J. Appl. Biol. Chem. **53**(2), 87-90 (2010)

Article

Phenolic Compounds Content and Tyrosinase Inhibitory Effect of Unripe Apple Extracts

Sang Yoon Choi[1], Sung Soo Kim[1], Yeonmi Lee[1], Bog-Hieu Lee[2], and Chankyu Han[1]*

[1]*Korea Food Research Institute, Seongnam 463-746, Korea*
[2]*Department of Food & Nutrition, Chung-Ang University, Anseng 456-756, Korea*

Received february 11, 2010; Accepted April 19, 2010

This study consisted of a quantitative analysis of five phenolic ingredients in differently sized unripe apple extracts, and their tyrosinase inhibitory effects were examined. In the HPLC analysis of phenolic ingredients, small (4±1 g per one) unripe apple extracts were observed to have significantly higher quercetin content than larger (8±1 g per one) unripe apple and ripe apple extracts. The amount of catechin, epicatechin, *p*-coumaric acid and chlorogenic acid contents were similar in both the small and large unripe apple extracts. For the results of the tyrosinase assay, small unripe apple extracts provided a potent tyrosinase inhibitory effect, showing 89.2% at 1000 ppm. The tyrosinase inhibitory effects of large unripe apple and ripe apple extracts were weaker than those of the small unripe apple extract. These results suggest that the small unripe apple extract could be useful for de-pigmenting material, while quercetin could be responsible for the potent tyrosinase inhibitory properties of small unripe apple extracts.

Key words: quercetin, tyrosinase, unripe apple

Apples have been cultivated in Europe and Central Asia since ancient times and are now widely produced as a major crop all around the world. In Korea, about 50 million tons of apples are produced annually and unripe apples, which account for 20~30% (18 kg/10 trees) of all apples, are singled out by a fruit-thinning procedure performed from May~June during the cultivation process, and discarded [Lee *et al*., 2000; Jung *et al*., 2002].

Recently, phenolic compounds in various fruits and vegetables have come under the spotlight for their excellent anticancer and antioxidant activities including tyrosinase inhibitory activity. Apples are rich in various functional ingredients and nutrients such as polyphenols, dietary fiber, vitamin C, sugars, and potassium [Lee *et al*., 2000; Won *et al.,* 2005; Park *et al*., 2009], and have also been reported to be effective in the prevention of cardiovascular diseases, arteriosclerosis, obesity, and cancer. [Choi *et al*., 2003; Nakazato *et al*., 2006; Miura *et al*., 2007; Auclair *et al*., 2008] The phenolic compounds found in apples are chlorogenic acid, catechin, epicatechin, and quercetin, and unripe apples in particular are known to have ten times more polyphenols compared to ripe apples, as well as being rich in organic acid and amino acids [Lee *et al*., 1972; Vrhovsek *et al*., 2004; Park *et al*., 2004].

This study was performed as part of a body of research on the utilization of unripe apples, which are usually discarded in huge amounts annually, despite their high content of active ingredients. Based on the previous report of rich polyphenol content in unripe apple and tyrosinase inhibitory effect of phenolic compounds, we evaluate whether unripe apple has a tyrosinase inhibitory effect. Tyrosinase inhibitory effect of unripe apple was not yet reported. In the study, ethanol extracts were prepared according to the sizes of unripe apples, and five kinds of phenolic compounds were then quantified, after which the inhibitory activity of tyrosinase was verified and compared.

Materials and Methods

Chemicals. Mushroom tyrosinase, L-dopa, catechin, epicatechin, chlorogenic acid, *p*-coumaric acid and quercetin were obtained from Sigma-Aldrich Co. (St. Louis, MO). HPLC grade distilled water and acetonitrile were purchased from SK chemical Co. (B&J, Ulsan, Korea).

Plant materials. The unripe apples used in this experiment, which were of the Hongro variety, were thinned out and collected during May 2009 at Yesan, Chungcheongnam-do and divided into two groups consisting of small (4±1 g per one) and large (8±1 g per one) sizes. For comparison, ripe apples of the Hongro variety (500±50 g per one) were also collected at Yesan, Chungcheongnam-do during September 2009 and used in the

*Corresponding author
Phone: +82-31-780-9236; Fax: +82-31-709-9876
E-mail: ckhan@kfri.re.kr

doi:10.3839/jabc.2010.016

experiment.

Preparation of the extract. Each 300 g sample was washed with distilled water and crushed, and then stir-extracted using 1.5 L of ethanol for 1 hour at room temperature. This process was repeated three times. The obtained extract was filtered through a filter paper and then completely concentrated using a rotary evaporator to prepare the apple extracts.

Quantification of phenolic compounds. Five phenolic compounds in unripe apples were quantified using an HPLC system (Jasco Co., Japan). The phenolic compounds were catechin, epicatechin, chlorogenic acid, *p*-coumaric acid, and quercetin. As regards the analytical conditions, the column used was a C18 (4 μm, 300×3.9 mm) bondpack, the mobile phase consisted of 2% acetic acid in distilled water (solvent A) and 0.5% acetic acid in 50% acetonitrile (rest is distilled water, solvent B), and with gradient elution of 80% after 70 minutes from 10% of the initial solvent B was performed at 0.8 mL/min. The temperature of the column was maintained at 40°C, and

Table 1. Yield of apple extracts

	Yields (%)
Unripe Apple (small)	1.15
Unripe Apple (large)	5.26
Ripe Apple	7.94

Each value represents % from total flesh apple (w/w).
Apples were extracted with ethanol.

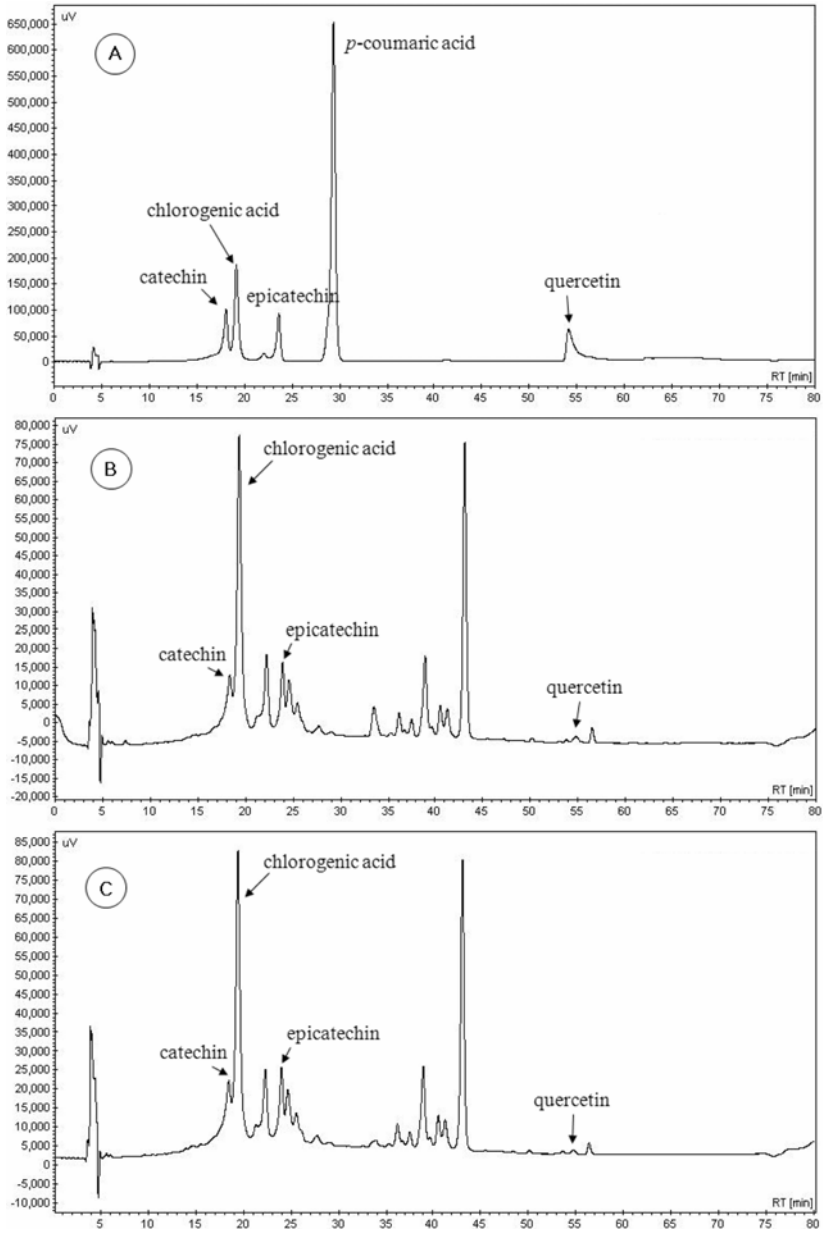

Fig. 1. HPLC chromatogram of phenolic compounds in apple extracts. A: Standard materials (0.125 mg/mL), B: Small unripe apple (10 mg/mL). C: large unripe apple (10 mg/mL)

Table 2. Quantitative analysis of phenolic compounds in apple extracts

	Unripe Apple (small)	Unripe Apple (large)	Ripe Apple
Catechin	0.667	0.701	0.014
Chlorogenic acid	1.006	0.970	0.272
Epicatechin	0.444	0.456	0.120
p-Coumaric acid	0.003	ND	ND
Quercetin	0.049	0.024	0.004

Each value represents % in ethanol extracts (w/w). ND: not detected.

Tyrosinase inhibitory activity measurement. Test samples of several concentrations were dissolved in methanol. 8.0 mM L-dopa 120 μL and test sample 40 μL and then mixed, and tyrosinase (125 U/mL) 40 μL was added in 96 well plates. After incubating them for 20 minutes at 37℃ for a reaction, the amount of dopachrome produced was measured for absorbance at 492 nm using microplate reader (Molecular Devices, USA) [Shin *et al*., 1998].

Results and Discussion

Comparison of the yield of unripe apple extracts by diameter size. The yield of ethanol extract by differently sized unripe apple extracts was shown in Table 1. The yield of the extract, obtained by stirring extraction using ethanol at room temperature, was the highest in the ripe apple extracts (7.94%), followed by the small unripe apple extracts (1.15%) and large unripe apple extracts (5.26%). Thus, showing higher values as the size of the fruit increased.

Comparison of phenolic compounds. Five phenolic compounds in unripe apple extracts (catechin, epicatechin, chlorogenic acid, *p*-coumaric acid, quercetin) were quantified using HPLC system (Fig. 1). After comparing the phenolic compounds in the extracts of unripe apples, the quercetin content was found to be over two times higher in the small unripe apple extracts (0.049%) than in the large unripe apple extracts (0.024%), and the contents of catechin and epicatechin in the large unripe apple extracts were observed to be slightly higher than those in the small unripe apple extracts, although not significantly different. The phenolic compound content in the extracts of ripe apples were found to be lower than in those of the unripe apples (Table 2).

Comparison of tyrosinase inhibitory activity. Tyrosinase is a major enzyme which induces the production of melanin pigment in the skin of animals. Melanin biosynthesis is started by the oxidation of tyrosine by tyrosinase. Melanin protects the skin from UV rays, but excessive production of melanin can cause skin pigmentation disorders such as chloasma, freckles, and dark spots. Jung *et al*. reported that water extract of unripe

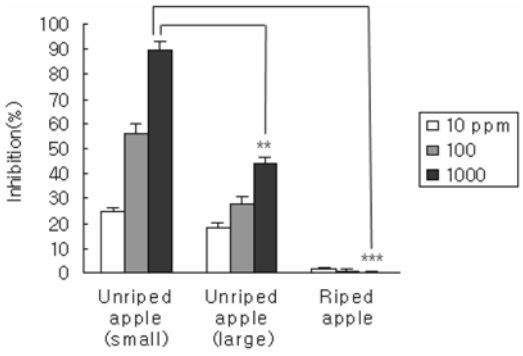

Fig. 2. Tyrosinase inhibitory effect of apple extracts. The data were obtained in triplicate and averaged (mean±SD). *p<0.05, **p<0.01, ***p<0.001

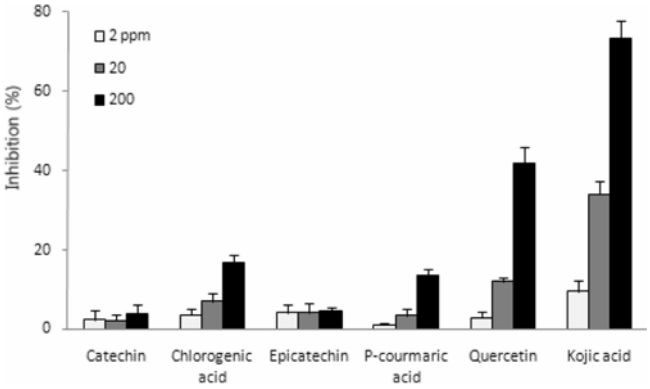

Fig. 3. Tyrosinase inhibitory effect of phenolic compounds in apple extracts. The data were obtained in triplicate and averaged (mean± SD). Kojic acid was used as positive control

apples did not exhibit tyrosinase inhibitory effect [Jung *et al*., 2002]. However, our results of the tyrosinase inhibitory activity of unripe and ripe apple ethanol extracts showed that the activity was 89.2% at 1000 ppm in small unripe apples and 43.6% and 0.3% in large unripe apples and ripe apples, respectively, demonstrating a higher inhibitory effect as the size of the fruit decreased (Fig. 2). Also, the tyrosinase inhibitory activity of the ingredients in apples was found to be high, in the order of quercetin, chlorogenic acid and *p*-coumaric acid (Fig. 3), and the quantification result of quercetin was 0.049% in small unripe apple extract and 0.024% in large unripe apple extract. These results suggesting that quercetin has a major effect in the tyrosinase inhibitory activity of unripe apple extract among these five phenolic ingredients.

Conclusion

Extracts of unripe apples, which were thinned out and discarded during the cultivation process, were prepared and compared for their tyrosinase inhibitory activity. The results showed higher activity as the size of the fruit decreased, and the extract of small unripe apples (4±1 g per one) showed tyrosinase

inhibitory activity as high as 89.2% at 1000 ppm. The extract of small unripe apples had twice as much quercetin as the extract of large unripe apples (8±1 g per one), which was seldom detected in ripe apples. Quercetin showed excellent tyrosinase inhibitory activity and was found in greater amounts in smaller fruits, tending to be proportional to the tyrosinase inhibitory activity of the unripe apple extract. Thus, it is considered that quercetin makes a major contribution to the tyrosinase inhibitory activity of unripe apple extract among these five phenolic ingredients.

Acknowledgments

This study was conducted with a research fund from the Yesan County Office, Chungcheongnam-do. The authors would like to thank the people concerned for their support.

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