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Chemical composition and antitumor activity of different polysaccharides from the roots of *Actinidia eriantha*

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ABSTRACT

Four polysaccharides (AEPA, AEPB, AEPC and AEPD) isolated and purified from the roots of *Actinidia eriantha* using gel filtration were subjected to composition analysis and valuated for the antitumor and immunomodulatory activity. The predominant neutral monosaccharides in four fractions were identified as galactose, arabinose and fucose, while the composition and ratio of the monosaccharides were different from one another. All four polysaccharides could significantly not only inhibit the growth of transplantable S180 sarcoma in mice, but promote splenocytes proliferation, natural killer cells activity, interleukin-2 production from splenocytes and serum tumor antigen-specific antibody levels in tumor-bearing mice, indicating that their antitumor activity might be achieved by improving immune response. Among four polysaccharides, AEPC and AEPD showed the higher antitumor and immunomodulatory activity. Taken together, the chemical composition of these polysaccharides could affect their antitumor and immunomodulatory activity, and AEPC and AEPD could be explored as antitumor agent with immunomodulatory activity.

1. Introduction

Malignancies are one of the most serious diseases that damage human health in the modern world and the second largest deadly disease just below heart disease. Currently chemotherapy is still the standard treatment method. However, most of the anticancer drugs currently used in chemotherapy are cytotoxic to normal cells, leading to multiple organ toxicity that includes hemopoetic suppression and immunotoxicity (Ehrke, 2003). There exists close relationship between the occurrence, growth and decline of tumor and immune states. The low immune function of an organism may not only result in the generation and development of a tumor, but also be one of the most important factors that prevent the tumor patients' recovery. The discovery and identification of new antitumor drugs, which can potentiate the immune function, has become an important goal of research in immunopharmacology and oncotherapy (Mitchell, 2003). A wide variety of compounds are capable of potentiating immune responses. Classical adjuvants of bacterial origin, such as Bacillus Calmette Guerin (BCG), have been shown to exert therapeutic effects in the treatment of cancer. However, the effect is limited due to a number of undesirable side effects in host, like liver dysfunction, induction of hepatic granuloma, and enhancement of tumor growth when large doses of BCG are administered. Although the immunomodulating property of IL-2, IL-4, and IL-7 promoted their use in the treatment of cancer patients, their diverse side effects, such as cardiovascular toxicity, pulmonary toxicity, hematological toxicity, etc., made limitations in their use (Ognibene et al., 1988; Rosenberg et al., 1994). Immunomodulators, which can be used for long period without or less side effects, are appreciable in the cancer therapy.

Most polysaccharides derived from higher plants are relatively nontoxic and do not cause significant side effects, which is a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds. Thus, plant polysaccharides are ideal candidates for therapeutics with immunomodulatory and antitumor effects and low toxicity (Schepetkin & Quinn, 2006). Recently, lentinan, schizophyllan and krestin have been accepted as immunoceuticals in several oriental countries (Wasser, 2002).

The genus *Actinidia* (Actinidiaceae) consists of over 58 species and widely distributed in the Asian continent. Most species are native to temperate regions of south-western China. *Actinidia* fruits are utritious fruits distinguishable from other fruits by the attractive green color of their flesh (Boldingh, Smith, & Klages, 2000; Nishiyama, Fukuda, & Oota, 2005). Some *Actinidia* species, such as *A. macrosperma*, are the important traditional medicine (Zhao et al., 2006). They were widely used as health foods and medical products (Yang, Bai, & Qiu, 2007). *A. eriantha* Benth is a liana plant

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that commonly grows in temperate climate zones. Its roots have been used for gastric carcinoma, nasopharyngeal carcinoma, breast carcinoma, and hepatitis in traditional Chinese medicine (Jiangsu New Medical College, 1977). Pharmacological experiments also indicated that the water extracts of this drug possessed the antitumor and immunopotentiating activities (Lin, Yu, Zhu, Wu, & Yu, 1987). However, the antitumor constituents of the roots of *A. eriantha* have not yet been reported. To elucidate the principles for the antitumor action and to acquire high-performance polysaccharide products, the crude polysaccharide from the roots of *A. eriantha* (CAEP) was isolated and purified using gel filtration to afford four polysaccharides, namely AEPA, AEPB, AEPC and AEPD, and the chemical composition and antitumor activity of these polysaccharides as well as their immunomodulatory potential on the immune response in tumor-bearing mice were investigated.

2. Materials and methods

2.1. Materials and reagents

The roots of *A. eriantha* were collected in Wuyi county, Zhejiang province, China in August 2007. A voucher specimen (No. 20070806) has been deposited at the Laboratory of Nature Drug, College of Animal Sciences, Zhejiang University, China. DEAE-Sephadex A-50 and Sephacryl S-400 was from Amersham Biosciences Co., Piscataway, NJ, USA. Arabinose, fucose, galactose, glucose, rhamnose, rhamnose, xylose, glucuronic acid, trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS) and rabbit anti-mouse IgG peroxidase conjugate were purchased from Sigma Chemical Co., Saint Louis, MO, USA; RPMI 1640 medium, fetal calf serum (FCS) and dimethyl sulfoxide (DMSO) were from Gibco, Grand Island, NY, USA; goat anti-mouse IgG1, IgG2a and IgG2b peroxidase conjugate were from Southern Biotech. Assoc., Birmingham, AL, USA. Cyclophosphamide (CTX) was provided by Jiangsu Hengrui Company, China. Inositol was purchased from Huamei Biochemistry Co., Shanghai, China. All other chemicals and solvents used were of analytical grade.

2.2. Isolation and purification of polysaccharides

The plant material (1 kg) was extracted with boiling water three times under reflux. The aqueous extract was filtered through Whatman filter paper. The filtrate was concentrated in a rotary evaporator under reduced pressure, and then centrifuged at 3000 rpm for 15 min. The supernatant was precipitated with three volumes of 95% ethanol, and stored overnight at 4 °C. The precipitate was collected by vacuum filtration in büshi funnel, and then washed sequentially with ethanol, acetone and petroleum ether to defat. The resulting precipitate was dissolved in distilled water and dialyzed against distilled water (cut-off M_w 7000 Da). The retentate portion was concentrated under a reduced pressure and lyophilized to afford crude *A. eriantha* polysaccharide (CAEP). CAEP was dissolved in 0.1 M NaCl solution and filtered through 0.45-μm Millipore filter, and then the solution was subjected to DEAE-Sephadex A-50 column chromatography and eluted with NaCl gradients (0.1–2.0 M). The elution fraction (5 ml) were collected and monitored for carbohydrate content based on phenol–sulfuric acid method at 492 nm absorbance. Finally, the eluted fractions were concentrated, dialyzed and lyophilized. The products were further chromatographed on a Sephacryl S-400 gel filtration column with water and lyophilized to afford four white polysaccharides, namely AEPA, AEPB, AEPC and AEPD. These polysaccharides were endotoxin free with Limulus amebocyte lysate (LAL) test.

2.3. Measurement of carbohydrate and protein contents

Total carbohydrate contents in purified samples were determined by phenol–sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith 1956). Uronic acid contents were measured by the carbazole–sulfuric acid method using glucuronic acid as standard (Bitter & Muir, 1962). In addition, proteins in the polysaccharides were quantified according to the Lowry method using bovine serum albumin (BSA) as the standard (Lowry, Rosebrough, Farr, & Randall, 1951), combined with the method of UV absorption on a TU-1800PC spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., China).

2.4. Molecular weight determination

The average molecular weight were determined by the HPGPC, which was performed on a Waters HPLC system (Allances 2695, Waters, USA) equipped with a Waters Ultrahydrogel 250 column (7.8 × 300 mm) and a Waters 2410 differential refractometer. The mobile phase was 0.1 mol/L $NaNO_3$, and the flow rate was 0.9 mL/min. The sample (2 mg) was dissolved in the mobile phase (0.2 ml) and centrifuged. A 20 μl sample was injected in each run. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (6100, 16,500, 26,290, 40,000, 84,000, 158,000) (Sun, Tang, Gu, & Li, 2005).

2.5. Analysis of monosaccharide composition

Neutral monosaccharide composition was analyzed according to the following procedure: the polysaccharide samples (5 mg) were hydrolyzed with 5 ml of 2 M TFA at 110 °C for 5 h, followed by evaporation and addition of MeOH to the residue. The hydrolyzate was reduced with $NaBH_4$ for 3 h at room temperature. The excess $NaBH_4$ was decomposed with HOAc and removed by repeated evaporation to dryness with the addition of 10% (v/v) HOAc in MeOH (Mawhinney, Feather, Barbero, & Martinez, 1980). Alditol acetates of the reduced sugars and authentic standards (rhamnose, fucose, arabinose, xylose, mannose, glucose and galactose with inositol as the internal standard) were prepared with AC_2O at 100 °C for 1 h and subjected to gas chromatography (GC) on an Agilent 4890D system (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame-ionization detector (FID) and a DB-35 capillary column (30.0 m × 0.32 nm × 0.25 μm). The injector temperature was kept at 250 °C (split injection 40:1). The operation was performed at a column temperature program from 110 °C to 190 °C at 5 °C/min, holding for 5 min at 190 °C, then increasing to 250 °C at 10 °C/min and finally holding for 5 min at 250 °C. The molar percentage of the component monosaccharides was calculated as follows. The correction factor is shown in the equation: $f_{i/s} = (W_i/W_s)/(A_i/A_s)$, where A_s and A_i are the values of peak areas for inositol and standard monosaccharide, respectively. W_s and W_i are the values of weights for inositol and standard monosaccharide, respectively. The molar ratio value is shown in the equation: $R_{i/s} = f_{i/s} \times (A_i/A_s)/M$, where A_i/A_s is the ratio value of peak area for the component monosaccharide of tested samples and inositol. M is the molecular weight of the monosaccharides and $f_{i/s}$ is the correction factor.

2.6. Animals and cell lines

ICR mice (Grade II, 5 weeks old) weighing 18–22 g were purchased from Zhejiang Experimental Animal Center (Certificate No. 22-2001001, Hangzhou, China) and acclimatized for 1 week before use. Half of them were male and the others were female. Ro-

dent laboratory chow and tap water were provided *ad libitum* and maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of 50 ± 10%, and a 12/12 h light/dark cycle. All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guidelines established by Institute for Experimental Animals of Zhejiang University and were approved by the University Committee for animal experiments.

Mouse sarcoma S180 cell lines (ATCC-TIB66) were provided by Zhejiang Academy of Medical Sciences. Human leukemia K562 cell lines obtained from ATCC (American Type Culture Collection) were maintained in the logarithmic phase of growth in RPMI 1640 complete medium (RPMI 1640 supplemented with 2 mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum) at 37 °C under humidified air with 5% CO_2. S180 tumor cells were adapted to *in vitro* culture in RPMI 1640 complete medium. Tumor cells were washed and resuspended at 10^7 cells/ml in PBS. The cells were lysed by five freeze/thaw cycles in liquid nitrogen and then in water. The mixture was centrifuged at 17,000*g* at 4 °C for 15 min and the supernatant (lysate) was collected, filtered through a 0.22 micron filter and frozen at −80 °C. Protein concentration in lysates was determined by the Bradford assay (Stoscheck, 1990).

2.7. Treatment and drug administration

Ascites of the S180-bearing mice were drawn out under aseptic conditions and then diluted 4-fold with aseptic saline. The diluted S180 cell suspension was inoculated subcutaneously into the armpit for 0.2 ml per mouse (Tu, Sun, & Ye, 2008). Twenty-four hours later, mice were divided into seven groups, each consisting of ten mice. The inoculated mice were administered per os (p.o.) with CAEP, AEPA, AEPB, AEPC and AEPD at a single dose of 10 mg/kg for 10 days or injected intraperitoneally (i.p.) with CTX at a dose of 10 mg/kg for 5 days once daily. Model control groups received the same volume of saline. The dose volume was 0.2 ml/10 g body weight. A normal control group without medicine administration and tumor inoculation was also used in this experiment. On day 11, mice were weighed, and sacrificed by cervical dislocation. The solid tumors were collected and weighed. Then the inhibitory rate against the growth of tumor was calculated. The inhibitory rate (%) = $[(C - T)/C] \times 100$, where C is the average tumor weight of the model control group; T is the average tumor weight of medicine groups. At the time of sacrifice, liver, kidney, heart, small intestine, and colon were removed and fixed in formalin for subsequent microscopic examination.

2.8. Splenocyte proliferation assay

Splenocytes collected from tumor-bearing mice was prepared as previously described (Tu et al., 2008). Splenocytes were seeded into 4 wells of a 96-well flat-bottom microtiter plate (Costar, USA) at 5×10^6 cell/ml in 100 μl complete medium, thereafter Con A (final concentration 5 μg/ml), LPS (final concentration 10 μg/ml), or RPMI 1640 medium were added giving a final volume of 200 μl. The plates were incubated at 37 °C in a humid atmosphere with 5% CO_2. After 44 h, 50 μl of MTT solution (2 mg/ml) were added to each well and incubated for 4 h. The plates were centrifuged (1400*g*, 5 min) and the untransformed MTT was carefully removed by pipetting. To each well 150 μl of a DMSO working solution (180 μl DMSO with 20 μl 1 N HCl) was added, and the absorbance was evaluated in an ELISA reader (Bio-Rad, USA) at 570 nm after 15 min. The stimulation index (SI) was calculated based on the following formula: SI, the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.9. Assay of natural killer (NK) cell activity

The activity of NK cells was measured as previously described (Tu et al., 2008). Briefly, K562 cells were used as target cells and seeded in 96-well U-bottom microtiter plate (Costar, USA) at 2×10^4 cells/well in RPMI 1640 complete medium. Splenocytes prepared as described above were used as the effector cells, and were added at 1×10^6 cells/well to give E/T ratio 50:1. The plates were then incubated for 20 h at 37 °C in 5% CO_2 atmosphere. Fifty microliters of MTT solution (2 mg/ml) was added to each well and the plate was incubated for another 4 h and subjected to MTT assay. Three kinds of control measurements were performed: target cells control, blank control and effector cells control. NK cell activity was calculated as following equation: NK activity (%) = $(OD_T - (OD_S - OD_E))/OD_T \times 100\%$, where OD_T, optical density value of target cells control, OD_S, optical density value of test samples, OD_E, optical density value of effector cells control.

2.10. Cytokine levels in the supernatants of cultured splenocytes

To analyze the effect of CAEP and its polysaccharides on the levels of IL-2 secreted by splenocytes of S180-bearing mice, splenocytes prepared as described above (5×10^6 cells/ml) were treated with Con A (5 μg/ml) for 48 h and the supernatants were collected by centrifugation for IL-2 measure. The stimulation index (SI) of the target cells was determined to measure IL-2 levels (Sun & Peng, 2008). The splenocytes of the normal ICR mice, maintained in RPMI 1640 complete medium at 37 °C in a humid atmosphere with 5% CO_2 for 48 h, were used as the target cells. Splenocytes were washed with RPMI 1640 medium, counted and finally diluted to the concentration of 5×10^6 cell/ml with the complete medium. The same volume of target lymphocytes and the supernatants containing IL-2 were added to the wells of 96-well microtiter plates. Each test was repeated four wells. After 20 h incubation at 37 °C in a humid atmosphere with 5% CO_2, 50 μl of MTT solution (2 mg/ml) was added to each well and the plate was incubated for another 4 h, and then subjected to MTT assay. The same concentration of Con A and RPMI 1640 were incubated as the normal control. The SI was calculated based on the following formula: SI = the absorbance value for cultures containing IL-2 divided by the absorbance value for normal cultures.

2.11. Measurement of antigen-specific antibody

The antigen-specific IgG, IgG1, IgG2a and IgG2b antibodies in sera were detected by an indirect ELISA as previously described (Sun, Ye, Pan, & Pan, 2004). In brief, microtiter plate wells (Costar, USA) were coated with 100 μl the S180 cell lysate (protein concentration 50 μg/ml, in 50 mM carbonate–bicarbonate buffer, pH 9.6) for 24 h at 4 °C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween), and then blocked with 5% FCS/PBS at 37 °C for 2 h. After three washings, 100 μl of a series of diluted sera sample or 0.5% FCS/PBS as control were added to triplicate wells. The plates were then incubated for 2 h at 37 °C, followed by three times of washing. Aliquots of 100 μl of rabbit anti-mouse IgG horseradish peroxidase conjugate diluted 1:16000, goat anti-mouse IgG1 peroxidase conjugate 1:8000, IgG2a peroxidase conjugate 1:8000, and IgG2b peroxidase conjugate 1:8000 with 0.5% FCS/PBS were added to each plate. The plates were further incubated for 2 h at 37 °C. After washing, the peroxidase activity was assayed as following: 100 μl of substrate solution (10 mg of *O*-phenylenediamine and 37.5 μl of 30% H_2O_2 in 25 ml of 0.1 M citrate–phosphate buffer, pH5.0) was added to each well. The plate was incubated for 10 min at 37 °C, and enzyme reaction was terminated by adding 50 μl/well of 2 N H_2SO_4. The optical density was measured in an ELISA reader at 490 nm, where sets of sera samples

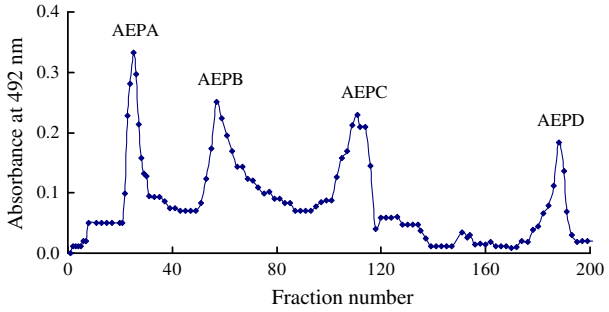

Fig. 1. Gel filtration chromatogram of the crude polysaccharide from the roots of *A. eriantha* (CAEP) on DEAE-Sephadex A-50 column (5 × 90 cm). The crude polysaccharide CAEP was dissolved in 0.1 M NaCl solution and applied to the column. The eluates were collected and the carbohydrate contents of collected fractions were monitored by phenol–sulfuric acid method.

have been subjected to within and between group comparisons, ELISA assays were performed on the same day for all of the samples.

2.12. Statistical analysis

The data were expressed as mean ± standard deviation (SD) and examined for their statistical significance of difference with ANOVA and a Tukey post-hoc test. *P*-values of less than 0.05 were considered to be statistically significant.

3. Result

3.1. Chemical composition of the polysaccharides

The crude polysaccharide from the roots of *A. eriantha* (CAEP) was further isolated and purified by DEAE-Sephadex A-50 and Sephacryl S-400 gel permeation chromatogram into the four polysaccharides AEPA, AEPB, AEPC and AEPD (Fig. 1). The yield of crude polysaccharide CAEP was 4.94% of the plant raw material, and the yields of four polysaccharides were 35.07%, 13.73%, 15.15% and 6.32% for AEPA, AEPB, AEPC and AEPD of the parent polysaccharide CAEP, respectively (Table 1). These polysaccharides showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that they didn't contain reducing sugar and starch-type polysaccharide (Yang, Zhao, & Lv, 2008). All four polysaccharides had negative responses to the Bradford test and no absorption at 280 nm in the UV spectrum, indicating the absence of protein. The average molecular weights were ca. 1.43×10^6, 2.06×10^6, 1.73×10^6 and 1.13×10^6 Da by HPPGC for AEPA, AEPB, AEPC and AEPD, respectively. The results of GC quantitative analysis of the acetylated of monosaccharides revealed that galactose, arabinose and fucose were the major sugars in four purified polysaccharides. Rhamnose was only found in AEPA and AEPB, but not present in AEPC and AEPD. AEPA and AEPB were composed of rhamnose, fucose, arabinose, xylose, mannose, glucose and galactose with molar ratios of 1.00:6.06:10.26:1.26:3.04:1.48:14.38 and 1.27:5.24:7.54:1.00:3.33:2.04:12.25, respectively. AEPC and AEPD consisted of fucose, arabinose, xylose, mannose, glucose and galactose with molar ratio of 5.76:9.95:1.00:3.60:2.77:14.16 and 5.51:8.29:1.00:3.49:3.99:13.59, respectively (Table 1). Furthermore, total uronic acid content was measured by carbazole–sulfuric acid method using glucuronic acid as the standard. The uronic acid contents in AEPA and AEPB were 22.3% and 15.1%, respectively, while AEPC and AEPD were not detectable for uronic acid.

3.2. Inhibition of polysaccharides on the growth of transplantable tumors in mice

The inhibitory effect of CAEP and its purified polysaccharides on the growth of transplantable S180 tumors in mice was shown in Table 2. As a positive control, CTX showed high inhibitory rate on S180 sarcoma in mice. The growth of transplantable S180 sarcoma in mice was also significantly inhibited by CAEP and four purified polysaccharides compared with the model controls ($P < 0.001$), with the inhibitory rate being 41.59%, 42.90%, 44.87%, 52.53% and 50.87% for CAEP, AEPA, AEPB, AEPC and AEPD, respectively. Among them, AEPC and AEPD showed the higher inhibitory effect against S180 sarcoma in mice. In addition, no signs of toxicity were observed in the mice treated with CAEP and its polysaccharides on base of body weight (Table 2) and microscopic examination of individual organs.

3.3. Effect of polysaccharides on splenocyte proliferation in S180-bearing mice

The effects of CAEP and its purified polysaccharides on Con A- and LPS-stimulated splenocyte proliferation in S180-bearing mice were shown in Fig. 2. CAEP and four purified polysaccharides significantly promoted Con A-induced splenocyte proliferation in the tumor-bearing mice ($P < 0.05$, $P < 0.01$ or $P < 0.001$). The cellular proliferation elicited by the B cell mitogen LPS was also significantly increased by CAEP and its four purified polysaccharides ($P < 0.05$ or $P < 0.01$). However, Con A- and LPS-stimulated splenocyte proliferations in the CTX-treated group were significantly lower than those of the model control ($P < 0.05$).

3.4. Effect of polysaccharides on natural killer (NK) cell activity in S180-bearing mice

Tumor cell elimination is known to be mediated in part by the cytotoxic activity of NK cells. We therefore measured the cytotoxic activity of splenocytes against NK cell-sensitive k562 cells using MTT colorimetric assay. As shown in Fig. 3, CAEP and four purified polysaccharides could significantly increase NK cell activity in the S180-bearing mice ($P < 0.05$, $P < 0.01$ or $P < 0.001$). However, NK

Table 1
Neutral monosaccharide composition, uronic acid content and yield of four purified polysaccharides from the roots of *A. eriantha*.

Sample	Neutral sugar (mol.%)[a]							Uronic acid (wt%)[b]	Yield (wt%) [c]
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Glucose	Galactose		
AEPA	2.67	16.16	27.38	3.36	8.12	3.96	38.36	22.33	35.07
AEPB	3.88	16.04	23.09	3.06	10.20	6.25	37.49	15.09	13.73
AEPC	ND[d]	15.47	26.73	2.69	9.66	7.43	38.03	ND	15.15
AEPD	ND	15.36	23.12	2.79	9.73	11.11	37.88	ND	6.32

[a] The quantities of the neutral sugars was given in mol.%.
[b] The uronic acid content was calculated as wt%.
[c] Value is expressed as wt% of the parent crude polysaccharide.
[d] ND indicates not detected.

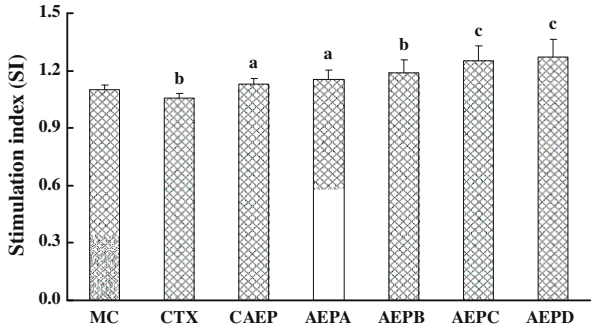

Fig. 4. Effect of the crude polysaccharide from *A. eriantha* (CAEP) and its four purified polysaccharides on IL-2 production from splenocytes in S180-bearing mice. S180-bearing mice were administered p.o. with CAEP and its four purified polysaccharides at the dose of 10 mg/kg for 10 days or injected i.p. with CTX at a dose of 20 mg/kg for 5 days once daily. Model control groups received the same volume of saline. On day 11, splenocytes were prepared and IL-2 level in the cultured supernatants were determined indirectly through the IL-2-dependent cells (mitogen stimulated lymphocytes) proliferation by the MTT method as described in the text. The values are presented as means ± SD ($n = 8$). Significant differences compared to model control group (MC) are designated as $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$.

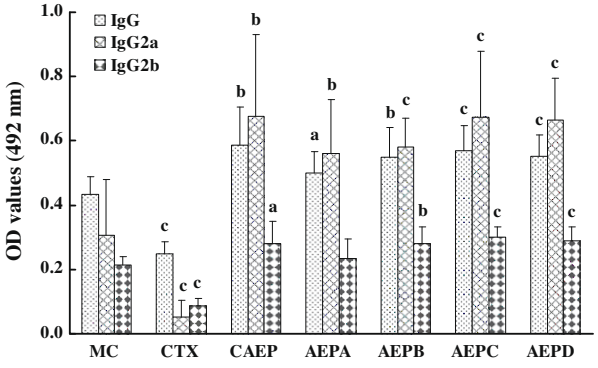

Fig. 5. Effect of the crude polysaccharide from *A. eriantha* (CAEP) and its four purified polysaccharides on tumor antigen-specific IgG, IgG2a, and IgG2b antibody in S180-bearing mice. S180-bearing mice were administered p.o. with CAEP and its four purified polysaccharides at the dose of 10 mg/kg for 10 days or injected i.p. with CTX at a dose of 20 mg/kg for 5 days once daily. Model control groups received the same volume of saline. On day 11, sera were collected and antigen-specific IgG, IgG2a, and IgG2b antibodies in the sera were measured by an indirect ELISA as described in the text. The values are presented as means ± SD ($n = 8$). Significant differences compared to model control group (MC) are designated as $^{a}P < 0.05$, $^{b}P < 0.01$ and $^{c}P < 0.001$.

cells is a specific antigen antibody reaction. Cell-mediated immune defense was mediated specifically by T cells. In addition to killing the tumor cells directly, T-cells can produce many lymphocyte factors consisting of macrophage mobile factor, lymphotoxin, transfer factor and interferon. Such factors could promote the proliferation and differentiation of immune cells, macrophage phagocytosis and the capacity of killing target cells, so that they play a role in preventing tumor (Kim et al., 2001). The capacity to elicit an effective T- and B-cell immunity can be shown by the stimulation of lymphocyte proliferation response (Marciani et al., 2000). It is generally known that Con A stimulates T-cells and LPS stimulates B-cell proliferation. The proliferation assay showed that CAEP and four purified polysaccharides could significantly promote the Con A- and LPS-stimulated splenocyte proliferation in S180-bearing mice, while the positive control CTX with high tumor inhibitory rate had suppressive effect on splenocyte proliferation (Fig. 2). The results indicated that CAEP and its purified polysaccharides could significantly increase the activation potential of T- and B-cells and enhance the humoral immunity and cell-mediated immunity in tumor-bearing mice.

NK cells are a type of lymphocytes and are part of the first line of innate defense against cancer cells and virus-infected cells (Moretta, Bottino, Cantoni, Mingari, & Moretta, 2001). With spontaneous cell-mediated cytotoxicity, they are also functionally similar to cytotoxic T lymphocytes (CTLs). Unlike CTLs, however, the killing by NK cells is non-specific, and NK cells do not need to recognize antigen/MHC on the target cell. NK cells can react against and destroy target cell without prior sensitization to it. NK cell activity assay is a routine method for analysis of a patient's cellular immune response *in vitro*, and can also be used to test the antitumor activities of possible drugs (Zhang, Sun, Wei, & Tian, 2005). In this study, CAEP and four purified polysaccharides were found to significantly enhance the killing activity of NK cells from splenocytes in tumor-bearing mice (Fig. 3), suggesting that they could enhance the non-specific cytolytic activities against autologous tumor cells.

Cytokines are peptides and low-molecular proteins, which affect cell functions and condition their interactions. By binding to specific cell surface receptors, they affect cell proliferation, differentiation and functions, and regulate both cellular and humoral immune responses. IL-2 is a important cytokine excreted by the activated T cells and has many immunopotentiating effects, such as proliferation of T-cells, B-cells, NK cells and monocytes, augmentation of cytotoxicites of T-cells and NK cells and *in vivo* generation of lymphokine-activated killer (LAK) cells, which exhibit high cytolytic activities against autologous tumor cells (Asano, Kaneda, Hiragushi, Tsuchida, & Higashino, 1997). In the present study, the level of IL-2 secreted by splenocytes in S180-bearing mice was significantly increased by CAEP and its purified polysaccharides, which implied that IL-2 may be involved in the immune response and induce NK cells production. The increase may also explain the antitumor properties of these polysaccharides.

The antibody-based therapy has proven to be more beneficial than other immunotherapy approaches, leading to complete regression of tumors in a good proportion of treated individuals (Melero, Hervas-Stubbs, Glennie, Pardoll, & Chen, 2007). Some antibodies can act either directly by blocking signal transduction pathways (i.e. when targeted to growth factor receptors) or indirectly via the activation of NK-mediated killing (ADCC: antibody-dependent cellular cytotoxicity). To investigate the effect of CAEP and its purified polysaccharides on the humoral immune responses in S180-bearing mice, the tumor antigen-specific antibody levels in serum were measured using ELISA. The amount of specific IgG in the serum was significantly increased by CAEP and four purified polysaccharides compared with model control. Th1 response is required for protective immunity against intracellular infectious agents, such as viruses, certain bacteria and protozoa, and presumably against cancer cells, while Th2 immunity is effective for protection against most bacterial as well as certain viral infections. Evaluation of IgG1 and IgG2a, IgG2b antibody isotypes in response to immunization provide a relative measure of the contribution of Th2 and Th1 humoral immune responses, respectively (Finkelman et al., 1990). In our studies, while model control mice produced the lower levels of IgG2a and IgG2b antibodies, the administration of CAEP and its purified polysaccharides resulted in significant increases in IgG2a and IgG2b antibody levels. This clearly demonstrated that CAEP and its purified polysaccharides modulated the quality of immune responses, and mainly proved a Th1 type of immune response in S180-bearing mice as associated sensitively with an enhancement of IgG2a and IgG2b levels (Germann et al., 1995). These results confirmed that CAEP and its purified polysaccharides could promote the specific humoral immune response.

GC quantitative analysis revealed that each polysaccharide contained similar neutral monosaccharide composition of fucose,

arabinose, mannose, glucose and galactose. However, the composition and the ratio of these monosaccharides greatly differed from one another. Rhamnose was only found in AEPA and AEPB, but not present in AEPC and AEPD. In addition, there were significant