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Antioxidant, Antimicrobial, and Antitumor Activities of Partially Purified Substance(s) from Green Tea Seed

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Abstract The aim of this study is to evaluate the antioxidant, antimicrobial, and antitumor activities of various concentrations of partially purified substance(s) from green tea seed (*Camellia sinensis* L.). The total polyphenol contents of each fraction (non-adsorption fraction: F-1, fraction eluted with 40% methanol: F-2, and fraction eluted with 100% methanol: F-3) purified by Diaion HP-20 column chromatography were, in the increasing order: F-1 (3.7 mg tannic acid equivalents, TAE/g) < F-3 (23.2 mg TAE/g) < seed extracts (26.2 mg TAE/g) < F-2 (42.7 mg TAE/g). The scavenging activities toward the 1,1-diphenyl-2-picyrylhydrazyl (DPPH) radical were, in decreasing order: F-2 (93.3%) > butylated hydroxytoluene (BHT; 89.8%) > ascorbic acid (89.3%) > leaf extracts (70.3%) > F-3 (15.9%) > seed extracts (15.8%) > F-1 (14.8%) at a 0.1% concentration. In studies on antimicrobial activities, the results indicate that the growth of yeast (*Candida albicans* KCCM 11282 and *Cryptococcus neoformans* KCCM 50544) was inhibited more so than that of other fungi (*Alternaria alternate* KCTC 6005 and *Rhizoctonia solani*). In addition, it appears that the antitumor activities of the F-1, F-2, and F-3 fractions at a concentration of 50 μg/mL showed 6, 7, and 23% growth inhibition of the HEC-1B cell line, 14, 11, 82% inhibition of the HEP-2 cell line, and 8, 16, and 81% inhibition of the SK-OV-3 cell line, respectively. Overall these results indicate that the antioxidant activity is greatest in the F-2 fraction, and the antimicrobial and antitumor activities are greatest in the F-3 fraction.

Keywords: *Camellia sinensis* L., green tea seed, antioxidant activity, antimicrobial activity, antitumor activity, Diaion HP-20 resin

Introduction

Green tea (*Camellia sinensis*) is an important commercial crop that is grown in over 30 countries and has been consumed world wide primarily as a beverage made from the processed leaf (1). It produces a variety of secondary metabolites that are valuable for human welfare. The main constituents of green tea are catechins that comprise up to 30% of the dry weight (2). The catechins found in green tea are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC), and catechin (3-7). These polyphenolic compounds in green tea are well known for their broad spectrum of biological activities such as antioxidant, antibacterial, antifungal, antigiardial, and antitumor functions (3-9). Green tea also contains volatile oils, vitamins, minerals, and many other useful secondary metabolites such as caffeine, theanine, and saponin (1, 10-14).

Some triterpenoid saponins such as camellidin I and hederin of green tea seed exhibit antifungal activity (15). Consumption of tea extract inhibited tumor cell growth and metastasis in mice (16, 17). Many mechanisms have been proposed for the inhibition of carcinogenesis by tea (18, 19). Although many studies on green tea leaf have been performed, the bioactive substances of green tea seed have not been sufficiently studied. Also, the antioxidant activities of tea seed have not been reported.

In a previous study (14), we reported the antitumor and antimicrobial activities of seed extracts of green tea. Our results showed that water extracts of the seed had stronger antifungal activities against yeast, and stronger growth inhibitory effects on human tumor cell lines than other solvent extracts. In this study, we determined the antioxidant, antimicrobial, and antitumor activities of various partially purified fractions of water extracts from green tea seed. *in vitro* using the scavenging test on 1,1-diphenyl-2-picyrylhydrazyl (DPPH) radicals, the paper disc method, and the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, respectively.

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Materials and Methods

Plant materials The seeds of green tea plant were kindly provided by Hankooktea Co., Gwangju, Korea in October 2004.

Analysis of general ingredients The moisture, crude ash, crude protein, and crude fat contents of green tea seed were quantified by the method of AOAC (20). The milled seeds were dried at 100°C to measure the moisture content. Crude ash and crude lipid were quantified after hexane extraction using the Electric Furnace (F-2F; Kong Soung Co., Seoul, Korea) and Soxhlet (Dong Ha-Tech., Seoul, Korea), respectively. Crude protein content was measured by the Kjeldahl method.

Sample preparation The dried and ground seeds (100 g) were extracted in distilled water (1 L) by shaking overnight at room temperature. The water extracts were filtered through Whatman No. 41 filter paper and then separated by centrifugation at 2,700×g, 4°C for 20 min. The supernatants were extracted three times in a

separatory funnel with a one half volume of ethyl ether. The aqueous layer was applied to a column (size; ϕ100× 400 mm) packed with Diaion HP-20 resin at a ratio of 1:2 (volume of Diaion HP-20 resin:filtrate). Elution was carried out in steps with 1 L each of 40% methanol in water and 100% methanol at a flow rate of 10 mL/min. Each fraction (F-1, F-2, and F-3) obtained by Diaion HP-20 column chromatography was concentrated using a rotary vacuum evaporator. After drying, samples were dissolved in phosphate buffered saline (PBS, pH 7.2), aseptically filtered with a 0.45 μm pore size filter, and used for assays. Also, seeds and leaves were extracted in distilled water by shaking overnight at room temperature to measure the polyphenol content and antioxidant activity.

Determination of total polyphenol content Total polyphenol content was measured by the Folin-Denis method (21). One mL of the diluted sample solution (5 mg/mL) was mixed with 9 mL of distilled water for 3 min. One mL of commercial Folin reagent was added to 10 mL of the diluted samples and set for 5 min before adding 10 mL of 7% sodium carbonate aqueous solution. The final volume was adjusted to 45 mL with distilled water. After holding the mixed solution for 1 hr, absor-bances were measured at room temperature at 760 nm using a UV-VIS spectrophotometer (Optizen 2120; Mecasys Co., Seoul, Korea). Total polyphenol content was determined from a standard curve obtained using tannic acid (Sigma Chemical Co., St. Louis, MO, USA) and was expressed as tannic acid equivalents (mg TAE/g sample).

Antioxidant activity The antioxidant activity of each sample was assessed on the basis of the radical scavenging effect toward the stable DPPH (Sigma Chemical Co.) free radical (22). One mL of 100 μM DPPH ethanol solution was added to 200 μL of each sample solution, butylated hydroxyltoluene (BHT; Sigma Chemical Co.) or ascorbic acid (Sigma Chemical Co.) at 0.1%, and allowed to react at room temperature. After 10 min, the absorbance values were measured at 517 nm using a spectrophotometer and converted into percentage antioxidant activity using the following formula:

Electron donating activity (EDA)
= [1-(absorbance of sample at 517 nm)/(absorbance of control at 517 nm)] × 100

The mean values were obtained from triplicate measurements.

Microorganisms and antimicrobial activity The yeast strains used for antimicrobial tests were *C. albicans* KCCM 11282 and *C. neoformans* KCCM 50544. The fungi used were *A. alternate* KCTC 6005 and *R. solani*. Media used for each test were YM Broth (Difco Laboratories, Detroit, MI, USA) and YM Agar (Difco Laboratories) for yeast, and Potato Dextrose Broth (Difco Laboratories) and Potato Dextrose Agar (Difco Laboratories) for fungi. Antimicrobial activity was measured using the paper (8 mm, Whatman, England) disc method (14, 23). To measure the antimicrobial activity, 50 μL of each sample solution (50 and 100 mg/mL) diluted with PBS was placed on a paper disc, and the diameter (mm) of inhibition was measured after incubation for 2 days at 30 and 27°C for yeast and fungus, respectively.

Tumor cell lines and antitumor activity Human cell lines from endometrial adenocarcinoma (HEC-1B), larynx carcinoma (HEP-2), and ovary carcinoma (SK-OV-3) were obtained from the Korean Cell Bank. The HEC-1B and SK-OV-3 cell lines were grown in RPMI 1640 medium (2.0 g/L, sodium bicarbonates, Gibco BRL, MD, USA). The HEP-2 cell line was grown in MEM medium (2.2 g/L, sodium bicarbonates, Gibco BRL). Each medium was supplemented with 10% fetal bovine serum (Gibco BRL), penicillin (10,000 unit/mL), and streptomycin (10 mg/mL). All cell lines were incubated at 37°C in a 5% CO_2 and 95% air chamber (MCO-18 AIC; Sanyo, Japan).

Cytotoxicity was examined using the MTT assay (24). Cells undergoing exponential growth were suspended in fresh medium (Gibco BRL) at a concentration of 2×10^5 cells/mL, dispensed in a 96-well flat plate in a volume of 0.1 mL per well, and stabilized by incubation at 37°C in a 5% CO_2 for 24 hr. Then 100 μL of each sample was added to the wells. After incubation at 37°C for 44 hr, 50 μL of MTT (0.5 mg/mL PBS) was added to each well, and the resulting 250 μL mixture was further incubated for 4 hr at 37°C. The formazan crystals formed in each well were dissolved in 100 μL of dimethyl sulfoxide (DMSO; Sigma Chemical Co.) solution by gentle mixing on a plate shaker. The optical density of the colored reaction samples was measured by reading at 570 nm on a multi-well scanning spectrophotometer (Microplate Autoreader; Bio-Tek Instrument, Winooski, VT, USA).

Inhibition rate
= [1-(absorbance of sample at 570 nm)/(absorbance of control at 570 nm)] × 100

Statistical analysis Data were expressed as means± standard deviation (SD). The statistical significance of differences between groups was determined by applying Student's *t*-test. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

Proximate composition of green tea seeds Table 1 shows the results of the analysis of green tea seeds. The moisture content was 3.69%. The crude protein composition was 12.02%. The crude lipid composition was highest at 22.98%. These measurements were different from those reported by Yang *et al.* (25) and Rah *et al.* (26) probably due to the differences in tea-producing districts, climatic properties, and soil.

Total polyphenol content The total polyphenol content of seed extracts, F-1, F-2, and F-3 of green tea were, in increasing order, F-1 (18.9 mg TAE/g) < F-3 (25.0 mg

Table 1. Proximate composition of green tea seeds

	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
Seed	3.69±0.03[1)]	12.02±0.05	22.98±0.04	3.63±0.02

[1)]The values are mean±SD (n=3).

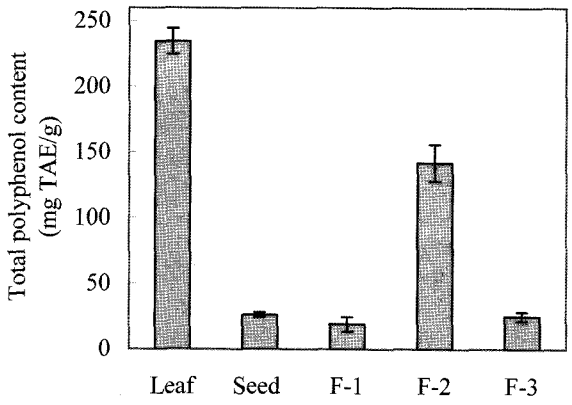

Fig. 1. Total polyphenol contents of water extracts and partially purified fractions from green tea seeds. Samples separated by Diaion HP-20 column chromatography; F-1: non-adsorption fraction, F-2: fraction eluted with 40% methanol, F-3: fraction eluted with 100% methanol. The values are mean±SD (n= 3).

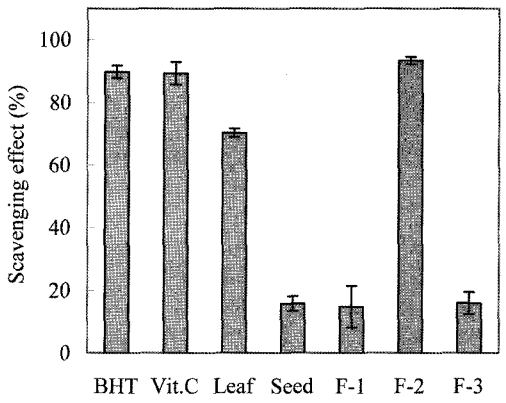

Fig. 2. Scavenging effect on the DPPH radical of partially purified fractions from green tea seeds at 0.1%. Samples separated by Diaion HP-20 column chromatography; F-1: non-adsorption fraction, F-2: fraction eluted with 40% methanol, F-3: fraction eluted with 100% methanol. The values are mean±SD (n=3).

TAE/g) < seed extracts (26.2 mg TAE/g) < F-2 (141.8 mg TAE/g) < leaf extracts (234.3 mg TAE/g) (Fig. 1). The total polyphenol content of seeds was lower than that of leaves. These results suggest that higher extraction yields of phenolic compounds were obtained with the fraction eluted with 40% methanol by Diaion HP-20 column chromatography.

Antioxidant activity Proton-radical scavenging action is known to be an important mechanism of antioxidation due to hydrogen donation (27). The scavenging of stable DPPH free radicals can be used to evaluate antioxidant activities in a relatively short time compared to other methods. The radical scavenging activities of all samples by the DPPH radical scavenging assay are shown in Fig. 2 and indicate that F-2 showed the highest antioxidant activity among the fractions. The scavenging activities toward the DPPH radical were, in the decreasing order, F-2 (93.3%) > BHT (89.8%) > ascorbic acid (89.3%) > leaf extracts (70.3%) > F-3 (15.9%) > seed extracts (15.8%) > F-1 (14.8%) at a 0.1% concentration. The F-1 and F-2 fractions had poor antioxidant activity when compared with other fractions and reference antioxidants such as BHT and ascorbic acid. These results suggest that the antioxidant activity of the water seed extract of green tea is partially attributable to the 40% methanol fraction (F-2). When the total polyphenol content of each sample is considered (Fig. 1), it is clear that the more polyphenols the fraction contained, the greater the antioxidant activity.

Antimicrobial activity The antimicrobial activity of green tea seed was evaluated by applying its various fractions to yeast and fungal cultures using a paper disc. There was a significant correlation between concentration level and inhibitory activity as measured by the size of clear zone. Consistent with our previous study (14), the inhibitory activities of the water and 70% ethanol extracts toward yeast and fungus increased with increasing extract concentration added to the paper disc, and the inhibitory effect was greater than is observed against bacteria. We then examined the antimicrobial activities of the fractions in this study (Table 2). The F-2 and F-3 fractions obtained by Diaion HP-20 column chromatography displayed the majority of inhibitory activity against yeast and fungus whereas no significant activity was detected in the F-1 fraction. In particular, the growth of yeast (*C. albicans*

Table 2. Antimicrobial activity of partially purified fractions from green tea seeds

Strains	Inhibition zone (mm)[1]					
	F-1[2]	F-2	F-3	F-1	F-2	F-3
	2.5 mg/disc[3]			5.0 mg/disc		
Yeast						
Candida albicans KCCM 11282	-[4]	18	26	-	22	31
Cryptococcus neoformans KCCM 50544	-	14	28	-	19	37
Fungus						
Alternaria alternate KCTC 6005	-	w[5]	14	-	w	17
Rhizoctonia solani	-	-	13	-	11	15

[1]Diameter, [2]Samples separated by Diaion HP-20 column chromatography; F-1: non-adsorption fraction, F-2: fraction eluted with 40% methanol, F-3: fraction eluted with 100% methanol,
[3]Concentration of testing sample, [4]No growth inhibition, [5]Weak inhibition zone.

KCCM 11282 and *C. neoformans* KCCM 50544) was inhibited more so than that of the fungi *A. alternate* KCTC 6005 and *R. solani*. The F-3 fraction displayed the largest clear zone (37 mm) against *C. neoformans* KCCM 50544 at 5 mg/disc.

Green tea contains much higher crude saponin content (12.2%) than that of sesame (0.29%) or peanut (0.63%) (26). Also, saponins prepared from tea seeds are known to have several physiological activities (15, 28, 29). It has been reported by Minoru *et al.* (30) that a mixture of saponins from tea seeds inhibits the growth of some yeast such as *Zygosaccharomyces rouxii*. These results suggest that components such as crude saponins in the F-3 fraction had the greatest antifungal activity.

Antitumor activity We examined the growth inhibitory effects of extracts made with various solvents in a previous study (14). This study reported that water extracts showed the highest effect compared to other extracts. Also, the inhibitory activity against the normal cell line NIH/3T3 was much lower than for human tumor cell lines. As shown in Fig. 3, the F-3 fraction obtained by Diaion HP-20 column chromatography displayed the majority of inhibitory activity against all human tumor cell lines tested except for HEC-1B cells, whereas no significant activity

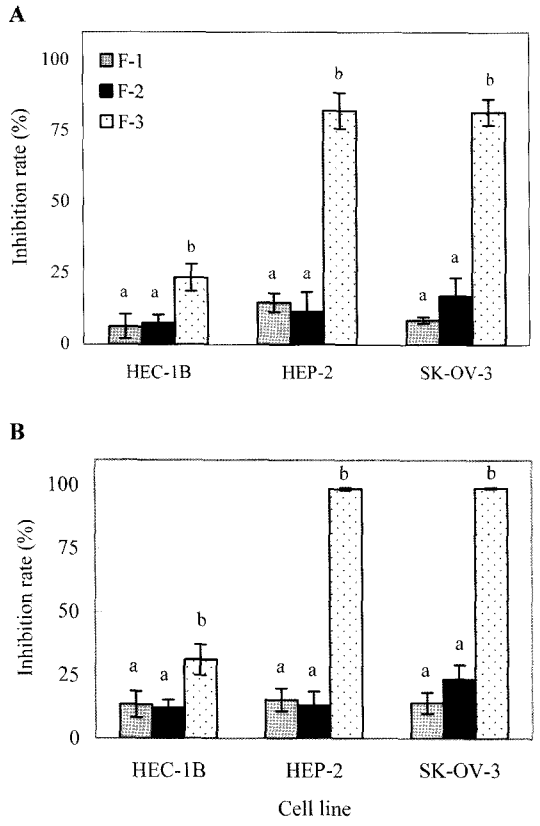

Fig. 3. Growth inhibition effect of fractions separated by Diaion HP-20 column chromatography from green tea seeds at 50 μg/mL (A) and 100 μg/mL (B). F-1: non-adsorption fraction, F-2: fraction eluted with 40% methanol, F-3: fraction eluted with 100% methanol. The values are mean±SD (n=3). [a-c]Values with different letters indicate significant differences among groups ($p<0.05$).

was detected in the F-1 and F-2 fractions. We also compared antitumor activities of the fractions at concentrations of 50 and 100 μg/mL as shown in Fig. 3A and 3B, respectively. As shown in Fig. 3A, at a concentration of 50 μg/mL, the F-1, F-2, and F-3 fractions inhibited cell growth by 6, 7, and 23% for the HEC-1B cell line, 14, 11, and 82% for the HEP-2 cell line, and 8, 16, and 81% for the SK-OV-3 cell line, respectively. In a similar trend, growth inhibition of each fraction at 100 μg/mL increased by 13, 12, and 31% for the HEC-1B cell line, 15, 13, and 98% for the HEP-2 cell line, and 14, 23, and 98% for the SK-OV-3 cell line, respectively (Fig. 3B). At a concentration of 0.5 mg/mL (data not shown), all but the F-1 fraction the showed growth inhibition against HEP-2 cells and SK-OV-3 cells. These results show that the antitumor activities of the F-2 and F-3 fractions obtained by Diaion HP-20 were over 95 %. Overall, the F-3 showed the highest antitumor activity at the lowest concentration. Notably, growth of the human larynx carcinoma cell line HEP-2 and human ovary carcinoma SK-OV-3 was strongly inhibited. It has been known that triterpenoid saponins have anti-inflammatory, antitumor, and immunomodulatory activities (31, 32). All these results suggest that bioassays involving the purification of the antitumor activity in the F-3 fraction have to be guided by growth inhibition of HEP-2 and SK-OV-3 cells in future experiments.

As a whole these results indicate that the antioxidant activity of green tea seeds is contained primarily in the F-2 fraction, whereas the antimicrobial and antitumor activities are contained primarily in the F-3 fraction. Therefore, it is presumed that the antioxidant substances are water-soluble or a little hydrophobic, and that the antimicrobial and antitumor substances are similar and likely to be hydrophobic saponins. In the future, follow-up studies should be done to purify and define the bio-active substances in green tea seed that function as antioxidant, antimicrobial, and antitumor agents.

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References

1. Chung SK, Kim MY, Kim YC, Iwai K, Matsue H. Antioxidant effects of Korean teabag teas by a simple and fast XYZ dish method. Food Sci. Biotechnol. 13: 197-201 (2004)
2. Millin DJ. Factors affecting quality of tea. pp. 127-160. In: Quality Control in the Food Industry. Herschduerter SM (ed). Academic Press, London, UK (1987)
3. Fassina G, Buffa A, Benellir R, Vamier OE, Noonan DM, Albini A. Polyphenolic antioxidant (-)-epigallocatechin-3-gallate from green tea as a candidate anti-HIV agent. AIDS 16: 939–941 (2002)
4. Mabe K, Yamada M. *In vitro* and *in vivo* activities of tea catechins against *Helicobacter pylori*. Antimicrob. Agents Ch. 43: 1788–1791 (1999)
5. Sakanaka S, Juneja L, Taniguchi M. Antimicrobial effects of green tea polyphenols on thermophilic spore-forming bacteria. J. Biosci. Bioeng. 90: 81-85 (2000)
6. Li X., Elsohly HN, Nimrod AC, Clark AM. Antifungal activity of (-)-epigallocatechin gallate from *Coccoloba dugandiana*. Planta Med. 65: 780 (1999)
7. Calzada F, Meckes M, Cedillo-River R. Antiamoebic and antigardial

activity of plant flavonoids. Planta Med. 65: 78–80 (1999)
8. Yang CS, Chung JY, Yang GY, Chhabra SK, Lee MJ. Tea and tea polyphenols in cancer prevention. J. Nutr. 130: 472S-478S (2000)
9. Cao Y, Cao R. Angiogenesis inhibited by drinking tea. Nature 398: 381 (1999)
10. Ogutuga DBA, Northcote DH. Caffeine formation in tea callus tissue. J. Exp. Bot. 21: 258-273 (1970)
11. Takeo T. L-Alanine as a precursor of ethylamine in *Camellia sinensis*. Phytochemistry 13: 1401-1406 (1974)
12. Matsuura T, Kakuda T. Effects of precursor temperature and illumination on theanine accumulation in tea callus. Agr. Biol. Chem. Tokyo 54: 2283-2286 (1990)
13. Shervington A, Shervington LA, Afifi F, El-omari MA. Caffeine and theobromine formation by tissue cultures of *Camellia sinensis*. Phytochemistry 47: 1535-1536 (1998)
14. Yoon WH, Choi JH., Lee KH, Kim CH. Antimicrobial and antitumor activities of seed extracts of *Camellia sinensis* L. Korean J. Food Sci. Technol. 37: 108-112 (2005)
15. Hiller K. New results on the structure and biological activity of triterpenoid saponins. pp. 167-184. In: Biologically Active Natural Products. Hostettmann K, Lea PJ (eds). Clarendon Press, London, UK (1987)
16. Das M, Sur P, Gomes A, Vedasiromoni, Ganguly DK. Inhibition of tumor growth and inflammation by consumption of tea. Phytother. Res. 16: 40-44 (2002)
17. Gupta S, Hastak K, Ahmad N, Lewin JS, Mukhtar H. Inhibition of prostate carcinogenesis in TRMP mice by oral infusion of green tea polyphenol. P. Natl. Acad. Sci. USA 98: 10350-10355 (2001)
18. Kuo P-L, Lin C-C. Green tea constituent (-)-epigallocatechin-3-gallate inhibits Hep G2 proliferation and induces apoptosis through p53-dependent and fas-mediated pathways. J. Biomed. Sci. 10: 219-227 (2003)
19. Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. Ann. Rev. Pharmacol. 42: 25-54 (2002)
20. AOAC. Official Method of Analysis of AOAC Intl. 14th ed. Method 876. Association of Official Analytical Communities, Washington, DC, USA (1980)
21. Folin O, Denis W. On phosphotungastic-phosphomolybdic compounds as color reagents. J. Biol. Chem. 12: 239-243 (1912)
22. Boo HO, Chon SU, Kim SM, Pvo BS. Antioxidant activities of colored sweet potato cultivars by plant parts. Food Sci. Biotechnol. 14: 177-180 (2005)
23. Bae JH. Antimicrobial effect of *Sophora angustifolia* extracts on food-borne pathogens. Food Sci. Biotechnol. 14: 311-316 (2005)
24. Strekova VY, Zagoskina NV, Subbotina GA, Zaprometev MN. Effect of prolonged illumination on synthesis of phenolic compounds and chloroplast formation in tea callus tissues. Sov. Plant Physiol. 36: 65-71 (1989)
25. Yang JK, Gang BK, Kim JM, Park YG, Choi MS. Physico-chemical properties and composition of fatty acids from seed oil of *Camellia sinensis* L. J. Korean Tea Soc. 6: 83-91 (2000)
26. Rah HH, Baik SO, Han SB, Bock JY. Chemical compositions of the seed of Korean green tea plant. J. Korean Soc. Appl. Biol. Chem. 35: 272-275 (1992)
27. Chen JH, Ho CT. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. J. Agr. Food Chem. 45: 2374-2378 (1997)
28. Sagesakc Y, Uemura T, Suzuki Y, Sugirua T, Yoshida M, Yamaguchi K, Kyuki K. Antimicrobial and anti-inflammatory actions of tea-leaf saponin. Yakugazu Zasshi 116: 238-243 (1996)
29. Kawai A, Mukai T, Horie H, Kohata K. Control effect of tea seed saponins against insect pests and mites. J. Tea Res. 87: 7-12 (1999)
30. Minoru T, Sumito Y, Kanoko Y, Hajime O, Tadaxhi YG, Katsunori K, Jan AB. Theasaponin E_1 destroys the salt tolerance of yeasts. J. Biosci. Bioeng. 90: 637-642 (2000)
31. Zhendan H, Chunfeng Q, Quanbin H, Ying W, Wencai Y, Hongzi Z. New triterpenoid saponins from the roots of *Platycodon grandiflorum*. Tetrahedron 61: 2211-2215 (2005)
32. Sparg SG, Light ME, Staden J. Biological activities and distribution of plant saponins. J. Ethnopharmacol. 94: 219-243 (2004)