Antitumor Activities of Red Ginseng Acidic Polysaccharide(RGAP) as an Immunomodulator

Jong Dae Park[a,*], Young Sook Kim[a], Han Jae Shin[a], Kyung Mee Park[a], Yi Sung Kwak[a] and Toshihiko Toida[b]

Division of Ginseng Efficacy, KT&G Central Research Institute, Taejon 305-345 KOREA[a],

Dept. of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, JAPAN[b]

Abstract

A red ginseng acidic polysaccharide(RGAP) with immunomodulating antitumor activities was isolated from Korean red ginseng. The molecular weight of RGAP was estimated to be 12-450 kDa by gel filtration chromatography. RGAP was found to increase survival rate and to inhibit of tumor growth significantly in a dose dependent manner in mice transplanted with tumor cells. RGAP significantly promoted nitric oxide(NO) production from peritoneal macrophages both*in vivo and in vitro*. Western blot analysis exhibited a newly synthesized inducible nitric oxide synthase(iNOS) protein band in the RGAP treated group. It seems likely that immunomodulating antitumor activities of RGAP are mainly mediated by NO production of macrophage. RGAP was further purified by ultrafiltration and anion exchange chromatography on DEAE-sepharose, followed by gel filtration on Sephacryl S-300 to give an active fraction(GFP) with stronger NO production in murine macrophages. GFP increased survival rate ten times compared to RGAP in male ICR mice transplanted with sarcoma 180 and also showed more potent tumoricidal activities of natural killer cells than RGAP. Sugar composition(mol %) of GFP was found to be arabinose:rhamnose:xylose:galacturonic acid:mannose:galactose:glucose(10:9:1:25:8:20:27) by GC/MS. The results suggest that clinical trials of RGAP in immunotherapy against cancer are highly feasible.

Introduction

Ginseng has been long reported to strengthen the organism's resistance to harmful physical, chemical and biological stresses. It is believed that immunomodulatory activities of ginseng is related to its regulatory effect on the immune system, which plays an important role in the pro-

tective mechanism of the body. Immunomodulatory activity constitutes a major arm of its biological effects. In the context of immunomodulatory activities of ginseng, more attention was paid on polysaccharide. Polysaccharide from the root of *Panax ginseng* has been recently known to have mitogenic activities[1], hypoglycemic activities[2-5] and antitumor activities[6-8] as well as immunostimulating activities in the cyclophosphamide-treated immunosuppressed mice[9]. Furthermore, acidic polysaccharides from ginseng root were found to inhibit toxohormone-L-induced lipolysis[10] and reduce the incidence rate of benzo[a]pyrene-induced autochthonous neoplasm[11]. Accordingly, acidic polysaccharide may be a primary candidate of medicinal applications of ginseng. However, while extensive studies on the immunomodulating activities[12-14] of polysaccharide from white ginseng were carried out, there is still scarce information about those of red ginseng, the steam-processed form of fresh ginseng. This result has led us to investigate in more detail in this regard. Therefore, the purpose of the present study is to revaluate immunomodulating antitumor potentials of red ginseng acidic polysaccharide(RGAP)[15,16], whose active substance is chemically to be elucidated, and also to search for new immunomodulatory compounds from water soluble non-saponin constituents of Korean red ginseng.

Experimental Methods and Results

Preparation and chemical characterization of RGAP

RGAP was isolated from Korean red ginseng(*Panax ginseng* C. A. Meyer) as described in the previous report[16]. RGAP was composed of 56.9% acidic sugar and 28.3% neutral sugar, as determined by carbazole and phenol sulfuric acid assays, respectively. Protein was scarcely detected, not more than 0.1% as determined by the Lowry method. Less than 0.006 EU(endotoxin unit) of endotoxin was present in 1 mg of RGAP as tested by *Limulus amebocyte* lysate assay (Sigma, USA). This level of endotoxin did not affect the experimental result obtained by RGAP. The molar compositin was identified as 51.8% glucuronic acid, 26.1% glucose, 5.1% galacturonic acid and 1.6% arabinose by GLC analysis of the corresponding alditiol acetate. The molecular weight is estimated to be a mixture of 12 KDa and 450 KDa by Sephacryl S-300 gel filtration chromatography.

Effect of RGAP on the distribution of spleen cell subtypes

RGAP was administered intraperitoneally to mice for seven consecutive days and the distribu-

tion of subtypes of spleen cells was analyzed by flow cytometry. Spleen cells were isolated and labeled for CD4+(helper/inducer T cell), CD8+(cytotoxic/suppressor T cell), B lymphocytes and macrophages-specific antibodies for the analytic flow cytometry. The proportions of CD4+, CD8+ were decreased, while the fraction of CD45R/B220+ B lymphocyte was marginally decreased by RGAP. However, CD11b cells such as macrophage and natural killer cells were significantly increased in the RGAP-treated group. As spleen weight and total spleen cell number were increased in the RGAP-administered mice, the increase of macrophages is thought to be a major contributor of immunomodulating activities of RGAP as well as splenic hyperplasia[16].

Effect of RGAP on NO(nitric oxide) production and iNOS(inducible nitrix oxide synthase)

Recent survey demonstrated that NO released from macrophage is a mediator of microbicidal and tumoricidal activities[17,18]. In order to investigate relationship between increased macrophages in spleen and tumoricidal effects, we have examined effect of RGAP on NO production and iNOS in BALB/c mice treated intraperitoneally with RGAP. As shown in Figure 1, Nitrite(NO) production of macrophages was increased at the dose of 100, 300 mg/kg. Western blot analysis showed a 130 kDa iNOS protein band in the RGAP treated group but no detectable 130 kDa band in control group. And also RGAP(50-500 μg/ml) was found to show to increase nitrite(NO)

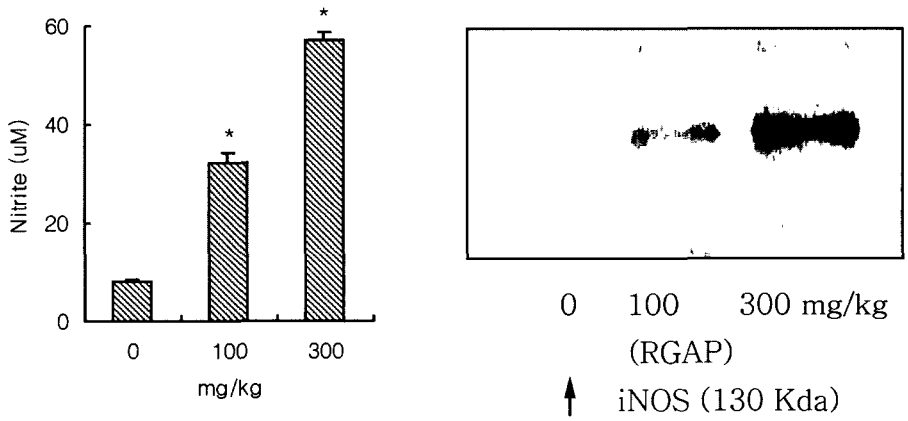

Fig. 1. Effect of RGAP on NO production and iNOS expression in the peritoneal macrophage in vivo. BALB/c mice were treated intraperitoneally with 100 and 300 mg/kg of RGAP for seven consecutive days. Zero denotes saline-treated control. NO was determined as nitrite by using Griess reagent. Represnted bands indicate 130 kDa iNOS in the peritoneal macrophages from RGAP -treated mice. The number of mice in each treatment group was fifteen. *indicates the value significantly different from control at $p<0.01$.

production in the peritoneal macrophages either in the absence or in the presence of IFN-γ *in vitro*. A transcription inhibitor, ATD(actinomycin), a translation inhibitor, CHX(cycloheximide) and an iNOS inhibitor, NMMA inhibited nearly NO production. Western blot analysis reveals that RGAP induced an iNOS protein in the presence of IFN-γ and the induction was inhibited by pretreatment of ATD and CHX. NMMA, due to its competitive inhibition on iNOS, did not affect the induction of 130 kDa iNOS whereas it completely abolished NO production. These results suggests that the immunomodulating activity of RGAP should be mediated by production of nitric oxide.

Effect of RGAP on tumoricidal activity of macrophage in vivo

Effect of RGAP on macrophage tumoricidal activity was also evaluated to elucidate antitumor activity, responsible for NO production. When RGAP were intraperitoneally administered to Balb/C mice for seven consecutive days, RGAP(100 mg/kg) induced low detectable tumoricidal activity, while RGAP at a dose of 300 mg/kg induced more potent tumoricidal activity to kill target WEHI 164 fibrocarcinoma cell in a ratio of target cell:effector cell (1:5, 1:10) when compared to control group[19]. This result further suggests that macrophage is thought to be a major contributor of antitumor activity of RGAP.

Antitumor activities of RGAP in animal model

The most important aim of cancertherapy is to increase the survival time of cancer patients enabling them to live in comfort. In our parallel study, survival rate of RGAP in male ICR mice transplanted with sarcoma 180 ascite tumor was evaluated. RGAP was intraperitoneally administered to mice once a day for seven consecutive days after tumor(1×10^6 cells) inoculation. RGAP was found to exhibit survial rate in a dose dependent manner at the dose of 50, 100, 300 mg/kg and longer activity than polysaccharide K (positive control), antitumor polysaccharide, isolated from mycelium of *Coriolus versicolor*. Especially, RGAP at a dose of 300 mg/kg showed similar survival effect to that of 5-fluorouracil(7.8 mg/kg). To further clarify the immunomodulating antitumor effects of RGAP, the inhibition of RGAP on the growth of sarcoma 180 tumor cells and 3LL Lewis lung carcinoma in a solid form was also examined. RGAP was intraperitoneally injected at doses of 100, 300 mg/kg daily for seven days starting one day after tumor transplantation to groin of ICR mice. RGAP showed significantly strong inhibitory effects against tumor growth in both sarcoma 180 tumor cells and 3LL Lewis lung carcinoma at the dose of 300 mg/kg

and the inhibition ratios were about 30 and 40%, respectively[19].

Isolation and characterization of active substances(GFP) from RGAP

To search for biologically active fraction from RGAP, RGAP was further purified by ultrafiltration system using 10 KDa membrane and anion exchange chromatography on DEAE-Sepharose, followed by gel filtration on Sephacryl S-300, to give an active fraction(GFP) with monitoring mitogenic activity in mouse spleen cells and NO production of murine macrophages. GFP showed NO production activity over five times as strong as that of RGAP, suggesting it to be a major active substance. To investigate sugar composition of GFP, it was methanolyzed in 1.0 M HCl/MeOH at 80°C for 24 hrs and trimethylsilylated by BSTFA/pyridine, and then analyzed by GC/MS. GC/MS was performed on a HP5868 equipment. GC conditions are as follows : column temp. 110 to 260°C, 10°C/min, injection port : 280°C, column : DB1 0.25 mm × 25 m capillary column. Sugar composition(mol %) of GFP was found to be composed of arabinose: rhamnose:xylose:galacturonic acid:mannose:galactose:glucose(10:9:1:25:8:20:27) by GC/MS, indicating that it is of great immunological significance in regard to the antitumor properties of RGAP.

Antitumor activities of active substance(GFP) isolated from RGAP

Comparative survival rates of RGAP and active substance(GFP) in male ICR mice transplanted with sarcoma 180 ascite tumor were evaluated. RGAP and GFP were treated in the same way as

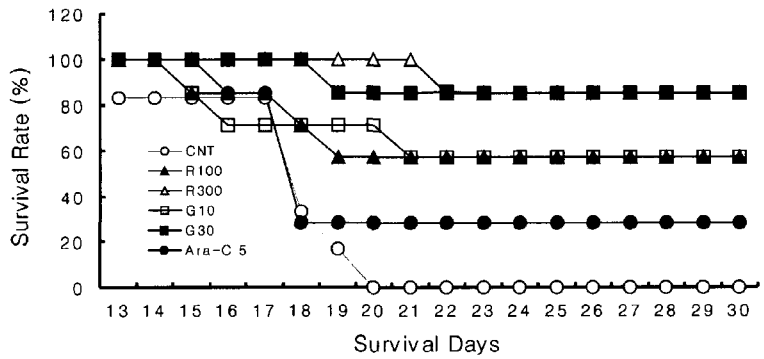

Fig. 2. Survival rate of RGAP and GFP in male ICR mice transplanted with sarcoma 180 ascite tumor. Sarcoma 180 cells(1×10^6) were inoculated intraperitoneally in ICR mice. Mice were treated with i.p. injections of GFP(10, 30 mg/kg) and RGAP(100, 300 mg/kg) for seven consecutive days. Results represent two experiments, wth a total of 14 mice each group.

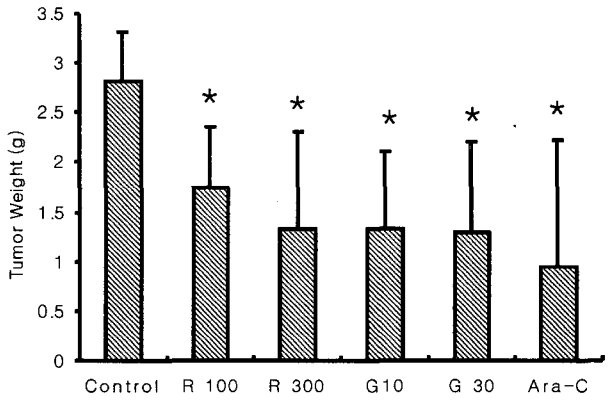

Fig. 3. Antitumor activity of RGAP and GFP against solid tumor. B16 melanoma cells (1×10^6) were subcutaneously in the groin of C57BL/6 mice. Mice were treated with i.p. injections of GFP(10, 30 mg/kg) and RGAP (100, 300 mg/kg) for seven consecutive days. On day 20, mice were sacrificed and tumorr weights were measured. Results represent two experiments, wth a total of 14 mice each group. $^*P<0.05$ *vs* control CNT : control, R : RGAP, G: GFP.

described above for antitmor activities in animal model. As shown in Figure 2,3, GFP was found to exhibit survial rate ten times as strong as that of RGAP in a dose dependent manner at the dose of 10, 30 mg/kg and longer activity than Ara-C(5 mg/kg) as positive control, commercial antitumor agent for acute lymphocytic leukemia. GFP at a dose of 10 mg/kg showed similar survival effect to that of RGAP(100 mg/kg). To further investigate the immunomodulating antitumor effects of GFP, the inhibition of GFP on the growth of B16 melanoma cells in a solid form was also examined. GFP and RGAP were intraperitoneally injected at doses of 100, 300 mg/kg daily for seven days starting one day after tumor transplantation to groin of ICR mice. GFP also showed significantly inhibitory effects about ten times as strong as that of RGAP against tumor growth and the inhibition ratios of GFP at the dose of 10 and 30 mg/kg were 47 and 46%, respectively.

And also, to evaluate potential role of NO in GFP-induced tumoricidal activity, GFP was intraperitoneally administered to Balb/C mice for seven consecutive days and cytotoxicity was measured in 18h supernatants of macrophages cultured with GFP. As shown in Fig. 4, 5, GFP(10, 30 mg/kg) was found to induce tumoricidal activity about ten times as strong as RGAP to kill target Yac-1 lymphoma and P815 mastocytoma cells when compared to RGAP group. This result further suggests that GFP is active substance with stronger antitumor activity mediated by NO production.

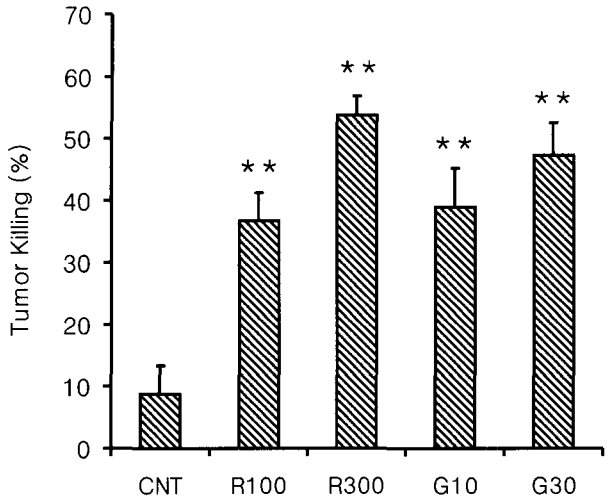

Fig. 4. Tumoricidal activities of RGAP and GFP against Yac-1 lymphoma cells in murine macropahages. BALB/c mice were treated intraperitoneally with 10, 30 mg/kg of GFP or 100, 300 mg/kg of RGAP for seven consecutive days. Macrophages were isolated and cultured for 48h. Afer 48h, the supernatant were cultured for additional 18h with Yac-1 lymphoma cells. Tumoricidal activity of treated macrophages was then determined by PMS/MTS method. *CNT: control, R: RGAP, G: GFP, **Indicates the values significantly different from control at $P<0.01$.

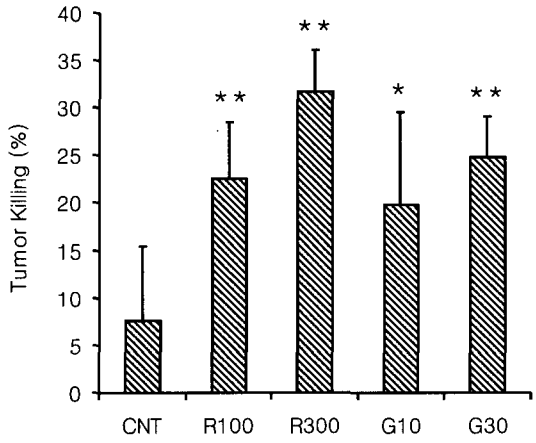

Fig. 5. Tumoricidal activities of RGAP and GFP against P815 mastocytoma cells in murine macropahages. BALB/c mice were treated intraperitoneally with 10, 30 mg/kg of GFP or 100, 300 mg/kg of RGAP for seven consecutive days. Macrophages were isolated and cultured for 48h. Afer 48h, the supernatant were cultured for additional 18h with P815 mastocytoma cells. Tumoricidal activity of treated macrophages was then determined by PMS/MTS method. CNT: control, R: RGAP, G: GFP, *Indicates the values significantly different from control at $P<0.05$, ** Indicates the values significantly different from control at $P<0.01$.

Discussions

Especially, it is noteworthy that RGAP was found to contain acidic sugar two folds as much as neutral polysaccharides, and also, one and half times as much as acidic sugar(43. 1%) of acidic polysaccharide fraction(Ginsan), purified from white ginseng by Yun *et al*[20]. As highlighted in the introductory section, it is surprising that very few are known to be effective on immunomodulating activities of RGAP despite an extensive knowledge of those of acidic polysaccharide of white ginseng, considering acidic sugar's importance to immune response. To determine the molar % composition of RGAP, the alditol acetates of hydrolysate were analyzed using reference alditol acetate by GLC. Four kinds of aldose and uronic acid were detected and glucuronic acid mainly comprized 51.8 molar %, indicating that glucuronic acid is important for the activity.

In the mice administered RGAP, there was a splenic hyperplasia ; spleen weights were increased as well as total spleen cell numbers. By analytic flow cytometry, macrophages were increased both in the proportion and total cell number. Correlation between splenic hyperplasia and increased splenic macrophages are well documented[21-24]. In the case of immunization of *Haemophilus influenza*, splenomegaly was induced and the number of splenic macrophages was increased[21]. One of major cytokines produced by macrophage, interleukin 1(IL-1), has been known to cause splenic granulocytic hyperplasia in mice[22]. Kim *et al.*[12] have recently reported that acidic polysaccharide fraction of white ginseng has been found to show increased antibody secreting cells to SRBC and phagocytic activity of reticuloendothelial system, as well as antitumor activity against the solid form of sarcoma 180 in ICR mice, suggesting it to exhibit stimulation on B and T cells together with macrophages. And also, Benxiang *et al.*[25] reported that saponin and polysaccharide of ginseng increased the phagocytosis of the reticuloendothelial system not only in normal mice but also in tumor bearing mice. Therefore, our present data represents the *in vivo* exposure of ginseng polysaccharides activates macrophages and induces a splenic hyperplasia. At the present, it seems likely that the immunomodulatory roles of RGAP rely on the activation of macrophages. Macrophage serves vital roles in host defense against against tumors, including tumor cytotoxicity and stimulation of antitumor lymphocytes[26]. Recent works showed that NO released from macrophage is a mediator of microbicidal and tumoricidal activity[27]. RGAP increased markedly NO release from macrophage in the absence and presence of IFN- in a concentration-dependent manner, suggesting that its anticancer effect is contributed by the augmented NO production. When the comparative effects on NO production was evalu-

ated between RGAP and Coplang(brand name), polysaccharide K from *Coriolus versicolo,* RGAP was two times as strong as Coplang, suggesting the possibility of the development of new immunomodulating anticancer drug from red ginseng. Recently Fan *et al.*[28] have reported that ginsenoside Rg1 enhanced the tumor cell killing by NO production from IFN-γ-activated macrophages. In that context, we compared the effects of RGAP and ginsenosides Rg1, Rb1 on the production of NO from the IFN-γ-stimulated macrophages and confirmed RGAP was effective ten times more than that of ginsenoside Rg1. These above described results suggest that RGAP activates macrophage to produce NO, which in turn suppresses further T-cell and B-cell proliferation so that decrease markedly PFCs response. This means that RGAP works as an immunomodulator responsible for anticancer activity through NO production to inhibit the growth of cancer cell lines. In our *in vivo* study, we demonstrated that RGAP induce pronounced tumoricidal activities of peritoneal macrophage *via* NO secretion as shown in Fig. 1. These results suggested that RGAP might serve as a second signal to induce IFN-γ treated murine macrophages to lyse tumor target cells through an NO-dependent pathway. For the purpose of isolation of active substance from RGAP, RGAP was subjected to anion exchange chromatography with NO producing activity-guided fractionation to give an active subfraction(GFP). GFP was found to exhibit immunomodulatory antitumor activities about ten times as strong as RGAP, suggesting that it is of great immunological significance in regard to the antitumor properties of RGAP. Sugar composition(mol %) of GFP was found to be composed of arabinose:rhamnose:xylose :galacturonic acid:mannose:galactose:glucose in the molar ratio described in the results section. Antitumor polysaccharide have been in many kinds of Basidiomycetes and Ascomycetes as lentinan, schizophyllan, PS-1426, TAK and PSK. Lentinan, schizophyllan and TAK were found to be composed of only carbohydrates, whereas, PSK require a protein moiety for activity. As early as 1969, it was ahown by Chihara *et al.* rhat two polysaccharide fractions from *Lentinus edodes* effected complete regression or growth inhibition of tumors developed in mice following subcuteneous injection of sarcoma 180 ascites. Further studies on the molecular structure of the active polysaccharides resulted in establishment of a single compound(lentinan) with a molecular weight around 1000 kDa and having a backbone of (1→3)-β-D-glucan[29]. In 1993, Tomaoda *et al.* have isolated two acidic polysaccharides, called ginsenans S-IA and S-IIA, from the root of *Panax ginseng* and structure of S-IA was identified to be composed of arabinose:galactose:galacturonic acid in the molar ratio of 8:8:1 and S-IIA, arabinose:galactose:glucose:galacturonic acid, in the molar ratio of 15:10:2:5. Both ginsenans S-IA and S-IIA was found to show remarkable

reticuloendothelial system-potentiating activity, having α-3,5-branched L-arabinose and β-1,4-linked D-galactose units[30]. It is especially characteristic of GFP that galacturonic acid, galactose and glucose might be mainly composed as major backbone. We are now investigating partial structure of GFP by acid-hydrolysis on the bais of molar sugar composition.

In conclusion, these above results suggest that RGAP works as an immunomodulator responsible for anticancer activity through NO production by macrophage to inhibit the growth of cancer cell lines and further clinical trials in immunotherapy against cancer are highly feasible. Extensive investigations on the roles of immunotherapy using biological response modifiers need to be performed in a more detail in conjunction with immunomodulating anticancer activities of RGAP. Additionally, we are currently engaged in carbohydrate sequencing analysis of an active fraction(GFP) fully purified from RGAP using chemical and spectral methods, and also study of structure-activity relationship of carbohydrate chains.

Refernces

1. Eun, S.M., Hung, N.K., Nam, L.K. and Cheung, K.Y., *Korean J. Ginseng Sci.* **13**, 215(1989)
2. Konno, C., Sugiyama, K., Kano, M., Takahashi, M. and Hikino, H., *Planta Medica* **50**, 443(1983)
3. Hikino, H., Oshima, Y., Suzuki, Y. and Konno, C., *Shoyakugaku Zasshi,* 331(1985)
4. Konno, C. and Hikino, H., *Int. J. Crude Drug Res.* **25**, 53(1987)
5. Konno, C., Murakami, M., Oshima, Y. and Hikino, H., *J. Ethnopharmacol.* **14**, 69(1985)
6. Moon, C. K., Sim, K. S., Lee, S. H., Park, K. S., Yun, Y. P., Ha, B. J. and Lee, C .C., *Arch. Pharm. Res.* **6**, 123(1983)
7. Wang, B.X., Lhi, J.C. and Cui, A.J., *5th, Int. Ginseng Symp.*(Abstract)(1988)
8. Quian, B.C., Zang, X.X., Li, B., Xu, C.Y. and Peng, X.Y., *Acta. Pharmacol. Sin.* **8**, 227(1987)
9. Yuan, X.N., Shang, X.H., Jin, M.L. and Shang, K., *Shenyang yaoxueyuan Xeuebao*, 162(1986)
10. Lee S. D. and Okuda, H., *Korean J. Ginseng Sci.* **14**, 67(1990)
11. Lee, Y.S., Chung, I.S., Lee, I.R., Kim, K.H., Hong, W.S. and Yun, Y.S., *Anticancer Res.* **17**, 323(1997)
12. Kim. Y.S., Kang K.S. and Kim, S. I., *Arch. Pharm. Res.* **13**, 330(1990)

13. Benxiang, W., Jingchao, C. and Aijing, L., *Advances in Chinese Medicinal Materials Res*, **519** (1985).
14. Fan, Z. H., Isobe, K. I., Kikuchi, K. and Nakashima, I., *American J. Chinese Medicine*, **23**, 279(1995)
15. Park, K. M., Jeong, T. C., Kim, Y. S., Shin, H. J., Nam, K. Y. and Park, J. D., *Natural Product Science*, 6(1), 31-35 (2000)
16. Park, K. M., Kim, Y. S., Jeong, T. C., Joe, C. O., Shin, H. J., Lee, Y. H., Nam, K. Y., and Park, J. D., *Planta Medica,* 67(2), 122-126 (2001)
17. Mullins, D. W., Burger, C. J. and Elgert, K. D., *The Journal of Immunology,* 6811(1999)
18. Stuher, D. J. and Nathan, C. F., *J. Exp. Med.*, **169**, 1543(1989)
19. Kim, Y. S., Park, K. M., Shin, H. J., Song, K. S., Nam, K. Y. and Park, J. D., *Yakhak Hoeji,* **46**(2), 113(2002)
20. Kim, K. H., Lee, Y. S., Jung, I. S., Park, S. Y.,Chung, H. Y., Lee, I. R. and Yun, Y. S., *Planta Medica*, **64**, 110(1998)
21. Ambrosino, D. M., Bolon, D., Collard, H., Van Etten, R., Kanchana, M. V. and Finberg, R. W., *J. Immunol.* **149**, 3978(1992)
22. Benjamin, W. R., Tare, N. S., Hayes, T. J., Becker, J. M. and Anderson, T. D., *J. Immunol.* **142**, 792(1989)
23. Gahan, C. G., Barrett, J.R., O'Brien, M.G., O'Sullivan, G.C., Shanahan, F and Collins, J. K., *J. Leukoc. Biol.* **62**, 726(1997)
24. Kharitonova, A. M., Grigor'eva, L. V., Bykovskaia, S. I., Ageeva, V. A., Stepanova, L. K. and Belaia, IuA, *Zh Mikrobiol. Epidemiol. Immunobiol.* **9-10**, 57(1992)
25. Benxiang, W., Jingchao, C. and Aijing, L., *Advances in Chinese Medicinal Materials Res*, **519** (1985).
26. Mullins, D. W., Burger, C. J. and Elgert, K. D., *The Journal of Immunology,* 6811(1999)
27. Stuher, D. J. and Nathan, C. F., *J. Exp. Med.*, **169**, 1543(1989)
28. Fan, Z. H., Isobe, K. I., Kikuchi, K. and Nakashima, I., *American J. Chinese Medicine*, **23**, 279(1995)
29. Chihara, G., Maeda, Y., Hamuro, J., Sasaki, T. and Fukoka, F., Natura, **222**, 687(1969)
30. Tomoda, M., Hirabayashi, K., Shimizu, N., Conda, R., Ohara, N. and Takada, K., *Biol. Pharm. Bull.*, **16,** 1087 (1993)