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Investigation of Antioxidative Activity from Apple (*Malus pumila* Miller) Peel by Various Extraction Solvents

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Abstract : Consumption of fruits and vegetables has been conducted to be effective in the prevention of chronic diseases. In this study, 70% methanol, 70% ethanol and chloroform-methanol mixture (CM, 2:1, v/v) were used as solvents in the extraction of apple peels. The total phenol content, total flavonoid content and antioxidant activity of various extracts were investigated using *in vitro* assays. The extract obtained by 70% methanol showed the highest total phenol content (20.87±0.17 mg CAE/g), ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical scavenging activity and ferric reducing antioxidant power. However, 70% ethanol extract possessed the strongest antioxidant activity assayed by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity and β-carotene bleaching method. And CM extract was found to show the highest total flavonoid content with the value of 9.26±0.06 mg QE/g. These results indicated that apple peels can be used in dietary applications with a potential to reduce oxidative stress.

Keywords : apple (Malus pumila Miller) peels, antioxidative activity, total phenol, flavonoid

1. Introduction

An increasingly important health problem in the world is the rising incidence of some diseases such as age-related neurodegenerative diseases, cardiovascular disease and cancer. The overproduction of oxidative radicals such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) results in oxidative stress, which is considered to contribute to these mentioned diseases [1,2]. Fruit and vegetables contain not only essential nutrients needed for daily life but also a wide variety of bioactive compounds (antioxidant phytochemicals) for health promotion and disease prevention [3,4]. There are several previous studies have conducted that people who have diets rich in natural water-soluble antioxidants such as fresh fruits and vegetables can improve the antioxidant activity and reduce the risk of chronic diseases [5]. Currently, a very promising way to overcome oxidative degradation is to increase the

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consumption of diets rich in fruit or vegetable.

Phenolic compounds are found to be abundant in apples, which has been investigated and ranges from 110 to 357 mg/100 g of fresh apple [6]. Previous study have found that apple with skin possessed higher total phenolic, total flavonoid and antioxidant activity as well as inhibited more growth of human cancer cells *in vitro* compared with apple without skin [7]. Based on the reports of Boyer and Liu [8], apples were most consistently associated with reduced risk of cancer, heart disease, asthma, and type II diabetes as well as increased lung function and increased weight loss. Apples also significantly lowered lipid oxidation and cholesterol levels in humans. These effects, which may be attributed to both the phenolics and the dietary fiber found in apples, may partially explain the inverse association of apple intake and risk of cardiovascular disease [8].

In this study, we investigated the total phenol contents, total flavonoid contents and antioxidant activity of 70% methanol, 70% ethanol and chloroform-methanol (CM, 2:1, v/v) extracts from apple (*Malus pumila* Miller) peels. The employed *in vitro* antioxidant activity assays were consisted of DPPH radical, ABTS radical scavenging activity, ferric reducing antioxidant power and β-carotene bleaching assays.

2. Materials and Methods

2.1. Materials

Apples (*Malus pumila* Miller) were purchased from Uiseong (Gyeongbuk, Korea). Apples were washed and peeled. Then peels were dried by a method of freeze drying with a freeze-dryer (EYELA, FDU-2000, Rikakikai Co., Tokyo, Japan) and ground with a blender (HMF-3250S, Hanil Electric Co., Seoul, Korea). The apple peel flours were stored at -80℃ (SW-UF-400, Sam-Won Co., Busan, Korea) for further analyses.

2.2. Preparation of apple peel extracts

Apple peel flours were mixed with extraction solvents including 70% methanol, 70% ethanol and chloroform-methanol mixture (CM, 2:1, v/v) in a ratio of 1:10 and kept in the dark about 24 h, and then used the Advantec No. 1 filter paper (Tokyo, Japan) to filter. The process of extraction was repeated 3 times. The filtrate was evaporated by rotary vacuum evaporator (EYELA, N-N series, Tokyo, Japan) until the solvents were completely removed. The extracts were collected and sealed in brown reagent bottles and frozen at -80℃ until required for further analyses.

2.3. Measurement of total phenol contents (TPC)

The total phenol contents were determined by using Folin-Denis' phenol reagent and caffeic acid was used as a standard for the calibration curve [6,9]. In brief, samples (0.5 mL) were mixed with 3.0 mL of distilled water and 0.5 mL of Folin-Denis' phenol reagent in the test tubes. After incubated for 3 min, 0.5 mL of 10% sodium carbonate (w/v) was added. Then the mixture was incubated in the room temperature for 60 min. The absorbance of the reaction mixture was measured at 700 nm using uv/vis-spectrophotometer (Specord 200, Analytikjena, Jena, Germany). Total phenol contents were expressed as mg of caffeic acid equivalents per g of extracts (mg CAE/ g extract).

2.4. Measurement of total flavonoids content (TFC)

Total flavonoid content was determined by aluminum chloride colorimetric method [10] with some modifications. Samples (0.5 ml) were mixed with 0.5 ml of 10% aluminum nitrate enneahydrate, 0.5 ml of 1 M sodium acetate and 2.0 ml of 80% ethanol (v/v). After this step, reaction mixture was incubated at

room temperature for 40 min, and then the absorbance was read at 415 nm. Quercetin was used as a standard. Total flavonoids contents were expressed as mg of quercetin equivalents per g of extracts (mg QE/g extract).

2.5. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

DPPH radical scavenging activity was measured according to the method of Blois and Duan et al [11,12]. Samples (2.0 mL) were mixed with 0.2 mM DPPH (2.0 mL) and then vigorously shaken. The mixture solution was stood in the dark for 30 min at 37℃ water bath. Ascorbic acid was used as positive control. Then the absorbance of the reaction mixture was read with spectrophotometer at 517 nm. The percentage inhibition of DPPH radical scavenging activity was calculated based on the control reading using the following calculation:

DPPH radical scavenging activity (%)

where A_s is the absorbance in the presence of sample or BHA, and A_c is the absorbance of control reaction.

2.6. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity assay

ABTS radical scavenging activity was evaluated according to the method of Sun et al [13]. The mixture of 15 mL of 7 mM ABTS and 15 mL of 2.45 mM potassium persulfate was stored at room temperature in the dark for 16 h to get the green-blue free radical $ABTS^{\cdot +}$. Then the solution was diluted with ethanol until the absorbance was 0.7±0.02 at 734 nm. Samples (0.4 mL) were mixed with 2.6 mL of ABTS working solution. After 6 min of reaction, the absorbance was taken at 734 nm. Ascorbic acid was used as positive control. The percentage of ABTS radical scavenging effect was calculated as follow:

ABTS radical scavenging effect (%)

where A_s is the absorbance in the presence of sample or ascorbic acid, and A_c is the absorbance of control reaction.

2.7. β-carotene bleaching assay

The antioxidant activity of different extract was evaluated according to the β-carotene bleaching method following the method of Elzaawely et al [14]. In brief, a solution of β-carotene was prepared by dissolving 1 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then added to a round-bottomed flask containing a mixture of 20 mg linoleic acid and 200 mg Tween 40. After the chloroform was removed under vacuum using a rotary evaporator at 40℃, 100 mL of aerated distilled water were added to the flask with vigorous shaking. The emulsion obtained was freshly prepared before experiment. An aliquot (3.0 mL) of the β-carotene-linoleic acid emulsion was mixed with 1.0 mL of sample extracts, positive control standards (ascorbic acid). Then the mixture was incubated at 50℃ for 120 min. Absorbance readings were performed immediately (t=0 min) and after 120 min of incubation at 470 nm with. Antioxidant activity (AOA) was calculated using the following formula:

A_0 and A'_0 are the initial absorbance of sample and control, whereas A_{120} and A'_{120} are the absorbance of sample and control after 120 min.

2.8. Ferric reducing antioxidant power (FRAP) assay

The working FRAP reagent was prepared by mixing 10 mL of 0.3 M sodium acetate buffer (pH 3.6), 1 mL of 10 mM TPTZ in 40 mM hydrochloride acid and 1 mL of 20 mM ferric chloride. The freshly prepared FRAP reagent (3.0 mL) was mixed with 0.5 mL of sample solution. After incubated at 37℃ water bath for 30 min, the absorbance was read at 593

nm. Ascorbic acid was used as the positive control. The FRAP values were expressed as the absorbance of samples [15].

2.9. Statistical analysis

The experimental data in triplicate were subjected to analysis of variance (ANOVA) and expressed as mean±SD (n=3). ANOVA was performed by using the one-way analysis of variance procedures. Duncan's multiple-range test was used to analysis the significant difference of means, and $p<0.05$ was considered to be statistically significant for all statistic procedures. IBM SPSS statistic 21 program was used for data analysis.

3. Results and Discussion

3.1. Yields

The various extraction yields of apple peels by 70% methanol, 70% ethanol and chloroform-methanol (CM, 2:1, v/v) were shown in Table 1. The extraction yield by 70% methanol and 70% ethanol was found to be high with the value of 58.62% and 60.57%. However, CM was found to be the lowest (21.51%).

3.2. Total phenol contents (TPC)

Apple peels were conducted to possess higher contents of phenolic compounds compared to flesh and whole fruit [16]. The scavenging ability of phenols is mainly due to the phenolic structure of hydroxyl substituent on the aromatic ring [17]. Furthermore, a positively and highly significant relationship between total phenolics and antioxidant activity was documented by Velioglu et al [18], which implied a compound with higher content of phenol possessed higher antioxidant activity. Total phenol contents were determined by according to the colorimetric Folin-Denis' method [6,9] with caffeic acid as a stand compound ($y=0.1141x+0.0076$, $R^2=0.9941$). The total phenol contents of apple peels by different extraction solvents (70% methanol, 70% ethanol and CM) were showed in Table 1. Total phenol contents of various extracts decreased in the order: 70% methanol extract

Table 1. Extraction yields, total phenol contents, total flavonoid contents and IC_{50} values in the antioxidant activity evaluation assays of apple (*Malus pumila* Miller) peels

Assays	70% methanol	70% ethanol	CM[2)]
Extraction yields (%)	58.62	60.57	21.51
Total phenol content (mg GAE/g)	20.87±0.17[c3)]	20.19±0.17[b]	18.17±0.17[a]
Total flavonoid content (mg QE/g)	7.77±0.11[a]	8.42±0.29[b]	9.26±0.06[c]
DPPH[1)] (IC_{50}, mg/mL)	0.48±0.00[b]	0.42±0.01[a]	0.88±0.06[c]
ABTS (IC_{50}, mg/mL)	0.65±0.01[a]	0.79±0.01[b]	1.57±0.04[c]
β-carotene bleaching (IC_{50}, mg/mL)	1.01±0.07[b]	0.78±0.08[a]	1.61±0.36[c]

1) DPPH [2,2-diphenyl-1-picrylhydrazyl] radical scavenging activity (DPPH), ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical scavenging activity (ABTS) and β-carotene bleaching assays.

2) CM: chloroform-methanol mixture (2:1, v/v).

3) The values are means±SD (n=3). Values with the different letters in the same row are significantly different ($p<0.05$) by Duncan's multiple range tests.

(20.87±0.17 mg CAE/g), 70% ethanol extract (20.19±0.17 mg CAE/g) and CM extract (18.17±0.17 mg CAE/g), respectively. The 70% methanol extract had the highest total phenolic contents in all the extracts. According to the reports of Vieira et al [16], total phenol content of apple peels was found to range from 569.57±3.12 to 640.85±7.41 mg gallic acid/100 g in different cultivars.

3.3. Total flavonoid contents (TFC)

Flavonoids family includes flavones, isoflavones, flavanones, anthocyanins, flavans, proanthocyanidins, and catechins, which have long been recognized to show antioxidant capacity and antiallergic, antiinflammatory, antiviral, antiproliferative and anticarcinogenic activities [4,19]. The content of flavonoid in extracts from various extraction solvents were calculated in accordance with the calibration curve of quercetin (y=0.1092x+0.0034, R^2 =9996). As can be seen in Table 1, total flavonoid contents of various extracts increased in the order: 70% methanol extract (7.77±0.11 mg QE/g), 70% ethanol extract (8.42±0.29 mg QE/g) and CM extract (9.26±0.06 mg QE/g), respectively. Wolfe et al [6] had conducted that peels were also highest in flavonoids, whose contents were in the range of 167.4±20.2-306.1±6.7 mg of catechin equivalents/100 g of peels.

3.4. DPPH radical scavenging activity

DPPH is a stable free radical and can be scavenged by antioxidants through donating hydrogen. The discoloration from purple to yellow induces the absorbance of reaction mixture decreases at 517 nm [20]. Fig. 1 described the DPPH radical scavenging abilities of various extracts from apple peels. The DPPH radical scavenging abilities of various extracts appeared to be associated with increasing concentrations (0.2 mg/mL, 0.4 mg/mL and 0.6 mg/mL). All extracts exhibited excellent DPPH radical scavenging ability even if their effects were lower than that of ascorbic acid. Results showed that the antioxidant activity of 70% ethanol extract (IC_{50}=0.42±0.01 mg/mL) was higher in all three extracts, followed by 70% methanol extract (IC_{50}=0.48±0.00 mg/mL) and CM extract (IC_{50}=0.88±0.06 mg/mL) (Table 1). Lu and Foo [21] reported that apple polyphenols were examined to possess good DPPH radical scavenging activity. DPPH radical scavenging activity in the skin was found to be higher than those in the flesh [22].

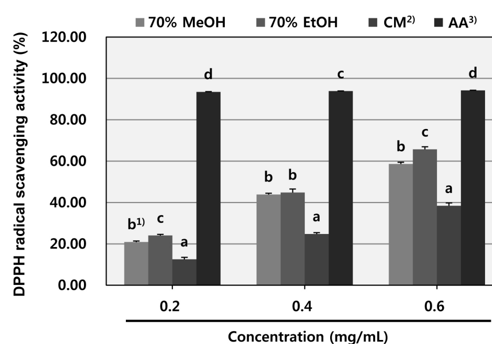

Fig. 1. DPPH radical scavenging activity of various extracts from apple (*Malus pumila* Miller) peels.

1) The values are means±SD (n=3). Bars with the different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.

2) CM: chloroform-methanol mixture (2:1, v/v) extract.

3) AA: ascorbic acid.

3.5. ABTS radical scavenging activity

The radical-cation $ABTS^{\bullet +}$ is produced by the oxidation of ABTS. In the absence of antioxidants, ABTS is rather stable, but it reacts actively with an H-atom donor (i.e. phenolics). Therefore, the blue/green chromophore would discolor gradually or be converted into a non-colored form of ABTS up to the antioxidant capacity of antioxidants [23]. Fig. 2 and Table 1 showed the inhibitory effect of various extracts on ABTS radical. As can be seen from results, ABTS radical scavenging activity was marked and

concentration-related. The IC_{50} values of different fractions increased in the following order: 70% ethanol extract (IC_{50}=0.79±0.01 mg/mL), 70% methanol extract (IC_{50}=0.65±0.01 mg/mL) and CM extract (IC_{50}=1.57±0.04 mg/mL), respectively. Obviously, the 70% ethanol extract exhibited the strongest scavenging activity against ABTS radical. Our results showed that the scavenging activity of apple peel extracts depended on their phenolic composition in a quantitative way, which was in consistent with the results of Chinnici et al [24].

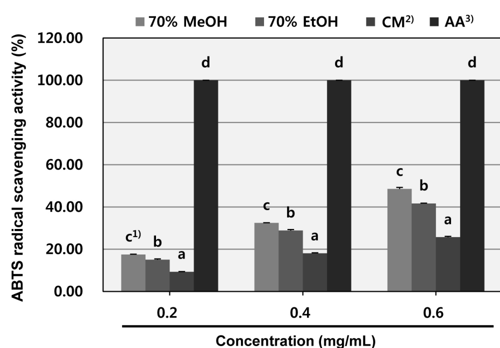

Fig. 2. ABTS radical scavenging activity of various extracts from apple (*Malus pumila* Miller) peels.

1) The values are means±SD (n=3). Bars with the different letters are significantly different (p<0.05) by Duncan's multiple range tests.

2) CM: chloroform-methanol mixture (2:1, v/v) extract.

3) AA: ascorbic acid.

3.6. β -carotene bleaching assay

The antioxidant activities of various extracts at 0.2 mg/mL to 0.6 mg/mL concentrations were compared with ascorbic acid measured by the bleaching of β-carotene were presented in Fig. 3. The highly unsaturated β-carotene molecules in this system can be attacked by free radicals generating from the oxidation of linoleic acid, and as a consequence, the characteristic orange color disappears. The presence of antioxidant can avoid the destruction of the β-carotene by neutralizing the free radicals formed in the system to keep the orange color [14]. As depicted in Fig. 3, the results exhibited concentration-dependent antioxidant activity by β-carotene bleaching method in all the tested concentrations of various extracts. The antioxidant activity of various extracts was found to decrease in the following order: 70% ethanol extract (IC_{50}=0.78±0.08 mg/mL), 70% methanol extract (IC_{50}=1.01±0.07 mg/mL) and CM extract (IC_{50}=1.61±0.36 mg/mL) (Table 1). Although ascorbic acid always showed the most effective antioxidant activity, 70% ethanol extract displayed stronger antioxidant activity compared with other extracts. Antioxidant activity of apple polyphenols including procyanidins and quercetin glycosides using β-carotene/linoleic acid method had been confirmed by Lu and Foo [21]. It was probable that the antioxidative components in extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals in this system.

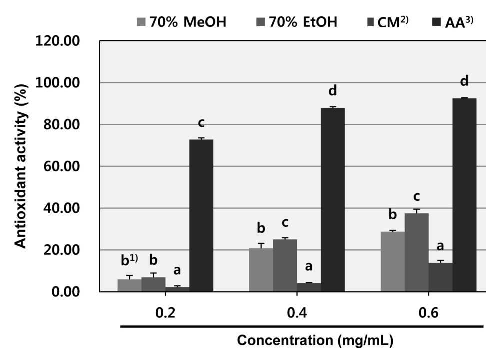

Fig. 3. Antioxidant activity of various extracts from apple (*Malus pumila* Miller) peels by using β-carotene bleaching method.

1) The values are means±SD (n=3). Bars with the different letters are significantly different (p<0.05) by Duncan's multiple range tests.

2) CM: chloroform-methanol mixture (2:1, v/v) extract.

3) AA: ascorbic acid.

3.7. Ferric reducing antioxidant power (FRAP)

Antioxidant potential of various fractions from apple peels was estimated from their ability to reduce the ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex to ferrous-tripyridyltriazine (Fe^{II}-TPTZ) at low pH, forming an intense blue color with an absorption maximum at 593 nm develops [15]. The antioxidant activities through the ferric reducing antioxidant power model system of apple peels extracts at 0.2 to 0.6 mg/mL concentrations compared with ascorbic acid were presented in the Fig. 4. The results revealed concentration-dependent ferric reducing antioxidant activities in all the tested concentrations of various extracts. At a concentration of 0.6 mg/mL, the FRAP values of various extracts increased in the following order: 70% methanol extract (0.44±0.01), 70% ethanol extract (0.39±0.00) and CM extract (0.28±0.00), respectively. The 70% methanol extract showed pronounced ferric reducing antioxidant power values compared with other extracts although lower than that of ascorbic acid. Significant differences in ferric

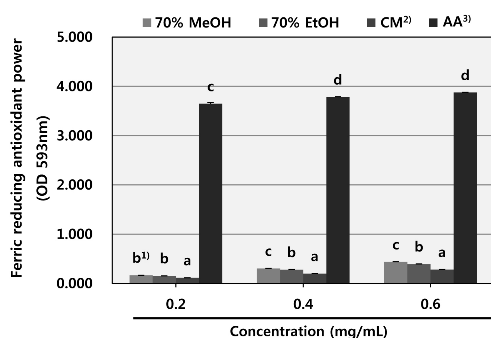

Fig. 4. Ferric reducing antioxidant power of various extracts from apple (*Malus pumila* Miller) peels.

1) The values are means±SD (n=3). Bars with the different letters are significantly different ($p<0.05$) by Duncan's multiple range tests.

2) CM: chloroform-methanol mixture (2:1, v/v) extract.

3) AA: ascorbic acid.

various extracts could be found. According to reducing antioxidant power values between the reports of Guo et al [25], apple peels displayed antioxidant activity by FRAP assay, whose FRAP value was 3.24±0.39 nmol/100 g wet weight. Similar to the results obtained from ABTS radical scavenging activity, correlation between FRAP values and total phenol contents could also be observed from our research. This significant correlation was in accordance with the reports of the Bertoncelj et al [26], who found total phenol content strongly correlated with antioxidant activity evaluated by FRAP assay in the honey.

4. Conclusions

Several methods have been developed to evaluate the antioxidative activity of 70% methanol, 70% ethanol and chloroform-methanol (CM, 2:1, v/v) extracts from apple peels. The antioxidant activity of various extracts all increased with the increasing concentrations. Total phenol contents were found to be correlated with ABTS radical scavenging activity and ferric reducing antioxidant power. Total phenol contents, ABTS radical scavenging activity and ferric reducing antioxidant power of various extracts decreased in this order: 70% methanol extract, 70% ethanol extract and CM extract. However, 70% ethanol extract possessed the strongest antioxidant activity in scavenging DPPH radical and lipid peroxidation inhibition. And CM extract was observed to possess the highest total flavonoid content with the value of 9.26±0.06 mg QE/g. Although apple peels are a waste product of applesauce and canned apple manufacture, their antioxidant potential to improve health when consumed should not be ignored.

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