JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Immunomodulatory and Antitumor Activities of Grape Seed Proanthocyanidins

Haibin Tong,[*,†] Xiangfu Song,[‡] Xin Sun,[†] Guangren Sun,[§] and Fengfuo Du[§]

[†]Life Science Research Center, Beihua University, Jilin 132013, China

[‡]School of Public Health, Jilin University, Changchun 130022, China

[§]College of Forestry, Beihua University, Jilin 132013, China

ABSTRACT: Proanthocyanidins are naturally occurring compounds that are widely available in many kinds of plants; particularly, the grape seeds are a rich source of proanthocyanidins. Grape seed proanthocyanidins (GSPs) have been demonstrated to possess a wide range of health beneficial properties. This study was carried out to elucidate the molecular mechanisms involved in the antitumor therapeutic and immunomodulating effects of GSPs through in vivo and in vitro models. The results showed that GSPs could significantly inhibit the growth of Sarcoma 180 tumor cells in vivo and remarkably increase thymus and spleen weight of Sarcoma 180-bearing mice and upgrade the secretion level of tumor necrosis factor-α (TNF-α) in serum. Moreover, GSPs could stimulate lymphocyte transformation, enhance lysosomal enzyme activity and phagocytic capability of peritoneal macrophages, and remarkably promote the production of TNF-α. These results suggested that GSPs could improve functional activation of the immune system, and the antitumor effects of GSPs were achieved by immunostimulating properties.

KEYWORDS: Proanthocyanidins, immunomodulation, antitumor activity, macrophage

INTRODUCTION

Proanthocyanidins are naturally occurring compounds that are widely available in fruits, vegetables, nuts, seeds, flowers, and bark.[1] They are a class of polymeric phenolic compounds consisting mainly of catechin, epicatechin, gallocatechin, and epigallocatechin units.[2] Grape seeds are a particularly rich source of proanthocyanidins. Grape seed proanthocyanidins (GSPs) are potent antioxidants and free radical scavengers, which are more effective than either ascorbic acid or vitamin E. Moreover, GSPs are believed to possess a wide range of health beneficial properties, including protection against UV light-induced carcinogenesis, prevention of immune suppression, increasing the production of cytokines, stimulation of nonspecific immunity, activation of humoral immunity, and enhancement of cell-mediated immunity.[3−8] Recently, a series of preclinical and mechanistic studies have showed that GSPs could have effective anticarcinogenic activity, inhibit proliferation, and induce apoptosis in a wide variety of tumor models via modulation of multiple cell signaling events involving critical molecular candidates associated with growth, differentiation, apoptosis, and oncogenesis.[9−13] Although the bioavailability and metabolism data on proanthocyanidins are still largely unavailable, these preclinical and mechanistic findings strongly implicate the use of proanthocyanidins as a novel, promising, and multitarget-based therapeutic agent against many cancer types.[14,15] At present, the possible antitumor mechanisms of proanthocyanidins are multifaceted and extremely complex and need to be further confirmed to expand the clinical application of GSPs.

Recently, antitumor and immunostimulating properties of many kinds of natural products were also widely reported.[16,17] Proanthocyanidins could stimulate humoral and cell-mediated immunity and exhibit a wider range of immunostimulating activities. At the same time, proanthocyanidins showed significant antitumor effects in both in vivo and in vitro models. Therefore, in this study, we carried out the research focusing on the relationship between antitumor and immunostimulatory activities of GSPs and proposed a new antitumor mechanism of GSPs.

MATERIALS AND METHODS

Chemicals. GSPs were purchased from Tianjin Jianfeng Natural Products Co. (proanthocyanidins contents >95%). The possible contaminants of endotoxin contained in GSPs were removed using Affi-Prep Polymyxin Matrix (Bio-Rad). RPMI-1640 medium was purchased from Gibco Invitrogen Co. Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. Bovine serum albumin (BSA), *p*-nitrophenyl phosphate, dimethyl sulfoxide (DMSO), concanavalin A (ConA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

Animals. Male BALB/c mice (18−22 g) were purchased from the Animal Experimental Center of Jilin University. The mice were housed in plastic cages in a room and kept under standardized conditions at a temperature between 22 and 24 °C and 20% humidity with a 12 h light/dark cycle, and they had free access to tap water and food throughout the study. They were allowed to acclimatize for 1 week before the experiments were started. Animal experiments were conducted under principles of good laboratory animal care and approved by the ethical committee for Laboratory Animals Care and Use of Jilin University.

In Vivo Antitumor Activity and TNF-α Secretion in Serum. Sixty mice were randomly divided into six groups of 10 each. Sarcoma 180 cells from the peritoneal cavities of tumor-inoculated mice were washed twice with phosphate-buffered saline (PBS) and resuspended in

Received: August 8, 2011
Accepted: September 29, 2011
Revised: September 29, 2011
Published: October 13, 2011

Table 1. In Vivo Antitumor Activities of GSPs[a]

				relative weight (mg/g)	
treatment	dose (mg/kg)	tumor weight (g)	inhibition rate (%)	spleen	thymus
NaCl		2.31 ± 0.37		4.23 ± 0.41	2.12 ± 0.31
GSPs	200	0.96 ± 0.33**	58.44	5.68 ± 0.73**	2.64 ± 0.48**
	100	1.11 ± 0.41**	51.95	5.11 ± 0.65**	2.42 ± 0.42*
	50	1.42 ± 0.38**	38.53	4.75 ± 0.77*	2.38 ± 0.29
5-FU	50	0.73 ± 0.27**	68.40	4.39 ± 0.81	2.15 ± 0.23

[a] Values are expressed as means ± SDs (n = 10); *p < 0.05, and **p < 0.01 as compared with the negative group.

PBS. Under sterile conditions, 0.2 mL of Sarcoma 180 cell suspension (1×10^6 cells/mL) was inoculated into the right hind limbs at day 0, while the normal group was not inoculated with tumor cells. After 24 h of the start of the experimental treatment, GSPs (50, 100, and 200 mg/kg, 0.2 mL), 5-fluorouracil (5-FU, 50 mg/kg, as a positive control), and sterile saline (as a negative control) were administered orally everyday. Ten days after the tumor inoculation, all mice were weighed and sacrificed, and solid tumors, spleens, and thymus were carefully extirpated and weighed. The tumor inhibition rate was calculated according to the following formula:

$$\%(\text{inhibition ratio of tumor growth}) = 100 - \text{mean solid tumor weight of the treated group} / \text{mean solid tumor weight of the negative control group} \times 100$$

The relative thymus (spleens) weight was measured in the ratio of the thymus (spleens) weight (mg) to body weight (g). Tumor necrosis factor-α (TNF-α) in sera collected from the tumor-bearing mice was measured using a murine enzyme-linked immunosorbent assay (ELISA) kit.

In Vitro Antitumor Activity of GSPs. The in vitro antitumor activity against Sarcoma 180 cells was determined by colorimetric MTT assay. Briefly, tumor cells were seeded in 96-well flat-bottomed plates (5000 cells/well in 100 μL) and allowed to adhere for 24 h at 37 °C with 5% CO_2 atmosphere. Sterilized sample solutions were added into 96-well plate to give a final concentration of 50, 100, and 200 μg/mL, while the negative control (NC group) was treated with the complete RPMI-1640 medium only, and the positive control was treated with 5-FU. After cultivation for 72 h, 20 μL of MTT solution (5 mg/mL) was added. After it was incubated at 37 °C for 4 h, the supernatant was aspirated, and 100 μL of DMSO was added to each well. The absorbance was measured at 570 nm by a microplate reader (Bio-Tek EXL800, American).

Lymphocyte Proliferation Assay. Lymphocyte proliferation was assessed according to the MTT method[18] with some modifications. Briefly, BALB/c mice were sacrificed, and the spleens were removed aseptically and then placed in cold Hank's balanced salt solution (HBSS). Spleen cells were obtained by gently teasing the organ in RPMI-1640 medium under aseptic conditions and centrifugation. The red blood cells were removed by hemolytic Gey's solution. Splenocytes (1×10^6 cells/mL) were suspended with complete RPMI-1640 medium. The purity and viability of splenocyte were over 95%. One hundred microliters of cells was plated in 96-well plates and incubated with or without ConA (5 μg/mL) or LPS (20 μg/mL), and various concentrations (50, 100, and 200 μg/mL) of GSPs were added. Cells were incubated for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cell concentrations were evaluated by MTT assay.

Preparation of Peritoneal Macrophages. Male BALB/c mice were injected intraperitoneally with sterile thioglycollate medium for three consecutive days, and then, the resident peritoneal macrophages were harvested by peritoneal lavage and centrifugation. Then, peritoneal macrophages were cultured in complete RPMI-1640 medium in a 96-well plate for 2 h. Nonadherent cells were removed by washing the plate with PBS, and the adhered macrophages were cultured for another 24 h with fresh RPMI-1640 medium containing 10% FCS.

Measurement of Phagocytosis Capacity of Peritoneal Macrophage in Vitro. To evaluate the phagocytic capability of mouse peritoneal macrophages, chicken red blood cells (cRBC) were used as antigen particles. Macrophages were treated with or without different concentrations of GSPs (50, 100, and 200 μg/mL) in 10% FCS-RPMI-1640 medium at 37 °C in a humidified incubator containing 5% CO_2 for 24 h and then incubated in 5% FCS-RPMI-1640 medium containing 0.5% cRBC for 1 h. Macrophages were then rinsed with PBS (pH 7.4). After fixation in 4% paraformaldehyde, the cells were stained with Wright−Giemsa dye. Phagocytosed cRBC were examined with light microscopy, and a minimum of 200 macrophages were counted in each well. The phagocytic rate (PR) and phagocytic index (PI) were calculated using the following formula:

$$\text{PR}(\%) = (\text{number of macrophages phagocyting cRBC} / \text{number of total macrophages}) \times 100$$

where PI = number of total ingested cRBCs/number of macrophage ingesting cRBCs.[19]

Assay of Macrophage Lysosomal Phosphatase Activity. The lysosomal phosphatase activity in peritoneal macrophages was determined according to the method described by Suzuki et al.[20] Briefly, peritoneal macrophage monolayers in 96-well culture plates (1×10^6 cells/well) were cultured with different concentrations of GSPs (50, 100, and 200 μg/mL) for 24 h, then solubilized with 25 μL of 0.1% Triton X-100, and incubated for 30 min at room temperature. Then, 100 μL of 10 mM p-nitrophenyl phosphate was added to each well as a substrate for acid phosphatase, followed by the addition of 0.1 M citrate buffer (50 μL, pH 5.0). The cultures were further incubated for 30 min at 37 °C, 0.2 M borate buffer (50 μL, pH 9.8) was added to the mixture to terminate the reaction, and the absorbance at 405 nm was measured using an ELISA reader.

Semiquantitative Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Peritoneal macrophages cells were cultured in the presence of 200 μg/mL GSPs in a six-well tissue culture plate (1×10^6 cells/well) for 6, 12, and 24 h. The total RNA (1 μg) isolated from the stimulated cells was reverse transcribed into cDNA using Promega RT-PCR kit. Primers used in the present study were as follows: TNF-α (forward, 5′-CTTCAGCCCCAGCAGTGTATTCTTT-3′; and reverse, 5′-AGAGAACCTGGGAGTAGACAAGGTA-3′) and β-actin (forward, 5′-CACCACACCTTCTACAATGAGCTGC-3′; and reverse, 5′-GCTCAGGAGGAGCAATGATCTTGAT-3′). The cDNAs from the reverse transcription reactions were amplified under the following conditions: denaturation at 94 °C for 30s, annealing at 50 °C for 30s, and extension at 72 °C for 90s with a final extension at 72 °C for 5 min. Amplified cDNA products were resolved on 1.5% agarose gel by electrophoresis and then stained with ethidium bromide.

Assay of TNF-α Secretion of Peritoneal Macrophages in Vitro. Peritoneal macrophages (2×10^6 cells/mL) prepared as above

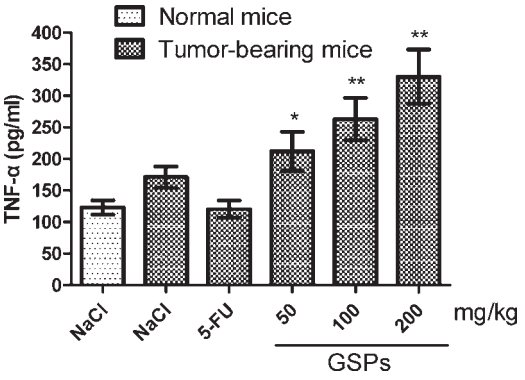

Figure 1. TNF-α release in serum of Sarcoma 180 tumor-bearing mice. Sarcoma 180 tumor-bearing mice were administered with NaCl, 5-FU, or GSPs for 10 days. Sera were collected after 10 days, and the TNF-α concentration was determined by ELISA. Values are means ± SDs; $^{*}p < 0.05$, and $^{**}p < 0.01$ vs negative control.

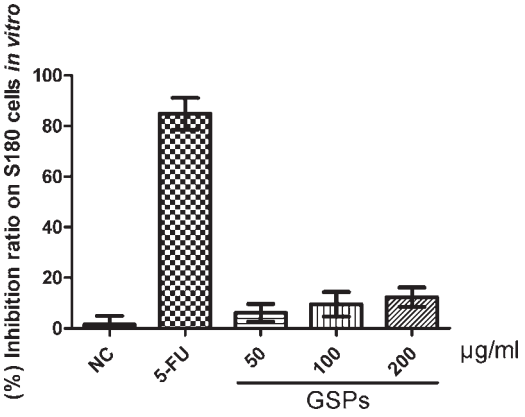

Figure 2. Inhibition ratio on in vitro proliferation of Sarcoma 180 cells by GSPs at different concentrations (50, 100, and 200 μg/mL). Values are means ± SDs.

were cultured in 48-well plates. After incubation with GSPs in different concentrations (5, 10, 50, 100, 200, and 400 μg/mL) for 24 h, TNF-α secretion was measured using a murine ELISA kit. LPS (20 μg/mL) was used as a positive control.[21]

Statistical Analysis. All statistical analyses were performed with SPSS version 13.0 for Windows. Data were expressed as means ± standard deviations (SDs) and examined for their statistical significance of difference with one-way analysis of variance. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

In Vivo and in Vitro Antitumor Test of GSPs. As shown in Table 1, GSPs showed excellent inhibitory activity against Sarcoma 180 solid tumor in vivo at concentrations of 50, 100, and 200 mg/kg body weight. The inhibitory rate reached 58.44% at the concentration of 200 mg/kg body weight. When treated with GSPs, a significant increase in relative spleen and thymus weights of Sarcoma 180-bearing mice were also observed (Table 1), and we also found that GSPs could increase body-weights of Sarcoma 180-bearing mice in the three drug-treated groups, although there was no statistical difference in body weights between negative control and treated groups (data not shown).

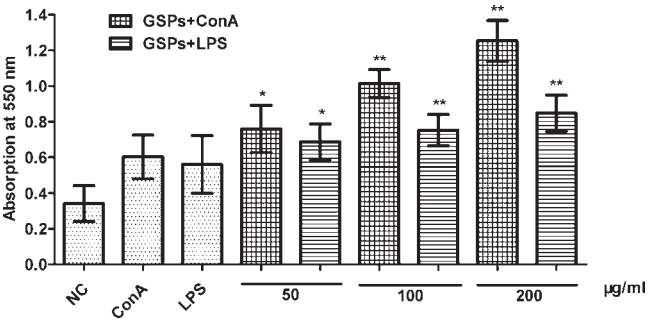

Figure 3. Effect of GSPs on ConA-induced or LPS-induced lymphocyte proliferation. The proliferation activity was expressed as the absorption at 570 nm. Values are means ± SDs; $^{*}p < 0.05$, and $^{**}p < 0.01$ vs ConA or LPS, respectively.

Moreover, the level of TNF-α in serum was raised in a dose-dependent manner of GSPs-treated Sarcoma 180-bearing mice (Figure 1).

5-FU is a chemotherapy drug usually used to treat several cancer types.[22] In the present study, 5-FU exhibited a high in vivo tumor inhibitory rate (68.40%), but it could not increase relative spleen and thymus weights of Sarcoma 180-bearing mice and the level of TNF-α in serum. Besides, 5-FU considerably decreased the body weight of Sarcoma 180-bearing mice.

Then, to evaluate the direct cytotoxicity against Sarcoma 180 tumor cells, in vitro tumor inhibitory effects of GSPs was determined by colorimetric MTT assay. As shown in Figure 2, GSPs exhibited only weak cytotoxic activity against Sarcoma 180 tumor cells; the highest inhibition ratio was 12.33% at the highest concentration (200 μg/mL). However, the inhibition ratio of 5-FU reached 84.83% at the concentration of 50 μg/mL, which indicated that GSPs had no significant cytotoxicity in vitro.

GSPs effectively prevented the formation of Sarcoma 180 tumor in vivo; consecutively, the level of TNF-α in serum was significantly increased in GSPs-treated groups. However, unlike 5-FU, GSPs had no significant direct cytotoxicity against Sarcoma 180 cells during in vitro antitumor test.

Much in vivo and in vitro evidence demonstrated that proanthocyanidins display an immunomodulating function by stimulating both cellular and humoral immunoresponse.[23] Furthermore, GSPs promoted the weight of immune organs of tumor-bearing mice, such as spleen and thymus. As we know, thymus and spleen were important immune organs, and the thymus index and spleen index reflect the immune function of the organism. GSPs could be an immunopotentiator to activate immune system. Therefore, the in vivo antitumor activity of GSPs may be due to activation of the mouse's own immune system to attack tumor cells.

Effects of GSPs on Immunomodulatory Function. The immunologic action of GSPs may begin with activating effector cells such as lymphocytes, macrophages, NK cells, and so on. Lymphocytes proliferation is an important indicator of immunoactivation. Therefore, we evaluated the effects of GSPs on lymphocytes proliferation. The colorimetric MTT assay was used for lymphocytes proliferation since the cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells, and the amount of MTT formazan generated is directly proportional to the cell number.[24] The presence of mitogens

Table 2. Effects of GSPs on Immune Function of Peritoneal Macrophages in Vitro[a]

treatment	PR (%)	PI	lysosomal enzyme activity (% of control)	TNF-α (pg/mL)
negative control	18.27 ± 4.23	1.00 ± 0.00	100.0 ± 8.6	151.26 ± 23.17
LPS	29.54 ± 6.38*	1.18 ± 0.12*	313.5 ± 45.3**	407.58 ± 69.22**
GSPs (μg/mL)				
50	26.52 ± 6.68*	1.19 ± 0.11*	286.3 ± 53.8**	214.47 ± 42.53*
100	37.14 ± 8.54**	1.45 ± 0.27**	360.1 ± 73.4**	279.65 ± 56.83**
200	46.94 ± 9.15**	1.59 ± 0.33**	434.9 ± 63.5**	387.31 ± 75.42**

[a] Values are expressed as means ± SDs. GSPs group and LPS group were compared against the negative control group using Student's *t* test; $*p < 0.05$, and $**p < 0.01$.

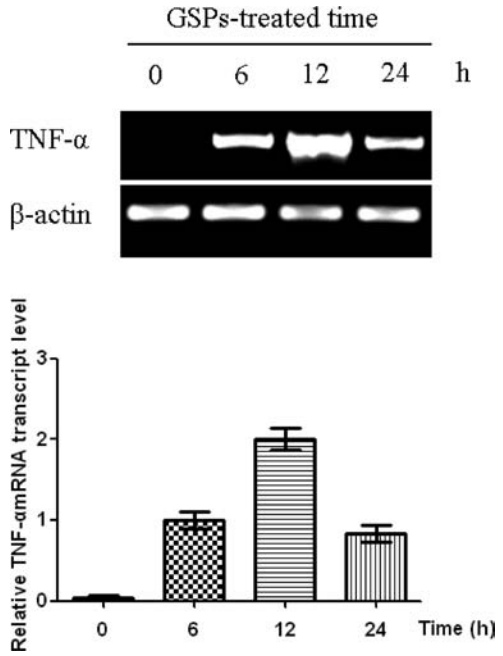

Figure 4. Effects of GSPs on the expression of mRNAs level of TNF-α. Peritoneal macrophages were cultured with or without GSPs (200 μg/mL) for 6, 12, and 24 h. The total RNA was isolated from the stimulated cells and subjected to semiquantitative RT-PCR. Transcripts of β-actin served as internal controls. A representative gel graph from three experiments is shown.

(ConA and LPS) in the system can postulate the possible activation pathway of GSPs.

As shown in Figure 3, GSPs could cause a significant increase in dose-dependent proliferation of the mouse spleen cells in the presence of ConA or LPS as mitogens for lymphocytes, as compared with only the ConA- or LPS-treated group. The results showed that GSPs could corporate with ConA or LPS to significantly augment ConA-induced and LPS-induced lymphocyte proliferation in vitro.

Macrophages are indispensable for keeping homeostasis and play an essential role in host defense against many types of invading tumor cells by phagocytosis and present antigens to lymphocytes and release numerous cytokine that regulate the activity of other cells.[25] Thus, we evaluated the effects of GSPs on the activation of peritoneal macrophage. Phagocytic activities are the most important functions of activated macrophages. To assess the direct effects of GSPs on macrophage phagocytosis, the phagocytic activities of macrophages to cRBC were examined in vitro. As shown in Table 2, GSPs significantly promoted the phagocytic activities of mouse peritoneal macrophages. As compared with negative control group, the PR and PI were both significantly elevated by GSPs treatment at doses of 100 and 200 μg/mL ($p < 0.01$). Moreover, we also found that GSPs-stimulated macrophages had obviously morphological changes from round cells to irregular cells. The above results revealed that by promoting phagocytic capacity, GSPs played a critical role in macrophage activation.

It is well-known that the phagocytosis action of a macrophage has a special merit. Namely, macrophages carry out their nonspecific defense function of the elimination stage of the phagocytic process by activating the lysosomal phosphatase in peritoneal macrophages.[26] GSP was found to enhance the phagocytosis capacity and also was shown to increase the lysosomal phosphatase activity (Table 2); the effects of GSPs were comparable with those of LPS. The results suggest that GSPs could activate the macrophages through modulation of lysosomal enzymes in peritoneal macrophages.

TNF-α is a principal mediator produced by macrophages that are involve in the regulation of necrosis, apoptosis, and proliferation of many cell types. To examine whether the stimulation of GSPs on peritoneal macrophages could influence the genes expression of cytokine, semiquantitative RT-PCR was performed for the mRNA level of TNF-α. As shown in Figure 4, the transcripts for TNF-α were hardly detectable in unstimulated peritoneal macrophages. The amounts of TNF-α transcript were increased significantly upon exposure to GSPs for 6 h and reached maximum at 12 h. When exposed to GSPs for 24 h, the amounts of TNF-α transcript were reduced gradually. Transcripts of β-actin served as internal controls. Furthermore, we examined the TNF-α production at the protein level. Peritoneal macrophages were stimulated by different concentrations of GSPs, the culture supernatants were collected at 24 h, and the amounts of TNF-α were measured by ELISA. As shown in Table 2, when treated with GSPs, the level of TNF-α was significantly increased as compared with the negative control level ($p < 0.05$). TNF-α was produced in a dose-dependent manner in response to GSPs stimulation. GSPs-activated peritoneal macrophages produced especially large amounts of TNF-α, reaching 387 pg/mL at a concentration of 200 μg/mL, which approximated the effects of LPS.

The development of novel therapeutic strategies for tumors has been considered particularly challenging for researchers due to tumors' aggressive underlying resistance to currently available therapies. More recently, the concept of enhancing the power of the immune system for cancer treatment has became an attractive concept to patients and clinicians alike, which has been

considered to be a promising therapeutic strategy for solid tumors.[27] In the present study, we carried out research focusing on the relationship between antitumor and immunostimulatory activities of GSPs and elucidated the molecular mechanisms involved in the antitumor therapeutic and immunomodulating effects. The results showed that GSPs exhibited significant antitumor effects by in vivo models, and GSPs could also stimulate humoral and cell-mediated immunity. Thus, we proposed a new antitumor mechanism of GSPs, which may indirectly play the role of antitumor activity through the functional activation of the immune system, such as promoting lymphocytes proliferation, enhancing peritoneal macrophages phagocytosis, and increasing the release of effecter molecules produced by macrophages.

AUTHOR INFORMATION

Corresponding Author

*Tel: + 86-432-64608351. Fax: + 86-432-64608350. E-mail: tonghaibin@gmail.com.

Funding Sources

This work was financially supported by the Foundation of Key Lab of Forestry and Ecological Environment of Jilin Province.

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