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Full Length Research Paper

Determination of antioxidant and α-glucosidase inhibitory activities and luteolin contents of *Chrysanthemum morifolium* Ramat extracts

Jinfeng Yang[1#], Ju-Sung Kim[2#], Hyun Ju Jeong[1], Hak-Hee Kang[3], Jun-Cheol Cho[3], Hun-Myeong Yeom[3] and Myong Jo Kim[1]*

[1]Department of Applied Plant Sciences, Kangwon National University, 200-701, Chuncheon, Korea.
[2]Majors in Plant Resource Sciences and Environment, College of Applied Life Sciences, Jeju National University, 690-756, Jeju, Korea.
[3]Skin Research Institute, AmorePacific Corporation R&D Center, Yongin, Gyeounggi-do, 446-729, Korea.

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Antioxidant and α-glucosidase inhibitory activities as well as luteolin contents were analysed in extracts of Chrysanthemum morifolium Ramat (CM), which is a traditional medicinal plant and an important oriental herbal medicine for the treatment of eye diseases in China and Japan. In this study, the results show that the maximum solvent extract yield (35.5%) and luteolin concentration (1.34 µg/ml) were achieved in methanol extract. In addition, 60% methanol extracts of CM exhibited high scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as significant reducing power. They also possessed a potent α-glucosidase inhibitory activity. Furthermore, the total phenolic content was 0.37 mg GAE/g. The total phenolic content of 60% methanol extract may have been the cause of its strong antioxidant ability. These results provide scientific support that the food industry could benefit from using extract containing this constituent.

Key words: Chrysanthemum morifolium Ramat, antioxidant, 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power, phenolic, α-glucosidase.

INTRODUCTION

Antioxidants serve to prevent the adverse effects of oxygen and can help capture and neutralize free radicals, which possess the ability to damage the human body. Carlsen and co-workers (2010) hypothesized that antioxidants from foods may also be active on their own *in vivo* as well as capable of bringing about beneficial health effects through other mechanisms, including induction of mechanisms related to antioxidant defense. In particular, some studies have shown that most bioactive food constituents are derived from plants. There are a large group of polyphenolic and phytochemical compounds found in fruits, vegetables, grains, bark,roots, stems, flowers and tea. Phytochemicals play important roles as antioxidants (Nijveldt et al., 2001), inflammatory mediators (Formica and Regelson, 1995) and antivirals (Wang et al., 1998). Most of the beneficial health effects of phytochemicals are attributed to their capacity to prevent injury caused by free radicals.

Interest in glucosidase inhibitors has grown due to their possible role to manage diabetes mellitus (DM). DM is an islet dysfunction syndrome characterized by insulin resistance-induced defects in the metabolism of sugars, protein, fat and electrolytes. DM affects approximately 4% of the population worldwide and is expected to increase to 5.4% in 2025 (Si et al., 2010). The rapid spread of DM can be attributed to people living in a modern industrial society who lead a sedentary lifestyle and consume large amounts of calories. Type 1 diabetes mellitus (T1DM) is

*Corresponding author. E-mail: kimmjo@kangwon.ac.kr. Tel: +82 33 250 6413. Fax: +82 33 253 6413.

#These authors contributed equally to this work.

due to injured human β-cell islets that can no longer normally secrete insulin. On the other hand, type 2diabetes mellitus (T2DM) is a metabolic disease that affects genetically susceptible individuals due to environmental factors, such as increased availability of food and decreased opportunity and motivation for physical activity (Park et al., 2005). T2DM is recognized as one of the most common metabolic diseases, and the number of patients is estimated to increase to about 300 million by 2025. Currently, treatment of type 2 diabetes involves improving patients' blood sugar levels as well as insulin secretion stimulated by drugs such as sulphonylureas, metformin, thiazolidinediones and α-glucosidase inhibitors (Fang et al., 2008). Glucosidases play an important role in the absorption of food. Specifically, α-glucosidase is located at the epithelium of the small intestine where it serves to reduce the absorption of glucose, thus inhibiting postprandial hyperglycemia. α-Glucosidase is the earliest metabolic abnormality that occurs in T2DM (Yang et al., 2010). Several α-glucosidase inhibitors such as acarbose, voglibose and miglitol (Scott and Spencer, 2000) are clinically used. Thus, natural products of great structural diversity are still good sources of such inhibitors.

Extraction is the first important step to recover and purify ingredients from plant materials. Therefore, the techniques and methods used for solvent extraction of plant materials are important. Wu and co-workers (2001) indicate that ultrasonic extraction of organic compounds from various plant materials can significantly improve the aid of intense ultrasound, thus achieving higher product yields with reduced processing time and solvent consumption. Additionally, the extraction method must enable the complete extraction of the compounds of interest while avoiding their chemical modification (Zuo et al., 2002). Goli et al. (2005) performed extraction of phenolic compounds from different matrices using solvents with different polarities, including methanol, water, ethyl acetate and petroleum ether. Therefore, improving the efficacy of the solvent extraction system is important. Here, different concentrations of various solvents were used in the ultrasonic extraction of *Chrysanthemum morifolium* Ramat (CM).

Dried flowers of CM is an important oriental herbal drug for the treatment of eye diseases or selected to process drinks in China, Korea and Japan (Zhou and Yang, 2009). In addition, dried CM flowers possess antioxidant activity (Kim and Lee, 2005), anti-tumor efficacy (Miyazawa and Hisama, 2003) and anti-inflammatory activities (Ukiya et al., 2001). These beneficial effects are believed to be due, at least in part, to the action of CM flower compounds. It was reported that the main active ingredients of CM flowers are flavonoids, volatile compounds, and chlo-rogenic acid (Wang et al., 2001). However, no studies have been conducted to investigate the α-glucosidase inhibitory activity of CM. Thus, the objectives of this research were to determine the α-glucosidase inhibitor activity, reducing power, scavenging ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and luteolin content of CM b-tography.

MATERIALS AND METHODS

DPPH, Folin-Ciocalteau reagent, gallic acid, butylated hydroxyannisole (BHA), 2,6-di-*tert*-butyl-4-methylphenol (BHT), 4-nitrophenyl-α-$_D$-glucopyranoside (*p*NPG) and α-glucosidase were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, ethanol, acetone and ethyl acetate were obtained from Dae Jung Chemicals & Metals Co.

Extract preparation

Dried chrysanthemum flowers were extracted in different solvents, including 60% methanol (60 M), 80% methanol (80 M), 100% methanol (100 M), 60% ethanol (60 E), 80% ethanol (80E), 100% ethanol (100 E), 100% acetone (A), aqueous (W) and 100% ethyl acetate (E). The filtered extracts were mixed and concentrated using a rotary evaporator. The extract was then stored under refrigeration for further analysis.

Determination of total phenolic content

Total phenolic content was measured following the method of Folin-Ciocalteu (Singleton and Rossi, 1965). Briefly, 0.1 ml each of the samples at different concentrations was mixed with 0.05 ml of Folin-Ciocalteau reagent and then mixed thoroughly. 3 min later, 0.3 ml of 20% sodium carbonate was added, followed by mixing with intermittent shaking. The reaction mixture was then incubated for 15 min at room temperature. Finally, 1 ml of distilled water was added and absorbance was measured at 725 nm using a spectrophotometer (V530, Jasco Co., Japan). Measurements were performed in triplicate, and the data were expressed as gallic acid equivalent (GAE) per mg of extract, based on the calibration curve of gallic acid.

DPPH radical scavenging activity

The DPPH radical scavenging activities of the extracts were determined by the method of Blois (1958) with some modifications. Initially, 4 ml of methanol solution containing 0.1 ml each of the samples at different concentrations (0.1, 1 and 10 mg/ml) was mixed with 1 ml of 0.15 mM DPPH (dissolved in methanol). The reaction mixture was then incubated for 30 min at room temperature. The control contained all reagents without the sample, whereas methanol was used as a blank. All measurements were performed in triplicate. DPPH radical scavenging activity was determined by measuring the absorbance at 517 nm and expressed as the inhibition percentage of free radicals by the sample after calculation using the following formula: Inhibition (%) = (absorbance of sample/1/2 absorbance of control) × 100.

Reducing power assay

Reducing power was measured according to the method reported by Oyaizu (1986) with some modifications. An aliquot of each extract (0.1 ml) was mixed with 0.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide, followed by incubation at 50°C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) was added, 0.5 ml aliquot of the upper layer was mixed

with distilled water (0.5 ml) and ferric chloride (0.1 ml, 0.1%), after which the absorbance was measured at 700 nm against a blank containing all of the reagents without the tested sample. BHT and BHA were used for comparison purposes.

Measurement of α-glucosidase activities

α-Glucosidase inhibitory activities were assayed as described (Oki et al., 1999) with some modifications. The reaction was initiated with 0.05 ml each of the samples at different concentrations in 0.2 mM phosphate buffer (pH 6.8), followed by incubation at 37°C for 15 min, after which 0.05 ml of enzyme solution was immediately added to the mixture before mixing and incubated at 37°C. Then, 3 mM *p*NPG (0.1 ml) was added, after which the reaction was stopped by the addition of 0.75 ml of 0.1 M Na_2CO_3. α-Glucosidase inhibitory activity was determined by measuring the release of *p*NPG at 405 nm.

The control (acarbose) contained all reagents without the tested sample. The reactions were conducted in triplicate. The α-glucosidase inhibitory activity was calculated as follows: Inhibitory ratio % = [1- (As- Ab)/ Ac] × 100, where Ac, As and Ab represent the absorbance levels of the control, sample and blank, respectively. The concentration of α-glucosidase inhibitor required to inhibit 50% of α-glucosidase activity under the assay conditions is defined as the IC_{50} value.

Apparatus and chromatographic conditions

The standard samples and CM extracts were separately dissolved in methanol and filtered (0.45 µm) for HPLC analysis. For HPLC, a Younglin liquid chromatograph (Hogye-dong, Anyang, Korea), which consisted of a pump, a column chamber, a multi-wavelength detector and a Midas for LC system, was used. Chromatographic separation was carried out at room temperature using a j'sphere ODS H80 analytical column (250 × 4.6 mm, 5 µl). The mobile phase consisted of methanol (A), 0.2% phosphatic acid in water (B) and acetonitrile (C); A:B:C was: 0 min, 20:80:0; 0.1 min, 20:50:30; 40 min, 100:0:0. The flow rate was 0.8 ml/min. The detector wavelength was set at 350 nm.

Statistical analysis

Data was subjected to the analysis of variance (ANOVA) and means were compared by Duncan's multiple range tests at $P < 0.05$ which were used to determine the significance of means.

RESULTS AND DISCUSSION

Extract yields with different extraction solvents

Extraction is the first important step for the recovery and purification of active ingredients from plant materials. The yields of CM extraction using different solvents indicated that 100 M extract yield was the highest (35.5%) (Table 1), possibly because the polarities of the extracts were similar to methanol. Numata et al. (1991) showed that extracts of different solvents have very different chemical properties. We can determine the polarity and other properties of the active ingredient by solvent experiments in order to provide a theoretical basis for further purification of active compounds (Harada and Kamei, 1997). According to Ibtissem et al. (2010), antioxidant activity depends on the method of extraction and solvent used. Hence, solvents used for extraction have dramatic effects on future experiments.

Total phenolic content

The total phenolic contents of the CM extracts using different solvents expressed as mg of gallic acid equivalents (GAE) per g of extract and E extract had the lowest phenolic concentration (0.25 mg GAE/g of extract) (Table 1). As expected, the 60 M extract showed higher phenolic content than the other extracts. The amount of total phenolic compounds in the 60 M extract was 0.37 mg GAE/g. The higher content of total phenolics in the 60 M extract might have accounted for the higher reducing power and DPPH radical scavenging activity. Duh et al. (1999) indicate that phenolic compounds may contribute directly to antioxidative action. Robards et al. (1999) also reported that more widespread and diverse phenolics, which possess multiple hydroxyl groups, have higher antioxidant activities against peroxyl radicals. Phenolic compounds from plants are known to be effective natural antioxidants (Shukla et al., 2009).

DPPH radical scavenging activity

In this study, the scavenging abilities of various extracts against DPPH radicals were measured, and the results are normalized and expressed as IC_{50} values (µg/ml) for comparison purposes (Table 1). The 60 M extract was found to have the highest DPPH radical scavenging activity. On the other hand, the lowest activity was found in E extract. Overall, the DPPH radical scavenging activities of all the extracts were superior to that of the positive control (BHT). The earlier findings are well correlated with the amount of phenolic constituents present in the respective extracts. Thus, 60 M extract had strong DPPH radical scavenging activity. These conclusions are in agreement with an earlier report by Li et al. (2007), who found that leaf lettuce possesses the highest total phenolic content and antioxidant activity. However, more detailed studies on the chemical compositions of those extracts, as well as studies using other models such as lipid peroxidation and *in vivo* assays, are essential to characterize them as biological antioxidants.

Reducing power assay

All extracts were measured spectrophotometrically by reading the absorbance at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. As shown in Figure 1, the extracts were tested at concentrations of 0.5, 1 and 2 mg/ml, and all samples showed

Table 1. Extract yield, total phenolic content and DPPH free radical scavenging activity of *C. morifolium* Ramat.

Solvent	Extract yield (%)	Total phenolic (mg GAE/g)	DPPHIC$_{50}$ (µg/ml)
Aqueous (W)	31.5^{c}	0.31^{e}	28.3^{d}
100% ethyl acetate (E)	13.4^{g}	0.25^{g}	30.5^{a}
100% acetone (A)	5.8^{h}	0.29^{h}	28.6^{c}
60% ethanol in water (60 E)	28.4^{e}	0.34^{c}	28.3^{e}
80% ethanol in water (80 E)	28.3^{e}	0.33^{d}	28.1^{f}
100% ethanol (100 E)	22.6^{f}	0.30^{f}	29.2^{b}
60% methanol in water (60 M)	30.1^{d}	0.37^{a}	25.9^{h}
80% methanol in water (80 M)	35.1^{b}	0.36^{b}	26.7^{g}
100% methanol (100 M)	35.5^{a}	0.36^{b}	28.4^{cd}
BHA			4
BHT			86
α-Tocopherol			1

Values with the same superscript are not significantly different by Duncan's multiple range test at $p<0.05$.

some degree of reducing power. However, the reducing power of the extracts increased with increasing concentration. In addition, 60 M extract had slightly higher absorbance values as compared to the other extracts. E extract appeared to be less effective with regards to reducing power. This result is in good correlation with our total phenolic content results, in which we demonstrated that 60 M extract possessed the highest phenolic content. Shimada et al. (1992) reported that antioxidant activity is concomitant with the development of reductones. Therefore, the significant antioxidant activity of 60 M extract from CM may be related to its reducing power. These results are in agreement with the results of Odabasoglu (2005), who found that methanol extract of *Peltigera rufescens* had very high reducing power.

α-Glucosidase activities

α-Glucosidase inhibitory activity was measured by calculating the IC_{50} values, and the results are summarized in Table 2. All extracts seemed to have potent inhibitory activity towards α-glucosidase. Among these, 60 M extract (IC_{50} values of 22.0 µg/ml) showed significantly higher inhibitory activity as compared to other extracts. The A extract had the lowest IC_{50} value (30.5 µg/ml). The results suggest that α-glucosidase inhibitory activities of the different extracts varied possibly due to the various phenolic contents of the extracts. Polyphenol-rich extracts of soft fruits (blueberry and blackcurrant), potato (*Ipomoea batatas* L.) roots and Morning glory (*Pharbitis nil* cv. Scarlett O'Hara) have been tested for their ability to inhibit α-glucosidase (McDougall et al., 2005). Further, polyphenols from Ecklonia inhibit glucosidase and showed promising anti-diabetic effects in mouse models (Iwai, 2008). It is widely accepted that dietary supplements can contribute significantly to improve health. In particular, plants with α-glucosidase inhibitory activity become more important as food materials in the treatment of postprandial hyperglycemia. According to the analyses earlier, we proposedthat CM may be a useful food material for treatment of type 2 diabetes patients.

HPLC analysis

Luteolin (3,4,5,7-tetrahydroxyflavone) is an important flavonoid and is widely distributed and present in many plant families, such as celery, sage, carrot and broccoli (Karrasch et al., 2007). Luteolin has the ability to inhibit lipopolysaccharide (LPS), induce TNF-α and IL-6, and prevent carcinogenesis in animal models (Kim et al., 2011). Furthermore, luteolin exhibits multiple biological activities such as antioxidant activities (Liu et al., 2009), and anabolic effects (Kim et al., 2011). To obtain a calibration curve, HPLC analysis of the calibration solutions ranged from 0.1 to 50 µg/ml. The retention time of luteolin was 27.616 min. Triplicate analyses for each solution were performed. According to the calibration curve, the luteolin concentrations of CM extracts using different solvents were calculated, and the analytical results are listed in Table 3. The data show that the luteolin concentrations changed when the CM extracts were changed. Specifically, the luteolin concentration of 80 M extract was the highest (1.34 µg/ml), followed by 60 M extract (1.19 µg/ml). W extract was the lowest of all the extracts with a luteolin concentration of only 0.38 µg/ml. Based on the earlier results, we can conclude that the extraction amount of luteolin reached its maximum using 80 M extract solvent.

In conclusion, methanol is the most effective solvent to extract the maximum luteolin concentration (1.34 µg/ml). In addition, we demonstrate that the 60 M extract of CMcontained the highest total phenolic content. The

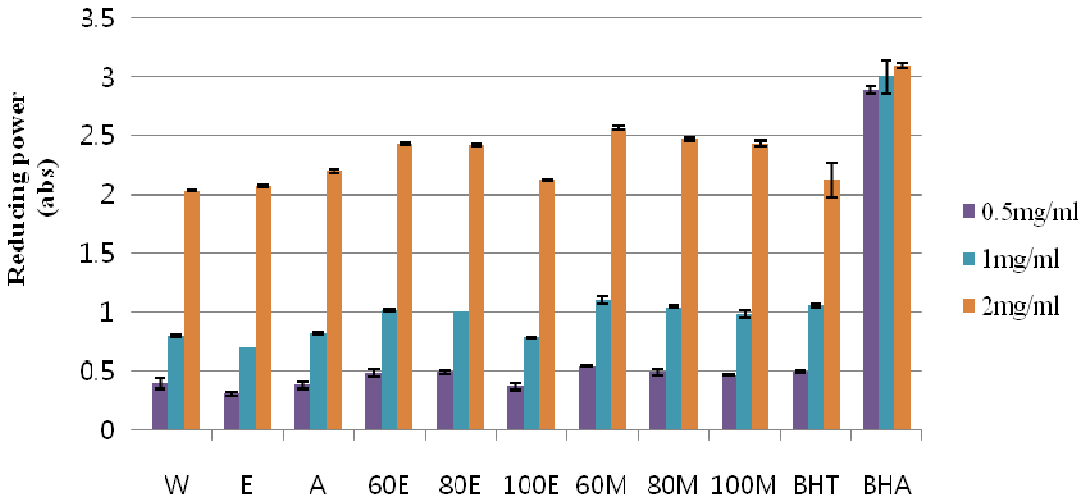

Figure 1. Reducing power activities of extracts from *C. morifolium* Ramat. CM was extracted using nine different solvents; 60% methanol in water (60 M), 80% methanol in water (80 M), 100% methanol (100 M), 60% ethanol in water (60 E), 80% ethanol in water (80 E), 100% ethanol (100 E), 100% acetone (A), aqueous (W) and 100% ethyl acetate (E).

Table 2. α-Glucosidase inhibitory activity of extract from *C. morifolium* Ramat.

Extract solvent	IC_{50} (µg/ml)
Aqueous (W)	25.6[d]
100% Ethyl acetate (E)	24.3[e]
100% Acetone (A)	30.5[a]
60% Ethanol in water (60 E)	25.6[e]
80% Ethanol in water (80 E)	22.8[f]
100% Ethanol (100 E)	27.8[c]
60% Methanol in water (60 M)	22.0[f]
80% Methanol in water (80 M)	28.3[b]
100% Methanol (100 M)	27.9[c]
Acarbose	3

Values with the same superscript are not significantly different by Duncan's multiple range test at $p<0.05$.

Table 3. Luteolin concentration in *C. morifolium* Ramat from different solvents.

Extract	Luteolin (µg/ml)
Aqueous (W)	0.38[f]
100% ethyl acetate (E)	0.87[d]
100% acetone (A)	0.91[d]
60% ethanol in water (60E)	1.12[b]
80% ethanol in water (80E)	1.00[c]
100% ethanol (100E)	0.47[e]
60% methanol in water (60M)	1.19[b]
80% methanol in water (80M)	1.34[a]
100% methanol (100M)	1.15[b]

Values with the same superscript are not significantly different by Duncan's multiple range test at $p<0.05$.

data also suggest that CM possess potent antioxidant activity. Especially, the DPPH radical scavenging activity and reducing power of CM were strong as compared to those of the control (BHA). In this study, a significant relationship was found between antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. Further studies should be carried out on extracts of CM, which should show that it has potent inhibitory activities towards α-glucosidase. So, our work provides the necessary information to exploit CM as a health food.

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