Immunomodulatory Activity of Polysaccharide from the Roots of *Actinidia kolomikta* on Macrophages

Di Guan (Corresponding author)
Graduate School of Life and Environmental Sciences, University of Tsukuba
1-1-1 Tennodai, Tsukuba 305-8577, Ibaraki, Japan
Tel: 81-29-853-6972 E-mail: kanteki09@hotmail.co.jp

Zhenya Zhang
Graduate School of Life and Environmental Sciences, University of Tsukuba
1-1-1 Tennodai, Tsukuba 305-8577, Ibaraki, Japan
Tel: 81-29-853-4712 E-mail: tyou6688@sakura.cc.tsukuba.ac.jp

Yingnan Yang
Graduate School of Life and Environmental Sciences, University of Tsukuba
1-1-1 Tennodai, Tsukuba 305-8577, Ibaraki, Japan
Tel: 81-29-853-8830 E-mail: yo.innan.fu@u.tsukuba.ac.jp

Guoqing Xing
Graduate School of Life and Environmental Sciences, University of Tsukuba
1-1-1 Tennodai, Tsukuba 305-8577, Ibaraki, Japan
Tel: 81-29-853-6972 E-mail: xingguoqing@hotmail.com

Jiqiang Liu
Biomedical Research Institute, Advanced Industrial Science and Technology
1-1-1 Higashi, Tsukuba 305-8566, Ibaraki, Japan
Tel: 81-29-861-6053 E-mail: tjljq1977jp@yahoo.co.jp

Received: December 29, 2010 Accepted: January14, 2011 doi:10.5539/ijb.v3n2p3

Abstract

Actinidia kolomikta is a species of wild plant that grows wildly in the northern part of Indochina. However there are no reports about its immunomodulatory activity. In this study, the immunomodulatory activities of crude polysaccharide (CP) fraction from the root of *Actinidia kolomikta* on macrophage RAW 264.7 cells were investigated. Results showed that treatment with 2.5-25 μg/ml of CP stimulated macrophage proliferation, nitric oxide production and phagocytosis in a dose-dependent manner. Treatment with 5 μM Doxorubicin (DOX) resulted in macrophages survival rate decrease (12.6%). However co-incubation with 25 μg/ml of CP, the DOX-induced macrophage survival rate was 89.8%. At the same time the apoptosis of macrophage decreased from 37.8% (5 μM DOX) to 21.5% (co-incubation with 10 μg/ml of CP). It is suggested that CP possesses immunomodulatory activity on macrophages and protects macrophages from DOX damage.

Keywords: Crude polysaccharide, Macrophages, Immunomodulation, Apoptosis

1. Introduction

The low immune function of an organism may not only result in the generation and development of a tumor, but

may also be one of the most important factors that prevent the tumor patient's recovery. Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of diseases. Macrophages play a significant role in the host defense mechanism. When activated, they activate phagocytic activity, produce and release reactive oxygen species (ROS) and nitric oxide (NO) in response to stimulation with various agents and can inhibit the growth of a wide variety of tumor cells and micro-organisms (Oliveira *et al.*, 2006; Schepetkin *et al.*, 2008). Macrophage also secrete cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1, IL-6β, IL-8, IL-12, IFN-γ and IFN-β2. Moreover, the immunomodulatory activity not only involves effects on macrophage activation but also on cell proliferation and differentiation (Schepetkin and Quinn, 2006).

Papers report that polysaccharide from the plant and mushroom can enhance and activate macrophage immune responses, leading to immunomodulation, anti-tumor activity, wound-healing and other therapeutic effects (Berner *et al.*, 2005; Sakurai *et al.*, 1997; Naeini *et al.*, 2010). Polysaccharides derived from higher plants are relatively nontoxic and do not cause significant side effects (Xu *et al.*, 2009a). Thus, plant polysaccharides are ideal candidates for therapeutics with immunomodulatory and antitumor effects and low toxicity.

The genus *Actinidia* consists of over 58 species and widely distributed in the Asian continent. Some *Actinidia* species such as *A. arguta* and *A. chinensis* Planch are used as health foods and medical products for cancer treatment (Du *et al.*, 2009; Graham *et al.*, 2000), it was also reported that the root of *A. eriantha* possessed antitumor and immunomodulatory activity (Xu *et al.*, 2009a; Xu *et al.*, 2009b; Sun *et al.*, 2009). *Actinidia kolomikta* (Rupr. et Maxim.) which grows in the wild throughout the northern part of Indochina, is a locally famous traditional medicine for diabetes. However there are few studies concerning its bioactivity. The purpose of this study was to investigate the immunomodulatory activity of the vine root extracts on macrophage RAW 264.7 cells.

2. Materials and methods

2.1 Chemicals and reagents

Minimum Essential Medium Eagle (MEM) medium, fetal bovine serum (FBS), penicillin-streptomycin solution and propidium iodide (PI) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA), Lipopolysaccharide (LPS) from *E. coli* 055 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), Doxorubicin (DOX) was purchased from TopoGEN, Inc. (Florida, USA).

2.2 Cell lines

The murine macrophage cell line, RAW 264.7 was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in MEM medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml of streptomycin at 37℃ in a humidified 5% CO_2 atmosphere (ESPEC CO_2 Incubator). Cells were cultured for 2-3 days to reach the logarithmic phase and used for experiment.

2.3 Extraction and isolation of crude polysaccharides

The roots of *Actinidia kolomikta* were collected from the northern part of Indochina. Briefly, the plant material was extracted with distilled water at 100℃ for 1 h, twice, at a ratio of 1:10 (w/v) (You *et al.*, 2009; Lavi *et al.*, 2006). The aqueous extract was centrifuged at 6000 rpm for 15 min and was filtered through a filter paper (GFA, 47 mm, Whatman). The filtrate was concentrated in a rotary evaporator under reduced pressure, and then it was precipitated by adding four volumes of 99.5% ethanol, and was stored overnight at 4℃. The precipitate was collected by centrifugation at 8000 rpm for 15 min. Then the precipitate was washed with 80% ethanol and dissolved in distilled water, after centrifugation the supernatant was concentrated under a reduced pressure and lyophilized to obtain 100℃ extraction crude polysaccharide (CP) fraction.

2.4 Activation assay

The effect of crude polysaccharide on RAW 264.7 cells proliferation was estimated by Cell Counting Kit-8. RAW 264.7 cells were cultured in 96-well plate at a density of 5×10^4 cells/ml at 37℃ in a 5% CO_2 atmosphere for 24 h. And then cells were incubated with various concentrations of CP (2.5, 5, 10, 25, 50 and 100 μg/ml) at 37℃ for 24 h. After incubation, 10 μl of CCK-8 solution was added and incubated at 37℃ for 4 h. The cell viability was determined by the optical density (O.D.) at a wavelength of 450 nm with a microplate reader (BIO-RAD Model 550). Data are expressed as percentage of control.

2.5 Measurement of nitric oxide production

Nitrite accumulation was measured by Griess reagent and used as an indicator of nitric oxide (NO) production in

the medium (Gamal-Eldeen *et al.*, 2007). Macrophage cells (1×10^5 cells/ml) were dispensed into 6-well plate for 24 h. And then cells were stimulated with LPS (1 μg/ml) and various concentrations of CP (5, 10 and 25 μg/ml) for 24 h. After incubation, 50 μl of culture supernatants were mixed with an equal volume of Griess reagent in 96-well plate and incubated at 25℃ for 10 min. The absorbance at 570 nm was measured on a microplate reader. Nitrite concentrations in culture supernatants were measured to assess NO production in RAW 264.7 cells. $NaNO_2$ was used as standard to calculated nitrite concentrations.

2.6 Phagocytosis assay

The phagocytic ability of macrophage was measured by neutral red uptake (Cheng *et al.*, 2008). RAW 264.7 cells were cultured in 96-well plate at a density of 5×10^4 cells/ml at 37℃ in a 5% CO_2 atmosphere for 24 h. And then the cells were incubated with various concentrations of CP (2.5, 5, and 10μg/ml) and LPS (1 μg/ml) at 37℃ for 48 h. 100 μl of 0.075% neutral red solution was added and incubated for 1 h. Then the supernatant was discarded and the cells were washed with PBS twice. Then 100 μl cell lysate solution (ethanol and 0.01% acetic acid at the ratio of 1:1) was added into 96-well plate to lyse cells at room temperature for 2 h. The optical density at 570 nm was measured by a microplate reader (BIO-RAD Model 550).

2.7 Protective activity

RAW 264.7 cells were cultured in 96-well plate at a density of 5×10^4 cells/ml for 24 h at 37℃ in a 5% CO_2 atmosphere. Then cells were incubated with DOX (5 μM) in the presence or absence of various concentrations of CP (2.5, 5 and 25 μg/ml) for 24 h. After drug exposure, 10 μl of CCK-8 solution was added and incubated at 37℃ for 4 h. The cell numbers were quantitated by reading the absorbance at 450 nm. Data are expressed as percentage of control.

2.8 Cell cycle analysis

The flow cytometry assay was performed after Hu *et al.*, (2009) with some modifications. Macrophage RAW 264.7 cells (1×10^5 cells/ml) were incubated in 6-well plate with DOX (5 μM) in the presence or absence of CP (2.5 and 10 μg/ml) for 24 h. Cells were harvested and washed with cold PBS (-), and then fixed in 70% ethanol at -4℃ over night. Cells were strained with PI solution (20 μg/ml) at 4℃ for 30 min. DNA histograms were generated by flow cytometry (BD-LSR, BD Biosciences). Data from 10 000 cells per sample were collected and the percentage of apoptotic cells was obtained with CellQuest software (Becton Dickinson).

2.9 Statistical analysis

Experiments were conducted in triplicate experiments and results were expressed as mean±SD. Statistical significance was calculated by a two-tailed Student's *t*-test.

3. Results and discussion

3.1 Effect of CP on macrophage proliferation

The stimulation effect of CP on macrophages proliferation was tested. Results showed that, exposure of CP activated macrophages proliferation (Figure 1). It was interesting to note that in the range of 2.5-25 μg/ml, CP stimulated RAW 264.7 cells proliferation in a dose-dependent manner. At the concentration of 25 μg/ml, the stimulation effect reached maximum, was 205.8%. Furthermore, high concentrations (50-100 μg/ml) were tested on macrophages. Though with the concentration increased, the cells survival rate decreased dose-dependently, the RAW 264.7 cells survival rate at 100 μg/ml was still higher than control, was 112.9%. It was suggested that, CP possessed stimulation effect on macrophage RAW 264.7 cells proliferation with low cytotoxic. The concentrations from 2.5 μg/ml to 25 μg/ml were used in the following experiments.

3.2 Effect of CP on nitric oxide production

It was reported that LPS showed strong immunomodulating activity (Tokunaka *et al.*, 2000; Avni *et al.*, 2010). It stimulates macrophages to produce pro-inflammatory cytokines and secondary mediatory, such as NO. NO is a gaseous molecule synthesized from L-arginine by nitric oxide systhase (NOS). It is a highly reactive free radical and it can form a number of oxidation products such as NO_2, NO_2^-, N_2O_3 and S-nitrosothiols. NO participates in the physiology and pathophysiology of many systems (Diouf *et al.*, 2009). It is an important mediator of the non-specific host defense against invading microbes and tumors. Thus NO can be used as a quantitative index of macrophage activation. Results of stimulatory effect of CP on macrophages NO production were showed in Figure 2. Data showed that incubation with CP stimulated RAW 264.7 cells NO production in a dose-dependent manner. Treatment with CP at the concentration of 25 μg/ml significantly stimulated NO production (19.7 μM) in comparison with control (7.2 μM) ($p<0.01$), and was higher than 16.0 μM of 1 μg/ml LPS (positive control) produced.

3.3 Effect of CP on phagocytosis activity

Because macrophages play an important role in host defense that phagocytize the pathogens (Zhao *et al.*, 2010). Thus phagocytosis is an important indicator of macrophage effector activity (Yu *et al.*, 2008) and it represents the final and most indispensable step of the immunological defense system (Campelo *et al.*, 2002). The phagocytic activity of macrophages was monitored by measuring the amount of neutral red internalized in macrophages. Results were shown in Figure 3. Results showed that CP significantly and dose-dependently increased the phagocytosis of RAW 264.7 cells in comparison with control ($p<0.01$). Moreover, the O.D. value of treatment with CP at 2.5 μg/ml was higher than that of positive control, which was treated with LPS at 1 μg/ml. The results demonstrated that administration of CP may result in the initiation of immune reaction against foreign materials such as pathogen and tumors (Chen *et al.*, 2010).

3.4 Protective effect of CP on DOX-induced macrophage viability

Doxorubicin (DOX) is a drug used in cancer chemotherapy. It is an anthracycline antibiotic, closely related to the natural product daunomycin, and like all anthracyclines it works by intercalating DNA. Treatment with DOX resulted in a decrease of macrophages survival rate, which was about 12.6% (Figure 4). But in the presence of CP, macrophages viability was significantly higher than incubation with DOX. For example, incubation with 25 μg/ml of CP, the cell survival rate (89.8%) was significantly higher than the cell survival rate of negative control (exposure of 5 μM DOX). When incubated DOX-induced macrophages with various concentrations of CP, cells survival rate increased in a dose-dependent manner. Moreover, CP protected macrophages from apoptosis associated with DOX induced toxicity. As shown in Figure 5, in comparison with control, treatment with DOX (5 μM) the RAW 264.7 cells apoptosis rate was 37.8%. However incubation of DOX-induced RAW 264.7 cells with CP, the apoptosis rate decreased. For example, incubation of DOX-induced macrophages with 2.5 and 10 μg/ml of CP, the apoptosis rates were 25.1% and 21.5%, respectively. Papers reported that, many anticancer drugs exert their effect by causing DNA damage. And reactive oxygen species (ROS), such as hydrogen peroxide and super oxide anion, play important roles in apoptosis induced by anticancer drugs (Mizutani, 2007; Islaih *et al.*, 2005; Atsumi *et al.*, 2006). In our study, CP showed high SOD-like activity and DPPH radical-scavenging activity (data not shown). So, maybe CP could reduce the ROS level in cells thereby mitigate the apoptosis induced by DOX.

4. Conclusion

In this study, CP stimulated macrophage proliferation, NO production and phagocytosis. Moreover, the toxicity of DOX on macrophages was attenuated by CP, which mitigated cells death and apoptosis induced by DOX. It is therefore suggested that CP possesses immunomodulatory activity on macrophages and could be explored as a potential agent that protects macrophages from DOX damage.

Acknowledgements

The materials were collected from their natural habitat by Dr Keo Intabon, Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan.

References

Atsumi, T., Tonosaki, K., & Fujisawa, S. (2006). Induction of early apoptosis and ROS-generation activity in human gingival fibroblasts (HGF) and human submandibular gland carcinoma (HSG) cells treated with curcumin. *Archives of Oral Biology*, 51, 913-921.

Avni, D., Ernst, O., Philosoph, A., & Zor, T. (2010). Role of CREB in modulation of TNFα and IL-10 expression in LPS-stimulated RAW264.7 macrophages. *Molecular Immunology*, 47, 1396-1403.

Berner, M. D., Sura, M. E., Alves, B. N., & Hunter, K. W. (2005). IFN-γ primes macrophages for enhanced TNF-α expression in response to stimulatory and non-stimulatory amounts of microparticulate β-glucan. *Immunology Letters*, 98, 115-122.

Campelo, P. M. S., Oliveira, M. B. M., Leão, A. M. A. C., Carbonero, E. R., Gorin, P. A. J., & Iacomini, M. (2002). Effect of a soluble α-D-glucan from the lichenized fungus *Ramalina celastri* on macrophage activity. *International Immunopharmacology*, 2, 691-698.

Chen, W. X., Zhang, W. Y., Shen, W. B., & Wang, K. C. (2010). Effects of the acid polysaccharide fraction isolated from a cultivated *Cordyceps sinensis* on macrophages *in vitro*. *Cellular Immunology*, 262, 69-74.

Cheng, A. W., Wan, F. C., Wang, J. Q., Jin, Z. Y., & Xu, X. M. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from *Glycyrrhiza uralensis* fish. *International Immunopharmacology*, 8, 43-50.

Diouf, P. N., Stevanovic, T., & Boutin, Y. (2009). The effect of extraction process on polyphenol content, triterpene composition and bioactivity of yellow birch (*Betula alleghaniensis* Britton) extracts. *Industrial Crops and Products*, 30, 297-303.

Du, G. R., Li, M. J., Ma, F. W., & Liang, D. (2009). Antioxidant capacity and the relationship with polyphenol and Vitamin C in *Actinidia* fruits. *Food Chemistry*, 113, 557-562.

Gamal-Eldeen, A. M., Amer, H., Helmy, W. A., Talaat, R. M., & Ragab, H. (2007). Chemically-modified polysaccharide extract derived from *Leucaena leucocephala* alters Raw 264.7 murine macrophage functions. *International Immunopharmacology*, 7, 871-878.

Graham, J. G., Quinn, M. L., Fabricant, D. S., & Farnsworth, N. R. (2000). Plants used against cancer-an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology*, 73, 347-377.

Hu, H. H., Zhang, Z. Y., Lei, Z. F., Yang, Y. N., & Sugiura, N. (2009). Comparative study of antioxidant activity and antiproliferative effect of hot water and ethanol extracts from the mushroom *Inonotus obliquus*. *Journal of Bioscience and Bioengineering*, 107, 42-48.

Islaih, M., Halstead, B. W., Kadura, I. A., Li, B. H., Hubbard, J. L. R., Flick, L., Altizer, J. L., Deahl, J. T., Monteith, D. K., Newton, R. K., & Watson, D. E. (2005). Relationships between genomic, cell cycle, and mutagenic responses of TK6 cells exposed to DNA damaging chemicals. *Mutation Research*, 578, 100-116.

Lavi, I., Friesem, D., Geresh, S., Hadar, Y., & Schwartz, B. (2006). An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces anti-proliferative and pro-apoptotic effects on HT-29 colon cancer cells. *Cancer Letters*, 244, 61-70.

Mizutani, H. (2007). Mechanism of DNA damage and apoptosis induced by anticancer drugs through generation of reactive oxygen species. *Yakugaku Zasshi*, 127, 1837-1842.

Naeini, A., Khosravi, A., Tadjbakhsh, H., Ghazanfari, T., Yaraee, R., & Shokri, H. (2010). Evaluation of the immunostimulatory activity of *Ziziphora tenuior* extracts. *Comparative Clinical Pathology*, 19, 459-463.

Oliveira, C. C. D., Oliveira S. M. D., Godoy, L. M. F., Gabardo, J., & Buchi, D. D. F. (2006). Canova, Brazilian medical formulation, alters oxidative metabolism of mice macrophages. *Journal of Infection*, 52, 420-432.

Sakurai, T., Kaise, T., Yadomae, T., & Matsubara, C. (1997). Different role of serum components and cytokines on alveolar macrophage activation by soluble fungal (1→3)-β-D-glucan. *European Journal of Pharmacology*, 334, 255-263.

Schepetkin, I. A., & Quinn, M. T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, 6, 317-333.

Schepetkin, I. A., Xie, G., Kirpotina, L. N., Klein, R. A., Jutila, M. A., & Quinn, M. T. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from *Opuntia polyacantha*. *International Immunopharmacology*, 8, 1455-1466.

Sun, H. X., Wang, H., Xu, H. S., & Ni, Y. (2009). Novel polysaccharide adjuvant from the roots of *Actinidia eriantha* with dual Th1 and Th2 potentiating activity. *Vaccine*, 27, 3984-3991.

Tokunaka, K., Ohno, N., Adachi, Y., Tanaka, S., Tamura, H., & Yadomae, T. (2000). Immunopharmacological and immunotoxicological activities of a water-soluble (1→3)-β-D-glucan, CSBG from *Candida* spp. *International Journal of Immunopharmacology*, 22, 383-394.

Xu, H. S., Wu, Y. W., Xu, S. F., Sun, H. X., Chen, F.Y., & Yao, L. (2009a). Antitumor and immunomodulatory activity of polysaccharides from the roots of *Actinidia eriantha*. *Journal of Ethnopharmacology*, 125, 310-317.

Xu, H. S., Yao, L., Sun, H. X., & Wu, Y. W. (2009b). Chemical composition and antitumor activity of different polysaccharides from the roots of *Actinidia eriantha*. *Carbohydrate Polymers*, 78, 316-322.

You, L., Yang, X. B., Zhao, Y., Ruan, Y., Yang, Y., & Wang, Z. Z. (2009). Separation and quantification of component monosaccharides of the tea polysaccharides from *Gynostemma pentaphyllum* by HPLC with indirect UV detection. *Food Chemistry*, 112, 742-746.

Yu, M. Y., Xu, X. Y., Qing, Y., Luo, X., Yang, Z. R., & Zheng, L. Y. (2008). Isolation of an anti-tumor polysaccharide from *Auricularia polytricha* (jew's ear) and its effects on macrophage activation. *European Food Research and Technology*, 228, 477-485.

Zhao, L. Y., Dong, Y. H., Chen, G. T., & Hu, Q. H. (2010). Extraction, purification, characterization and antitumor activity of polysaccharides from *Ganoderma lucidum*. *Carbohydrate Polymers*, 80, 783-789.

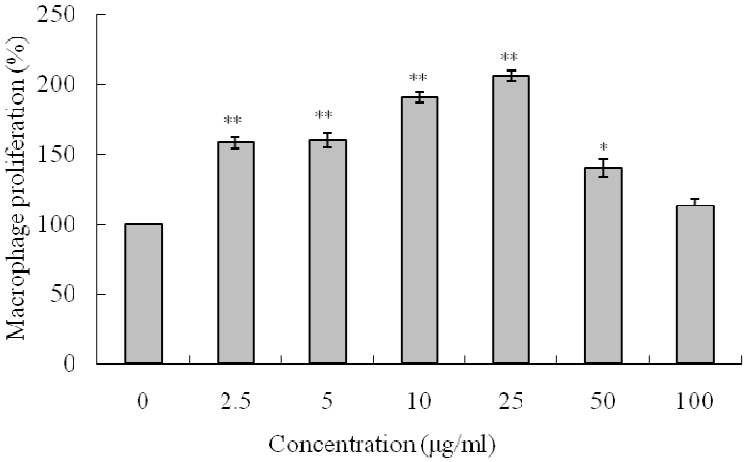

Figure 1. Effect of CP from the root of *A. kolomikta* on macrophage RAW 264.7 cells proliferation.

Cells were incubated with various concentrations of CP (2.5, 5, 10, 25, 50 and 100 μg/ml). Data are expressed as means±S.D. of three independent experiments. ($^{*}p<0.05$, $^{**}p<0.01$ in comparison with control).

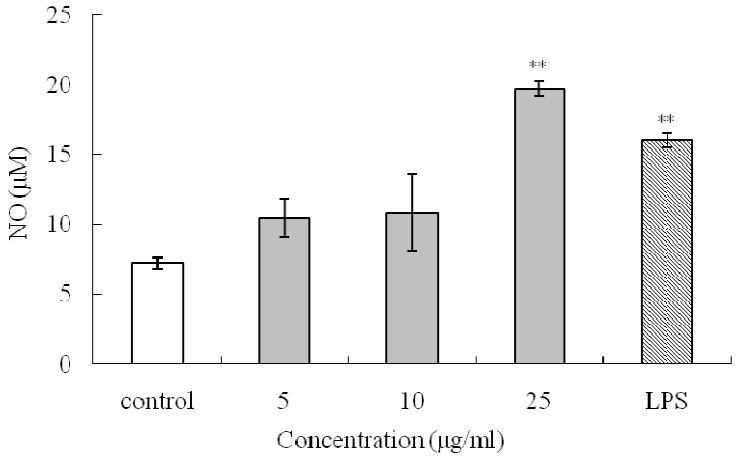

Figure 2. Effect of CP from the root of *A. kolomikta* on macrophage RAW 264.7 cells NO production.

Cells were incubated with various concentrations of CP (5, 10 and 25 μg/ml) and 1 μg/ml of LPS for 24 h. LPS was the positive control. Data are expressed as means±S.D. (n=3). ($^{**}p<0.01$ in comparison with control).

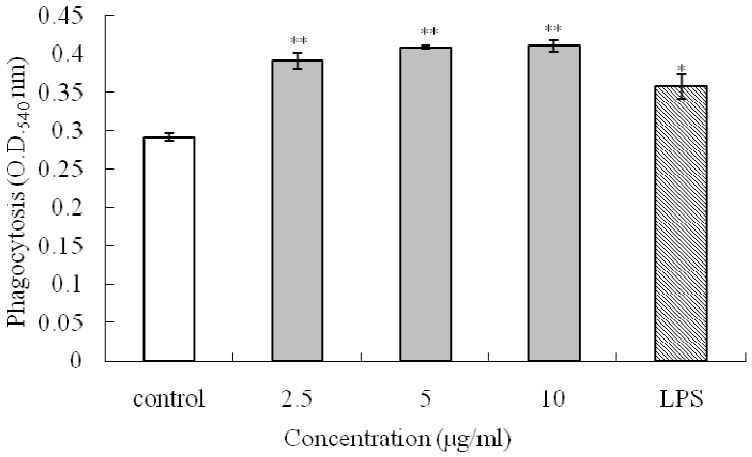

Figure 3. Effect of treatment with CP for 48 h on phagocytosis of macrophage RAW 264.7 cells.

Cells were incubated with various concentrations of CP (2.5, 5 and 10 μg/ml) and 1 μg/ml of LPS for 48 h. Data are expressed as means±S.D. (n=3), ($^{*}p<0.05$, $^{**}p<0.01$ in comparison with control).

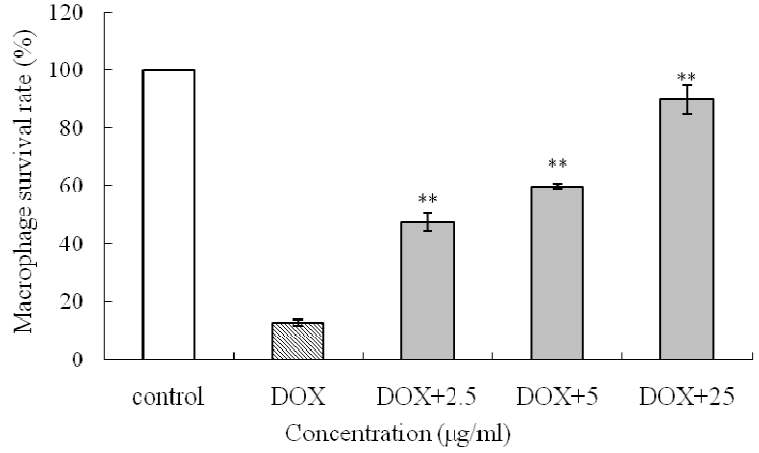

Figure 4. Effect of CP from the root of *A. kolomikta* on DOX-induced RAW 264.7 cells survival.

RAW 264.7 cells were incubated with DOX (5 μM) in the presence or absence of various concentrations of CP (2.5, 5 and 25 μg/ml) for 24 h. Data are expressed as means±S.D. of three independent experiments. ($^{**}p<0.01$ in comparison with DOX).

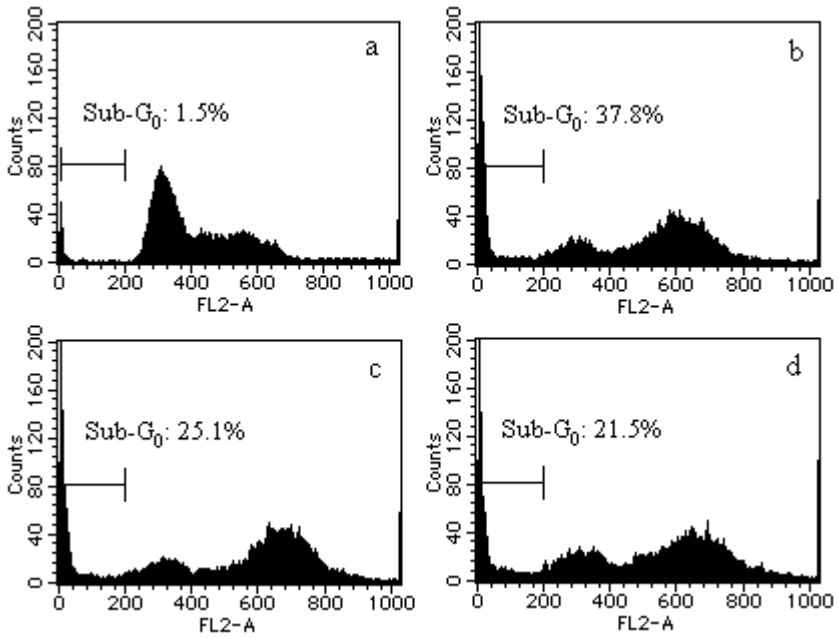

Figure 5. Effect of CP on the percentages of apoptosis of DOX-induced RAW 264.7 cells.

RAW 264.7 cells were incubated with DOX (5 μM) in the presence or absence of different concentrations of CP (2.5 and 10 μg/ml) for 24 h. a: control; b: 5 μM DOX; c: 5 μM DOX+2.5 μg/ml CP; 5 μM DOX+10 μg/ml CP. Cells were fixed with ethanol and stained with PI. The DNA content of cells was measured by flow cytometry and cell cycle profiles were analysed using CellQuest software.

 ISSN 1916-9671 E-ISSN 1916-968X