Prunus mume* extract in cooked chicken breast meat, Int. J. Food. Sci. Tech. 41: 15-19.

Mahakunakorn P, Tohda M, Murakami Y, Matsumoto K, Watanabe H (2004). Antioxidant and free radical-scavenging activity of Choto-san and its related constituents. Biol. Pharm. Bull. 27: 38-46.

Matsuda H, Morikawa T, Ishiwada T, Managi H, Kagawa M, Higashi Y (2003). Medicinal flowers. VIII. Radical scavenging constituents from the flowers of *Prunus mume*: structure of prunose III. Chem. Pharm. Bull. 54: 440-443.

Nakatani N, Kayano S, Kikuzaki H, Sumino K, Katagiri K, Mitani T (2000). Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). J. Agr. Food. Chem. 48: 5512-5516.

Ng CC, Lin CY, Tzeng WS, Chang CC, Shyu YT (2005). Establishment of an internal transcribed spacer (ITS) sequence-based differentiation identification procedure for mei (*Prunus mume*) and plum (*Prunus salicina*) and its use to detect adulteration in preserved fruits. Food Res. Int. 38: 95-101.

Pari L, Suresh A (2008). Effect of grape (*Vitis vinifera* L.) leaf extract on alcohol induced oxidative stress in rats. Food. Chem. Toxicol. 46: 1627-1634.

Shi JY, Gong JY, Liu JE, Wu XQ, Zhang Y (2009). Antioxidant capacity of extract from edible flowers of *Prunus mume* in China and its active components, LWT-Food Sci. Technol. 42: 477-482.

Shirwaikar A, Shirwaikar A, Rajendran K, Punitha ISR (2006). *In Vitro* Antioxidant Studies on the Benzyl Tetra Isoquinoline Alkaloid Berberine. Biol. Pharm. Bull. 29: 1906-1910.