Purification of Total Ginsesides with Macroporous Resins and Their Biological Activities

Huayue Li, Haizhu Jin[1], Dong Geun Lee, Jae Hwa Lee, Sang Hyeon Lee, Bae Jin Ha, Jong-Myung Ha*

Department of Pharmaceutical Engineering, College of Medical Life Science, Silla University, Busan, Korea,
1: Department of Biochemistry, Yantai University, Yantai 264005, China

Total ginsenosides were purified and their antioxidant, antibacterial and anticancer activities were measured. The crude extracts of ginseng, which were extracted with 75% ethanol by ultrasonification method, were firstly purified on AB-8 macroporous adsorption column to remove water soluble impurities, and decolored on Amberlite IRA 900 Cl anion-exchange column. Then, they were purified on Amberlite XAD16 adsorption column to delete the non-polar impurities. Total ginsenosides contents of the purified extracts were 79.4%, 71.7% and 72.5% in cultured wild ginseng, red ginseng and white ginseng, which were significantly increased than those of crude extracts. All of the three extracts showed concentration-dependant scavenging activities against DPPH radicals, among which white ginseng showed the most powerful activity. Cultured wild ginseng roots showed strongest effect against both *B. subtilis* PM125 (Gram-positive) and *E. coli* D31 (Gram-negative) bacteria, while red ginseng and white ginseng only showed the activity against *B. subtilis*. According to the result of the MTT assay, all of the three extracts inhibited the growth of U-937 human hohistiocytic lympma cell, which were significantly different ($P < 0.05$) when compared to the control.

Key words : ginsenoside, macroporous resin, purification, antioxidant, antibacterial, anticancer

Introduction

Ginseng (Panax ginseng, C.A. Meyer) has long been used as a tonic, anti-fatigue, sedative and anti-gastric ulcer drug and many studies have suggested that its pharmacological effects are due to ginseng saponins. Nowadays, wild ginseng is rarely available and the ginseng roots on the market are mostly collected from cultivation in the farms. Field cultivation is a time-consuming from seeding to the final harvest, which takes 4-6 years. Cultured wild ginseng roots were obtained by using plant cell culture process to produce more efficient production of ginseng and its active ingredients such as ginsenosides[1]. With the cell culture method, production is more controllable in terms of the product quality and quantity with few natural limitation such as seasonal climate and geographical environment, and the culture conditions and process variables are more easily optimized. This kind of ginseng product is very competitive in the market, however, the ginsenosides contents and many kinds of biological activities should be determined compared to other cultivated ginseng.

More than 30 kinds of ginseng saponins have been reported from ginseng and allied plants and novel ginsenosides continue to be reported[2,3]. Many attempts have been made to separate ginseng saponins[4-8]. Traditionally, purification of the ginsenosides was usually done by using organic solvents such as methanol and water saturated butanol[9], which production was deep colored and still contained many impurities. And what's more, it was so difficult to remove toxic organic solvents completely that they were unsuitable to be used as food or medicine directly. Another traditional purification method was using adsorbents such as active carbon, Sephadex G and DEAE-Sephadex. These adsorbents were often used following organic solvents to purify crude ginsenosides extracts. But they were limited in specialty, which were not able to adsorb the ginsenosides effectively. So, a more effective purification method needs to be developed.

In this study, cultured wild ginseng roots, red ginseng (steamed ginseng) and white ginseng (cultivated raw ginseng) were simultaneously used for analysis. Three different macroporous resins were used for purification of total

* To whom correspondence should be addressed at : Jong-Myung Ha, Department of Pharmaceutical Engineering, College of Medical Life Science, Silla University, san 1-1, Kwaebop-dong, Sasang-gu, Busan, Korea
· E-mail : jmha@silla.ac.kr, · Tel : 051-999-5467
· Received : 2006/07/23 · Revised : 2006/08/28 · Accepted : 2006/09/22

ginsenosides. In order to compare biological activities of each product, antioxidant activity, antibacterial activity and anticancer activity were determined.

Materials and Methods

1. Plants and adsorbents

Cultured roots of Panax ginseng C.A. Meyer (cultured wild ginseng roots) was purchased from VitroSys Incorporation (Korea). Red ginseng and white ginseng were obtained from a local herbal shop in Busan, Korea. Standard ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rh1, Rg1, Rg2 and Rg3 were purchased from Hongjiu Ginseng Co.,Ltd. (Jilin, China). AB-8 macroporous adsorption resin was purchased from The Chemical Plant of Nakai University (Tianjin, China), Amberlite® IRA 900 Cl anion-exchange resin and Amberlite® XAD 16 adsorption resin was obtained from Sigma-Aldrich Korea (Yongin, Korea). Their physical properties were listed in Table 1.

Table 1. Physical characteristics of macroporous resins.

Resin	Particle size (mm)	pH range	Max. Op. Temp. (℃)	Polarity
AB-8	0.3-1.25	4-14	120	Moderate polarity
IRA 900	0.65-0.82	0-14	77	-
XAD 16	0.56-0.71	0-14	150	Non-polar

2. Preparation of crude extracts

Ten gram of each ground sample was transferred to 250 ml flasks followed by 200 ml 75% ethanol and then was placed in an ultrasonic bath (Model 5210R-DTH, Branson Ultrasonics Corporation, Danbury, USA). Ultrasonication was carried out for 120 min and the extraction temperature was set at 39℃[10]. The extraction was repeated two additional times. The combined extracts were centrifuged at 3000 rpm × 15 min in a centrifuge (Model MF-600, Hanil Science Industrial Co. Ltd., Inchun, Korea) and the upper layer was concentrated to 40 ml with an rotary evaporator (Model N-1000, Rikakikai Co. Ltd., Tokyo, Japan) at 37°C.

3. Purification of total ginsenosides with macroporous resins

AB-8 polar adsorbing resin was used for crude ginsenosides extracts (40 ml) to eliminate water soluble impurities at the flow-rate of 1 BV/h and desorb the adsorbed ginsenosides with 70% ethanol (v/v) (2 BV/h). Then the collected solution was transferred to the column packing with Amberlite IRA 900 Cl strong base anion exchange resin at the flow-rate of 1 BV/h to remove the colour. Finally, the solution was poured into the column which was packed with Amberlite XAD 16 resin to remove non-polar compounds. The final solution was collected and evaporated to dry in a CentriVap concentrator (LABCONCO Corporation, Kansas, USA).

4. Total yield and purity of the ginsenosides

Total weights of each extract were measured correctly to calculate the yield. Purity of the ginsenosides was calculated using Re as the calibration standard[11]. A good linear relationship was obtained over the range of 0.005-0.03 mg/ml, and the regression equation was y=30.376x-0.0258 (R2=0.9983, n=5), where y was the absorbance at 544 nm, and x was the concentration of total ginsenosides (mg/ml).

5. DPPH radical scavenging activity

The free radical scavenging activity of the extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH•) method proposed by Brand-Williams et al.[12] with slight modification. Briefly, 1 ml of DPPH solution (0.1 mM, in ethanol anhydrous) was added to 0.5 ml of sample solution. The mixture was shaken vigorously and left to stand (25℃) in the dark for 20 minutes. Then the absorbance was measured at 525 nm in a spectrophotometer. The capability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH\bullet \text{ scavenging effect } (\%) = [(A0 - A1) / A0] \times 100$$

where A0 was the absorbance of the control reaction and A1 was the absorbance of the sample. L-ascorbic acid, was used as a positive control.

6. ROS (Reactive Oxygen Species) scavenging activity

The level of generation of ROS was measured by the method popularized by Thomas et al.[13]. All tissues were homogenized in ice-cold homogenization solution. The homogenate was centrifuged at 900 × g for 15 min, at 4℃. In order to obtain a postmitochondrial fraction, the supernatant was centrifuged at 12,000 × g (4℃, 15 min). The supernatant was regarded as a postmitochondrial fraction and the mitochondrial pellet was resuspended in homogenization solution and used as the mitochondrial fraction[14]. 125 μM DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) was added to homogenate for 250 μl of final volume. Changes in fluorescence intensity were measured at 5, 10, 15, 20 and 25 min on Fluorescence Plate Reader (Bio-Tek, USA) with excitation and emission wavelengths at 485 nm and 530 nm, respectively.

7. Total antioxidant activity in linoleic acid emulsion

The antioxidant activity of the extracts on inhibition of linoleic acid peroxidation was assayed using the thiocyanate method[15] with some modification. Each sample solution (2.5 ml, 0.01 mg/ml) was added to 2.5 ml linoleic acid emulsion.

The mixed solution (5 ml) was incubated at 37℃ in the dark. The degree of oxidation was measured by sequentially adding ethanol (5 ml, 75% v/v), ammonium thiocyanate (0.5 ml, 30% w/v), and ferrous chloride (0.5 ml, 0.02 M v/v) to sample solution (0.5 ml) which was taken after five days' incubation. Finally, the absorbance was read at 500 nm and the data used were the average of triplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by the following equation: Inhibition (%) = [(A0 − A1) / A0] × 100

where A0 was absorbance of the control reaction and A1 was the absorbance in the presence of the sample. α-tocopherol was used as a positive control.

8. Determination of antimicrobial effects

Antibacterial activity for the purified ginsenosides extracts of CWG (cultured wild ginseng roots), RG (red ginseng) and WG (white ginseng) (8 mg/ml in 0.01% acetic acid, v/v) were tested by radial diffusion method[16] against *B. subtilis* PM125 (Gram-positive) and *E. coli* D31 (Gram-negative). Bacteria were distributed on 1.5% LB agar media. Then wells were made on the medium with 3 mm punch. Different volumes (5, 10, 15, 20 μl) of the sample was placed into the wells of the agar plates. The plates were incubated at 37℃ for 24 h. The diameters of the inhibition zones were measured in millimeters.

9. Determination of anticancer effect

The potential anticancer effect of three purified ginsenosides extracts was investigated by determining their effects on the viability of a human hohistiocytic lympma cell line (U-937). Different numbers (500-10,000) of cells were plated in 200 μl media per well in a 96 well plate and they were incubated (37℃, 5% CO_2) overnight to allow the cells to attach to the wells. Two μl of extracts dissolved in PBS was added to each well. Then they were incubated (37℃, 5% CO_2) for 1-5 days to allow the extracts to take effect. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (20 μl, 5 mg/ml, in PBS) was added to each well and they were incubated (37℃, 5% CO_2) for 1-5 hours to allow the MTT to be metabolized. The formazan (MTT metabolic product) was resuspended in 200 μl DMSO and mixed them thoroughly with the solvent. Finally, optical density at 560 nm and subtract background at 670 nm were read. Optical density should be directly correlated with cell quantity. The cell death was expressed as percent mean(±S.D.) dead cells compared to untreated cells (taken as 100% viable) at different concentrations of the extracts.

10. Statistical analysis

The experimental data were expressed as mean±S.D. of triplicate measurements. The results were processed by Microsoft Office Excel 2003 software. Significant differences between means were determined by Duncan's multiple range tests. $P < 0.05$ were regarded as significant.

Results

1. Total yield and purity of the ginsenosides

According to Table 2, total yield of the crude extracts were 31.2%, 37.2% and 35.0% for CWG (cultured wild ginseng roots), RG (red ginseng) and WG (white ginseng), respectively. And for purified extracts, they were 1.4%, 4.6% and 3.9%, respectively. Purity of the ginsenosides was measured by Re standard calibration. Crude extracts of CWG, RG and WG were containing 4.0%, 12.1% and 11.3% of the ginsenosides and purified extracts were containing 79.4%, 71.7% and 72.5%, respectively.

Table 2. Yield and purity of total ginsenosides from 10 gram of ground ginseng.

		Yield (g)	Purity (%)
Crude Extracts	CWG	3.12±0.14	4.0±0.8
	RG	3.72±0.37	12.1±1.2
	WG	3.50±0.22	11.3±0.9
Purified Extracts	CWG	0.14±0.05	79.4±1.3
	RG	0.46±0.04	71.7±0.9
	WG	0.39±0.02	72.5±1.5

2. DPPH radical scavenging activity

Total DPPH radical scavenging potential of purified extracts of CWG, RG and WG at the concentrations of 0.01, 0.05, 0.2 and 1 mg/ml were measured using L-ascorbic acid as the positive control. The results were shown in Fig. 1. All of the three samples possessed the strong scavenging ability on DPPH radical and their abilities were concentration-dependant. At the concentration of 1 mg/ml, their sequence was L-ascorbic acid (85.1%), WG (73.1%), CWG (68.7%) and RG (62.1%).

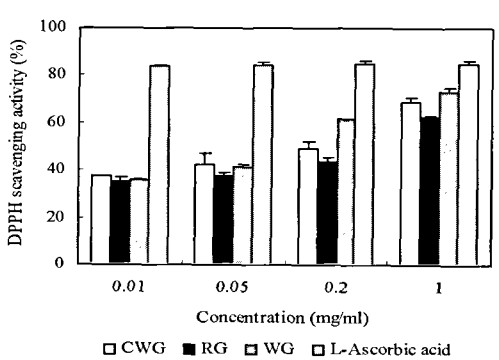

Fig. 1. Free radical scavenging capacity of the purified extracts at different concentrations as determined by the DPPH method. Results were mean±S.D. of three parallel measurements.

3. ROS (Reactive Oxygen Species) scavenging activity

ROS inhibition in postmitochondrial fraction of the LPS-induced rat was determined at the concentrations of 1 and 10 μg/ml, which was showed in Fig. 2. All of the three samples demonstrated better scavenging activity at the concentration of 10 μg/ml. Their activities were decreased in the order of RG, WG and CWG, which were 50.6%, 46.0% and 40.7%, respectively.

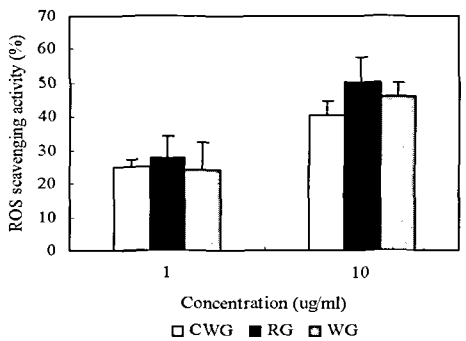

Fig. 2. ROS scavenging activity of the purified extracts at different concentrations. Results were mean±S.D. of three parallel measurements.

4. Total antioxidant activity in linoleic acid emulsion

The results for linoleic acid peroxidation were plotted in Fig. 3. CWG, RG and WG showed good lipid peroxidation inhibition activity at the concentration of 0.01 mg/ml, although they were much lower than that of α-tocopherol at the same concentration. The inhibition effect was decreased in the order of α-tocopherol, RG, WG and CWG, which were 90.4%, 41.1%, 40.7% and 34.5%, respectively.

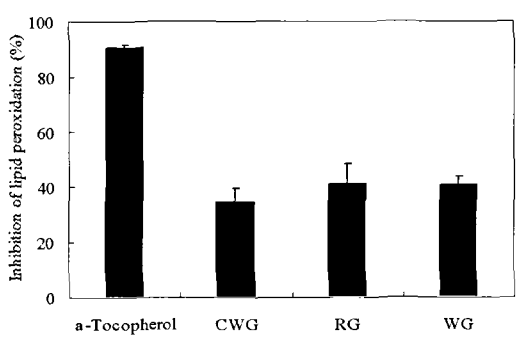

Fig. 3. Lipid peroxidation inhibition activity of the purified extracts at the concentration of 0.01 mg/ml. Results were mean±S.D. of three parallel measurements.

5. Determination of antimicrobial activities

According to the results in Table 3, all of the three samples showed antibacterial activity against *B. subtilis*. Inhibitory effect increased with increase of the sample's concentration from 5 to 20 μl/well. The purified extracts of CWG was shown to have the most effective antimicrobial activity among three samples. Against *E. coli*, CWG showed good antimicrobial activity while RG and WG showed no activity.

Table 3. Antimicrobial activity of the purified extracts by the radial diffusion method against *B. subtilis* PM125 and *E. coli* D31.

Bacteria	Sample volume (μl/well)	Antibacterial agents zone inhibition (mm)		
		CWG	RG	WG
B. subtilis PM125 (Gram-positive)	5	12	10	10
	10	15	12	11
	15	17	15	13
	20	20	16	15
E. coli D31 (Gram-negative)	5	8	-	-
	10	10	-	-
	15	12	-	-
	20	15	-	-

6. Determination of anticancer effect

The potential anticancer effect obtained with the MTT assay are shown in Fig. 4. All of the three purified extracts showed to be a concentration-dependant potent inhibitor of U-937 human hohistiocytic lympma cell proliferation and the % cell death was 33.8%, 36.8% and 40.7% after treatment with 5 mg/ml of CWG, RG and WG, respectively.

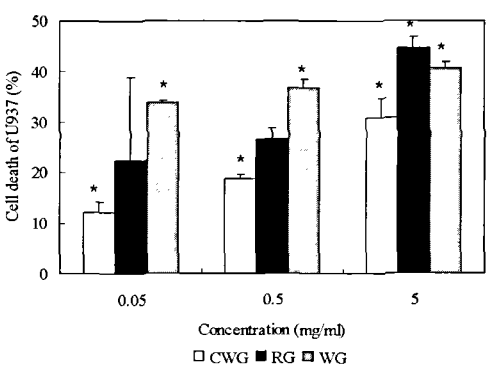

Fig. 4. Anticancer activity of purified extracts measured by MTT assay. Y axis means death percentage of U-937 human hohistiocytic lympma cell and x axis means concentrations of the extracts treated in the assay. Results were mean±S.D. of three parallel measurements. *Significantly different at $P < 0.05$ as compared with the control.

Discussion

The results presented in this study provide the first evidence that purifying with three macroporous resins was significantly increased the contents of the total ginsenosides (Table 2). Macroporous resin is an artificially synthetic polymer adsorbent which has multi hole three dimension structures. It is a new type resin developed from application of ion exchange and other adsorbent. Macroporous resin is widely used in medicine manufacturing and in extraction of active ingredients in natural plants such as ginsenosides, gypenosies, saponin on diosgenin, seviosides and notoginsenosides etc.[17-20] This process has the advantages of nontoxic, good specialty, easy operation, low cost and repeated use of the resin. So it

may be a trend method for industrial production instead of toxic organic solvents. The purifying process included using the first resin (AB-8) to adsorb water solution of coarse ginsenosides to eliminate water soluble impurities and desorbing the adsorbed resin with organic solution or its water solution; and using the second (Amberlite IRA 900 Cl) and the third resins (Amberlite XAD 16) to adsorb impurity selectively from the organic eluent of ginsenosides. Standard ginsenosides informed that there was some impurities and some of the ginsenosides were missed in purified samples (data not shown). These problems could be resolved by improving chromatography conditions. He et al.[21] improved the purity of the ginsenosides to 96% by changing the pH of the solvent and adding salt such as sodium chloride.

The main pharmacologically active constituents of ginseng are believed to be ginsenosides, derivatives of the triterpene dammarane structure[22]. In order to compare biological activities of the purified CWG, RG and WG extracts, of which main components were ginsenosides, antioxidant activity, antimicrobial activity and anticancer activity were determined. Antioxidant activities measured in this study including DPPH radical scavenging activity, ROS scavenging activity and lipid peroxidation inhibition activity.

According to the result of DPPH radical scavenging activity (Fig. 1), the scavenging effect was increased with the concentration increasing in all of the three extracts, however, they were lower than that of L-ascorbic acid. At the concentration of 1 mg/ml, the scavenging effects were L-ascorbic acid > WG > CWG > RG. This activity was much higher than the earlier observation done on North American ginseng extracts with similar method at the same concentration[23] and similar with the result researched by Kim et al[22]. Also, all of the three extracts were showed good ROS scavenging activity and their activities were better at the higher concentration (Fig. 2). Their ROS scavenging activities at 10 μg/ml were RG > WG > CWG. Fig. 3 demonstrated lipid peroxidation inhibition activity of three purified extracts. The inhibition of three extracts was RG > WG > CWG, but all of them were much lower than that of α-tocopherol at the same concentration. This result was better than EtOAC extracts of wild ginseng leaves and cultivated ginseng at the same concentration, which were observed by Jung et al.[14] But whether the inhibition activity of three purified extracts was concentration-dependant, it needs further research.

Antibacterial activity of the ginsenosides was not reported much. In this study, two different kinds of bacteria were used to determine the antibacterial activity with radial diffusion method. Purified extracts of CWG, RG and WG were all showed good antibacterial activity against *B. subtilis* PM125, while only CWG showed activity against *E. coli* D31 (Table 3).

Anticancer activity of the three purified extracts was determined by observing cell viability of human hohistiocytic lympma cell (U-937) by MTT analysis in this study. According to the result, cell viability of U-937 was showed significant difference compared to the control, which was not treated with the extracts (Fig. 4). All of the three extracts caused significant cell death at the concentration of 5 mg/ml as compared with the control.

In conclusion, this study showed that purifying with three linked macroporous resins was significantly increased the purity of the total ginsenosides. Cultured wild ginseng roots showed good biological activities as well as red and white ginseng, however, their activities were quite different. These results should assist the development of new ginsenosides purification method and provide a possibility of high quality but low price ginsenosides products. In further study, how to improve the purification efficiency much better and the relations between ginsenosides distribution and their biological activities should be researched.

References

1. Bulgakov, V.P., Khodakovskaya, M.V., Labetskaya, N.V., Chernoded, G.K., Zhuravlev. Y.N. The impact of plant rolC oncogene on ginsenoside production by ginseng hairy root cultures. Phytochemistry 49: 1929-1934, 1998.
2. Jung, J.D., Park, H.W., Hahn, Y., Hur, C.G., In, D.S., Chung, H.J., Liu, J.R., Choi, D.W. Discovery of genes for ginsenoside biosynthesis by analysis of ginseng expressed sequence tags. Plant Cell Rep. 22: 224, 2003.
3. Xie, J.T., Mehendale, S.R., Wang, A.B., Han, A.H., Wu, J.A., Osinski, J., Yuan, C.S. American ginseng leaf: ginsenoside analysis and hypoglycemic activity. Pharmacol. Res. 49: 113-117, 2004.
4. Fuzzati, N., Gabetta, B., Jayakar, K., Pace, R., Peterlongo, F. Liquid chromatography-electrospray mass spectrometric identification of ginsenosides in Panax ginseng roots. J. Chromatogr. A 854: 69-79, 1999.
5. Kwon, S.W., Han, S.B., Park, I.H., Kim, J.M., Park, M.K., Park, J.H. Liquid chromatographic determination of less polar ginsenosides in processed ginseng. J. Chromatogr. A 921: 335-339, 2001.
6. Lau, A.J., Woo, S.O., Koh, H.L. Analysis of saponins in raw and steamed Panax notoginsengusing high-performance liquid chromatography with diode array detection. J. Chromatogr. A 1011: 77-87, 2003.

7. Lau, A.J., Seo, B.H., Woo, S.O., Koh, H.L. High-performance liquid chromatographic method with quantitative comparisons of whole chromatograms of raw and steamed Panax notoginsen. J. Chromatogr. A 1057: 141-149, 2004.
8. Li, L., Zhang, J.L., Sheng, Y.X., Ye, G., Guo, H.Z., Guo, D.A. Simultaneous quantification of six major active saponins of Panax notoginseng by high-performance liquid chromatography-UV method. Journal. Pharmaceut. Biomed. 38: 45-51, 2004.
9. Park, M.K., Park, J.H., Han, S.B., Shin, Y.G., Park, I.H. High-performance liquid chromatographic analysis of ginseng saponins using evaporative light scattering detection. J. Chromatogr. A 736: 77-81, 1996.
10. Wang, H.C., Chen, C.R., Chang, C.J. Carbon dioxide extraction of ginseng root hair oil and ginsenosides. Food Chem. 72: 505-509, 2001.
11. Liu, C.P., Luo, G.Q., Dai, M., Wang, Z.S. Study on the adsorption of soyasaponins by macroporous resins. Ion Exchange and Adsorption 19: 318-323, 2003.
12. Brand-Williams, W., Cuvelier, M.E., Berset, C. Use of a free radical method to evaluate antioxidant activity. Lebensm.-Wiss. u.-Technol. 28: 25-30, 1995.
13. Thomas, P., Herbert, D.G., James, P.K. Production of reactive oxygen by mitochondrial from normoxic and hypoxic rat heart tissue. Free Rad. Biol. Med. 13: 289-297, 1992.
14. Jung. C.H., Seog, H.M., Choi, I.W., Cho. H.Y. Antioxidant activities of cultivated and wild Korean ginseng leaves. Food Chem. 92: 535-540, 2005.
15. Gulcin, I., M. Oktay, E. Kirecci, O.I. Kufrevioglu. Screening of antioxidant and antimicrobial activities of anise (Pimpinella anisum L.) seed extracts. Food Chem. 83: 371-382, 2003.
16. Lehrer, R.I., Rosenman, M., Harwig, S.S.S.L., Jackson, R., Eisenhauer, P. Ultrasensitive assays for endogenous antimicrobial activity. J. Immunol. Methods 137: 167-173, 1991.
17. Ma, T.J., Tu, P.F., Lu, F.J. Isolation and purification of total tubeimosides from Bolbostemma paniculatum by macroporous resin. China Journal of Chinese Material Medica 30: 744-746, 2005.
18. Ou, L.L., Shi, Z.Q., Shi, R.F., Wang, Z., Wang, R.F. Studies on separation and purification of saponins from Panax notoginseng with strongly polar macroporous adsorption resin. Chinese Traditional and Herbal Drugs 34: 905-907, 2003.
19. Wang, R.F., Ou, L.L., Kong, D.L., Shi, Z.Q. Isolation and purification of camptochecine and vincoside-lactam from Camptocheca acum inata fruit by macroporous adsorption resins. Chinese Traditional and Herbal Drugs 36: 849-851, 2005.
20. Wu, H., Liang, H., Wu, D.C., Yuan, Z.H. Isolation and purification of total saponins from cornus officinalis by column chromatography of macroreticular resin. J. Fourth Mil. Med. Univ. 24: 689-691, 2003.
21. He, K.J., Han, W., Luan, H.W., Yang, L. A facile determination method for total ginsenosides combined purification and spectrometry. Chin. J. Pharm. Anal. 23: 284-288, 2003.
22. Kim, Y.K., Guo, Q., Packer, L. Free radical scavenging activity of red ginseng aqueous extracts. Toxicology 172: 149-156, 2002.
23. Kitts, D., An, D.W., Hu, C. Antioxidant properties of a North American ginseng extract. Mol. Cell. Biochem. 203: 1-10, 2000.