Vaccine 27 (2009) 3984–3991

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Novel polysaccharide adjuvant from the roots of *Actinidia eriantha* with dual Th1 and Th2 potentiating activity

Hong-Xiang Sun[a,*], Hui Wang[a], Hai-shun Xu[a,b], Yang Ni[a,c]

[a] *Key Laboratory of Animal Epidemic Etiology & Immunological Prevention of Ministry of Agriculture,*

(Alum) are the only vaccine adjuvants licensed by the Food and Drug Administration (FDA) for use in humans [15]. While Alum is safe, it is a relatively weak adjuvant particularly when used with subunit antigens. Moreover, the Alum is a mild Th2 adjuvant that can effectively enhance IgG1 antibody responses, but it is rarely associated with Th1 type immune responses [16]. Furthermore, Alum is poor at stimulating cell-mediated immune responses, and may actively block activation and differentiation of cytotoxic T-lymphocytes [17]. Hence, there is a major unmet need for a safe and efficacious adjuvant capable of boosting cellular plus humoral immunity [18].

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 recognized as an effective biological response modifier with low toxicity [19]. Recently, many polysaccharides have been shown to possess adjuvant potential on specific cellular and humoral immune responses against antigen and vaccine and be excellent candidates to replace Alum as the adjuvant of choice for many vaccines [20–29]. Particular advantages offered by plant polysaccharide adjuvants in inducing cellular in addition to humoral immunity offer excellent safety, tolerability, ease of manufacture and formulation. Thus, the plant polysaccharide adjuvants have enormous potential for use in vaccines against both pathogens and cancer [18].

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 nutritious fruits distinguishable from other fruits by the attractive green color of their flesh [30]. Some *Actinidia species*, such as *Actinidia macrosperma*, are the important traditional medicine [31]. The roots of *A. eriantha* Benth have been used for gastric carcinoma, nasopharyngeal carcinoma, breast carcinoma, and hepatitis in traditional Chinese medicine [32]. The modern pharmacological experiments also proved that the water extracts of this drug possessed antitumor and immunopotentiating activities [33]. We previously reported that the water-soluble polysaccharide (AEPS) was the main active principles of the antitumor and immunomodulatory effect of this drug [34]. Ovalbumin (OVA) is commonly used as the model antigen to detect the adjuvant effect of chemicals [35]. We have previously used OVA as the model antigen to screen the saponins with the adjuvant properties from Chinese traditional medicines [36]. The obtained saponins with the adjuvant properties have also been verified their adjuvant effects on recombinant hepatitis B surface vaccine, Newcastle disease virus-based recombinant avian influenza vaccine, and recombinant fowlpox virus vector-based avian influenza vaccine in the mice and chickens [37,38]. In this study, in order to investigate the immunoadjuvant property of the isolated polysaccharide AEPS, its toxicity and adjuvant potential on the cellular and humoral immune responses to OVA in mice were investigated.

## 2. Materials and methods

### 2.1. Mice

Female 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 prior to use. Rodent 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 the Institute for Experimental Animals of Zhejiang University and were approved by the University Committee for animal experiments.

### 2.2. Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS), RPMI-1640 medium, and rabbit anti-mouse IgG peroxidase conjugate were purchased from Sigma Chemical Co., Saint Louis, MO, USA; goat anti-mouse IgG1, IgG2a and IgG2b peroxidase conjugate were from Southern Biotech. Assoc., Birmingham, AL, USA; cytokine (IL-2, IL-10, and IFN-γ) detecting ELISA kits were from Wuhan Boster Biological Technology Co. Ltd., Hubei, China. Quil A was kindly provided by Brenntag Nordic A/S, Denmark. Trizol was from Invitrogen, USA; revert Aid$^{TM}$ M-MuLV reverse transcriptase was from Fermentas, USA; diethylpyrocarbonate (DEPC) and ribonuclease inhibitor were from Biobasic, Canada; oligo(dT)$_{18}$ were from Sangon, China. Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp., Hangzhou, Zhejiang, China; aluminum hydroxide gel (Alum) was from Zhejiang Wanma Pharm Co. Ltd., Hangzhou, Zhejiang, China.

Human leukemia K562 cell lines, sensitive to natural killer (NK) cells, were purchased from Institute of Cell Biology, Chinese Academy Sciences. They were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with 2 mM L-glutamine (Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma), and 10% fetal calf serum at 37 °C under humidified air with 5% $CO_2$.

### 2.3. Extraction, purification and characterization of polysaccharide AEPS

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. The plant material (1 kg) was extracted with boiling water three times under reflux. The aqueous extract was filtered through filter paper. The filtrate was concentrated 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 resulting precipitate (49.4 g) was dissolved in distilled water and dialyzed against distilled water (cut-off $M_w$ 7000 Da). The retentate portion was subjected to DEAE–Sephadex A-50 column chromatography, washed with $H_2O$, and eluted with 1.0 M sodium chloride solution. The eluates collected were concentrated, dialyzed and lyophilized to afford a total *A. eriantha* polysaccharide (AEPS, light off-white powder, 34.7 g).

Total sugar content was estimated by the phenol–sulfuric acid analysis using glucose as standard [39]. Uronic acid content was determined by the carbazole–sulfuric acid method using glucuronic acid as standard [40]. Neutral monosaccharide composition was analyzed according to the following procedure: AEPS (5 mg) were hydrolyzed with 5 ml of 2 M TFA at 110 °C for 5 h to release component monosaccharides. The hydrolyzed monosaccharides (inositol as the internal standard) were derivatized to acetylated aldononitriles [41] and isothermally separated by gas chromatography (GC) in 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) [34].

A stock AEPS solution with a concentration of 10 mg/ml was prepared by dissolving in 0.89% saline. The solution was sterilized by passing it through a 0.22-μm Millipore filter, and then analyzed for endotoxin level by a gel-clot *Limulus* amebocyte lysate assay (Zhejiang A and C Biological, Zhejiang, China).

### 2.4. Toxicity assays

Six-week-old female ICR mice were divided into five groups, each consisting of five mice. Animals were injected twice subcu-

**Table 1**
Sequences of primer used for RT-PCR.

| Gene | Primer sequence | Product size (bp) |
|---|---|---|
| GAPDH | 5′-CCCACAGTAAATTCAACGGCAC-3′<br>5′-CATTGGGGTTAGGAACACGGA-3′ | 570 |
| IL-2 | 5′-CTCTACAGCGGAAGCACAGC-3′<br>5′-CATCTCCTCAGAAAGTCCACCA-3′ | 381 |
| IFN-γ | 5′-TGAACGCTACACACTGCATCTTGG-3′<br>5′-CGACTCCTTTTCCGCTTCCTGAG-3′ | 459 |
| IL-4 | 5′-ATGGGTCTCAACCCCCAGCTAGT-3′<br>5′-GCTCTTTAGGCTTTCCAGGAAGTC-3′ | 399 |
| IL-10 | 5′-CCAGTTTTACCTGGTAGAAGTGATG-3′<br>5′-TGTCTAGGTCCTGGAGTCCAGCAGACTCAA-3′ | 324 |
| T-bet | 5′-AACCAGTATCCTGTTCCCAGC-3′<br>5′-TGTCGCCACTGGAAGGATAG-3′ | 436 |
| GATA-3 | 5′-GAAGGCATCCAGACCCGAAAC-3′<br>5′-ACCCATGGCGGTGACCATGC-3′ | 255 |

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

incubated for 5 min at 37 °C. The tubes then received 200 U of M-MuLV reverse transcriptase and were incubated for 60 min at 42 °C. Finally, the reaction was stopped by heating at 70 °C for 10 min. The samples were stored at −20 °C until further use.

Amplification was carried out in a total volume of 20 μl containing 0.5 μl (10 μM) of each cytokine- or transcription factor-specific primers (Table 1), 2 μl of 10× PCR buffer, 1.2 μl of $MgCl_2$ (25 mmol/l), 0.5 μl of dNTP (10 mM), 1 μl of transcribed cDNA, and 0.25 μl of Taq DNA polymerase. PCR was performed for 27 (GAPDH and IFN-γ), 29 (GATA-3), 31 (IL-2 and T-bet), or 33 (IL-4 and IL-10) cycles using a PTC-200 Thermal Cycler (MJ Research, Watertown, MA) with the following program of denaturation at 94 °C for 1 min, annealing at 55 °C (IL-2, GATA-3, and T-bet), 57 °C (GAPDH), or 58 °C (IL-4, IL-10, and IFN-γ) for 50 s, and elongation at 72 °C for 0.5 min. Semi-quantitative RT-PCR was performed using GAPDH as an internal control to normalize gene expression for the PCR templates. The PCR products were studied on a 1.5% agarose gel and the amplified bands were visualized using ImmageMaster VDS Software (Phamacia Biotech, USA) after staining with GoldView. The size of the amplified fragments was determined by comparison with a standard DNA marker.

### 2.11. Statistical analysis

The data were expressed as mean ± standard errors (S.E.) 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. Results

### 3.1. Isolation, characterization and composition of AEPS

The crude polysaccharide was extracted with hot water from the roots of *A. eriantha* and purified by means of DEAE–Sephadex A-50 gel chromatography, leading to a water-soluble polysaccharide AEPS. AEPS showed positive sulfuric acid–carbazole reaction, suggesting that it contained uronic acid and negative Fehling's reagent and iodine–potassium iodide reactions, indicating that it did not contain reducing sugar and starch-type polysaccharides. UV analysis and triketohydrindene hydrate reaction indicated that AEPS was not contaminated with proteins. GC quantitative analysis with derivatization revealed that AEPS was composed of rhamnose (3.18 mol.%), fucose (14.85 mol.%), arabinose (26.87 mol.%), xylose (4.49 mol.%), mannose (7.83 mol.%), glucose (7.72 mol.%), and galactose (35.05 mol.%) with the molar ratio of 1.00:4.67:8.45:1.41:2.46:2.43:11.02. The uronic acid content in AEPS was 3.70%.

### 3.2. Toxicity of AEPS

The endotoxin level in a stock AEPS solution with a concentration of 10 mg/ml was measured to be less than 0.5 endotoxin units (EU)/ml. Therefore, the AEPS sample used in this study was excluded from endotoxin contamination. When the animals were administered subcutaneously twice ranging from 0.5 to 5.0 mg at weekly intervals, there is no lethality observed. Local swelling or loss of hair was not observed in mice at the tested doses. The results suggested that the safety dose of AEPS used for human and animal was at least up to 200 mg/kg.

### 3.3. Effect of AEPS on splenocyte proliferation in OVA-immunized mice

The effects of AEPS on mitogen- and OVA-stimulated splenocyte proliferation in the immunized mice are shown in Fig. 1. As a positive control, Quil A markedly enhanced Con A-, LPS-, and OVA-stimulated splenocyte proliferation in the immunized mice. AEPS also significantly increased Con A- and LPS-stimulated splenocyte proliferation in the OVA-immunized mice ($P < 0.05$ or $P < 0.01$). The OVA-induced splenocyte proliferation in the immunized mice was also similarly increased by AEPS at three doses compared to OVA group ($P < 0.01$ or $P < 0.001$).

### 3.4. Effect of AEPS on the OVA-specific serum antibody response

The OVA-specific IgG, IgG1, IgG2a, and IgG2b antibody levels in the serum were measured 2 weeks after the last immunization using ELISA and the results were shown in Fig. 2. OVA

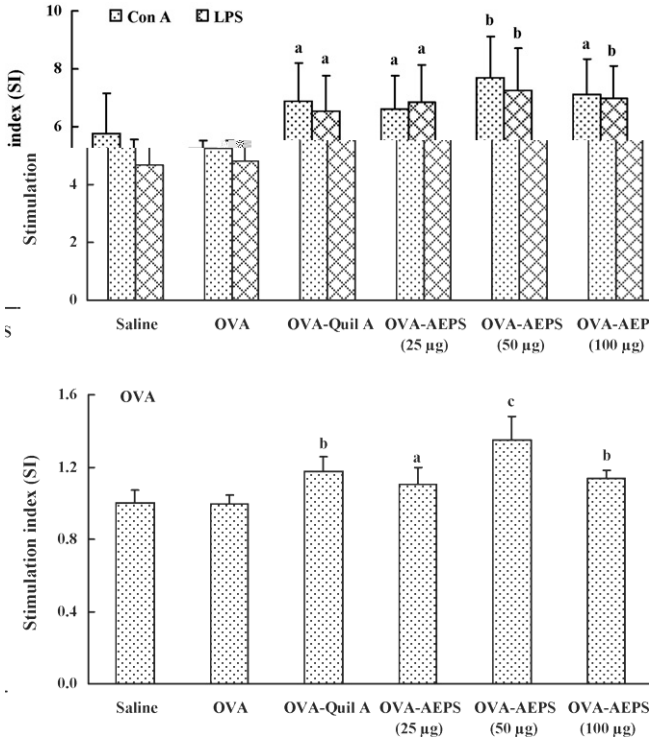


**Fig. 1.** Effect of AEPS on mitogen- and OVA-stimulated splenocyte proliferation in the mice immunized with OVA. Significant differences with OVA alone group were designated as $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

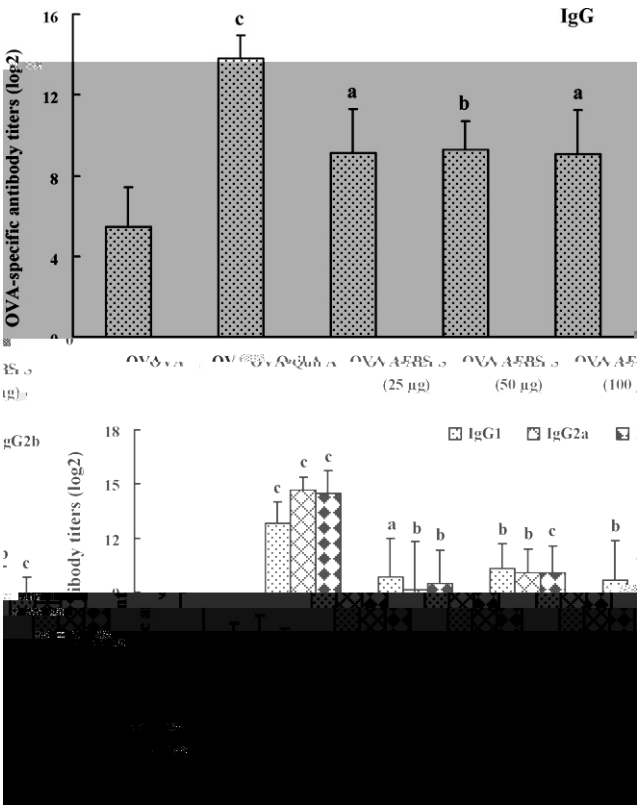


**Fig. 2.** Effect of AEPS on OVA-specific IgG, IgG1, IgG2a, and IgG2b antibodies in OVA-immunized mice. Significant differences with OVA alone groups were designated as $^{a}P<0.05$, $^{b}P<0.01$, and $^{c}P<0.001$.

alone induced low levels of OVA-specific IgG antibody. However, addition of Quil A and AEPS to OVA resulted in a significant increase in total IgG antibody titers ($P<0.05$, $P<0.01$, or $P<0.001$). The OVA-specific serum IgG1 titers in OVA-immunized mice were also significantly increased by Quil A and AEPS ($P<0.05$, $P<0.01$, or $P<0.001$). With regard to OVA-specific serum IgG2a and IgG2b titers, mice immunized with OVA/Quil A and OVA/AEPS had a dramatic increase compared with OVA alone group ($P<0.01$ or $P<0.001$). The findings indicated that AEPS significantly enhanced serum OVA-specific antibody production in immunized mice.

### 3.5. Effects of AEPS on NK cell activity in OVA-immunized mice

The effects of AEPS on NK cell activity in OVA-immunized mice were shown in Fig. 3. AEPS and Quil A significantly enhanced the killing activity of NK cell in the OVA-immunized mice ($P<0.01$ or $P<0.001$). The findings indicated that AEPS could promote lytic activity of NK cells in mice immunized with OVA.

### 3.6. Effect of AEPS on cytokine secretion by splenocytes from OVA-immunized mice

The calibration curves of IL-2, IFN-γ and IL-10 were constructed with mouse cytokine standards, and their correlation coefficients were all bigger than 0.9980. As shown in Fig. 4, the contents of cytokines IL-2, IFN-γ, and IL-10 in the supernatants from cultured splenocytes in the mice immunized with OVA/AEPS and OVA/Quil A were significantly higher than those in OVA control mice ($P<0.01$ or $P<0.001$), suggesting that AEPS significantly enhanced the production of the Th1 and Th2 cytokines in OVA-immunized mice.

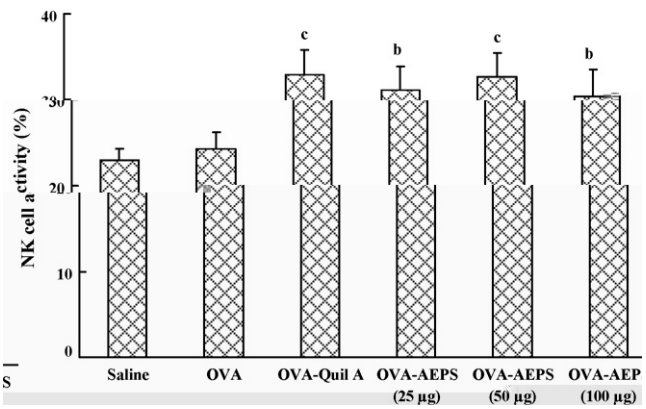


**Fig. 3.** Effect of AEPS on NK cell activity in mice immunized with OVA. Significant differences with OVA alone group were designated as $^{b}P<0.01$ and $^{c}P<0.001$.

### 3.7. Effect of AEPS on mRNA expression of cytokines and transcription factors in splenocytes from the immunized mice

As shown in Fig. 5 and Table 2, AEPS and Quil A significantly not only increased the mRNA expression of Th2 cytokines IL-4 and IL-10 and transcription factor GATA-3 ($P<0.01$ or $P<0.001$), but enhanced that of Th1 cytokines IL-2 and IFN-γ and transcription factor T-bet ($P<0.05$, $P<0.01$, or $P<0.001$) in splenocytes from the immunized mice. Thus, findings showed that AEPS up-regulated the gene expression of Th1/Th2 cytokines and transcription factors in splenocytes from the immunized mice.

## 4. Discussion

Despite technological advances in molecular biology and in genetic engineering, allowing identification of antigens with

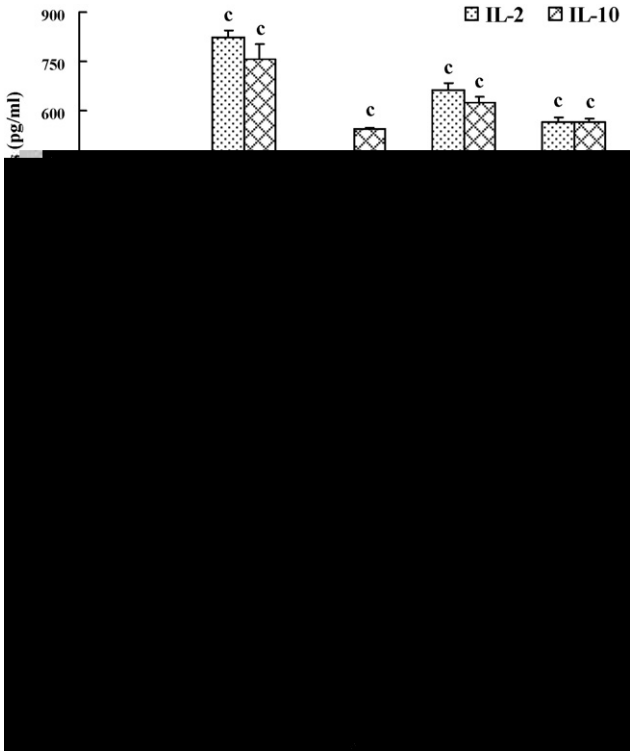


**Fig. 4.** Effects of AEPS on cytokine production in splenocytes from the OVA-immunized mice. Significant differences with OVA alone group were designated as $^{c}P<0.001$.

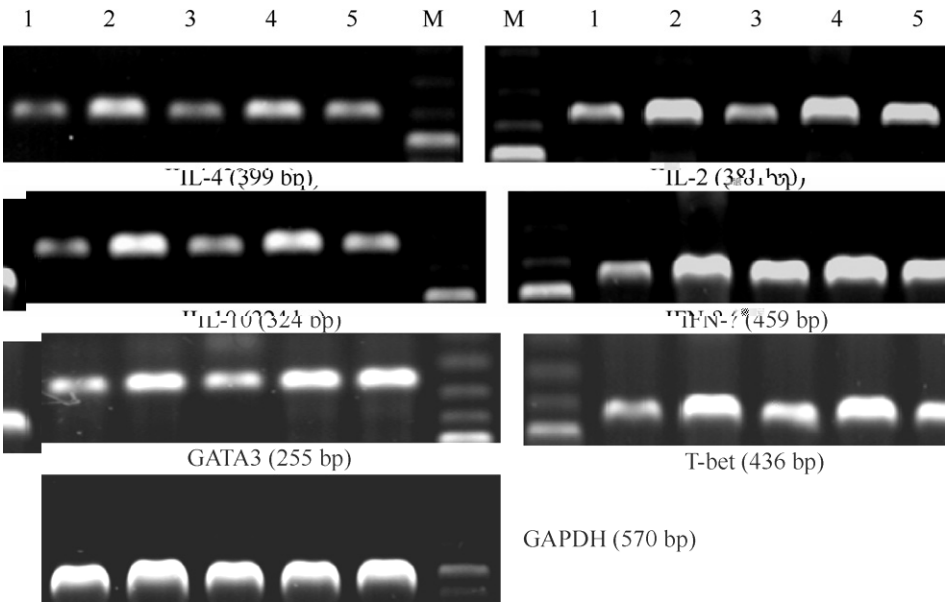


**Fig. 5.** Effect of AEPS on the mRNA expression of GAPDH, cytokines and transcription factors in splenocytes from the OVA-immunized mice. Lane M, DNA marker; lane 1, OVA; lane 2, OVA–Quil A; lane 3, OVA–AEPS (25 μg); lane 4, OVA–AEPS (50 μg); lane 5, OVA–AEPS (100 μg).

immunogenic potential, the majority of vaccines requires association with adjuvants capable of increasing the potency or stimulating the appropriate immune response [4,44]. An efficient adjuvant should have negligible toxicity and enhances the humoral or/and cellular immune response to a specific antigen [45]. Ideally, adjuvants should promote an appropriate immune response, be stable with long shelf life, biodegradable, cheap to produce and not themselves immunogenic [46]. While several hundred different adjuvants have been proposed over the last few decades, the vast majority have not been successful in being approved for human use, with limitations including lack of efficacy, unacceptable local or systemic toxicity, manufacturing difficulties, instability, and prohibitive cost [47].

In our previous work, the saponins from the root of *Achyranthes bidentata* [48], *Astragalus membranaceus* [49], *Bupleurum chinense* [50], *Glycyrrhiza uralensis* [51], *Panax notoginseng* [42], *Platycodon grandiflorum* [52] and the herb of *Gynostemma pentaphyllum* [53] were shown to possess the adjuvant potentials on the cellular and humoral immune response of mice against OVA. Although the haemolytic activities and toxicities of these saponins were lower than that of Quil A, most saponins still had some haemolytic effects. In addition, the saponins have a strong adjuvant activity when administered parenterally, in general, while they have a low or no activity when delivered orally [36]. The extensive studies on plant polysaccharide adjuvants indicate that these are excellent candidates to replace Alum as the adjuvant of choice for many vaccines. Meanwhile, most polysaccharides could exert the similar pharmacological effects when administered parenterally and orally. AEPS have been proved to significantly increase splenocytes proliferation, NK cell and CTL activity, IL-2 production from splenocytes, and serum antigen-specific antibody levels in S180 sarcoma-bearing mice by oral administration route [34]. To further search the safer adjuvant, the current study was undertaken to evaluate the toxicity of AEPS and its adjuvant potential on the cellular and humoral immune responses of mice against OVA.

The cellular immune response plays an important role in the host response to intracellular pathogens by limiting replication and accelerating clearance of infected cells and in the generation of both humoral and cell-mediated responses to vaccination. Among the T-lymphocytes, helper T cells induce B-lymphocytes to secrete antibodies, and cytotoxic T-lymphocytes help phagocytes to destroy ingested microbes and to kill intracellular microbes. Humoral immunity, however, mediated by antibodies, which are produced by B-lymphocytes, functions by neutralizing and eliminating extracellular microbes and microbial toxins. The capacity to elicit an effective T- and B-lymphocyte immunity can be shown by the stimulation of lymphocyte proliferation response. It is generally known that Con A stimulates T cells and LPS stimulates B cell proliferation. The proliferation assay showed that AEPS could significantly promote the Con A-, LPS-, and OVA-stimulated splenocyte proliferation in the immunized mice. The results indicated that AEPS could significantly increase the activation potential of T and B cells, and induce the humoral immunity and cell-mediated immune response in the OVA-immunized mice.

**Table 2**
The mRNA expression level of cytokines and transcription factors in splenocytes from the OVA-immunized mice.

| Gene | OVA | OVA–Quil A | OVA–AEPS | | |
|---|---|---|---|---|---|
| | | | (25 μg) | (50 μg) | (100 μg) |
| IL-4 | 0.28 ± 0.02 | 0.48 ± 0.04[b] | 0.29 ± 0.06 | 0.46 ± 0.05[b] | 0.43 ± 0.08[a] |
| IL-10 | 0.34 ± 0.02 | 0.70 ± 0.02[c] | 0.43 ± 0.02[b] | 0.66 ± 0.01[c] | 0.47 ± 0.03[c] |
| GATA-3 | 0.40 ± 0.03 | 0.66 ± 0.04[c] | 0.46 ± 0.02 | 0.55 ± 0.01[b] | 0.58 ± 0.05[b] |
| IL-2 | 0.57 ± 0.01 | 0.92 ± 0.01[c] | 0.58 ± 0.01 | 1.00 ± 0.01[c] | 0.81 ± 0.00[c] |
| IFN-γ | 0.52 ± 0.01 | 0.91 ± 0.01[c] | 0.87 ± 0.01[c] | 1.05 ± 0.02[c] | 0.92 ± 0.05[c] |
| T-bet | 0.47 ± 0.04 | 0.94 ± 0.01[c] | 0.63 ± 0.07[a] | 0.87 ± 0.04[c] | 0.73 ± 0.01[c] |

[a] Significant differences with OVA alone group were designated as $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

[25] Lu Y, Wang DY, Hu YL, Huang XY, Wang JM. Sulfated modification of epimedium polysaccharide and effects of the modifiers on cellular infectivity of IBDV. Carbohydr Polym 2008;2:180–6.
[26] Huang XY, Hu YL, Zhao XN, Lu Y, Wang JM, Zhang F, et al. Sulfated modification can enhance the adjuvanticity activity of *Astragalus* polysaccharide for ND vaccine. Carbohydr Polym 2008;2:303–8.
[27] Sun YX, Liu JC. Adjuvant effect of water-soluble polysaccharide (PAP) from the mycelium of *Polyporus albicans* on the immune responses to ovalbumin in mice. Vaccine 2008;26(31):3932–6.
[28] da Silva BP, de Medeiros Silva G, Parente JP. Chemical properties and adjuvant activity of a galactoglucomannan from *Acrocomia aculeata*. Carbohydr Polym 2009;75(3):380–4.
[29] Guo ZH, Hu YL, Wang DY, Ma X, Zhao XN, Zhao BK, et al. Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. Vaccine 2009;27(5):660–5.
[30] Nishiyama I, Fukuda T, Oota T. Genotypic differences in chlorophyll, lutein, and β-carotene. J Agric Food Chem 2005;53:6403–7.
[31] Zhao YP, Wang XY, Wang ZC, Lu Y, Fu CX, Chen SY. Essential oil of *Actinidia macrosperma*, a catnip response kiwi endemic to China. J Zhejiang Univ Sci B 2006;7(9):708–12.
[32] Jiangsu New Medical College. Dictionary of Chinese Materia Medica. Shanghai: Shanghai People's Press; 1977. p. 440.
[33] Lin SQ, Yu P, Zhu SM, Wu XC, Yu BS. Preliminary study on anticancer effect and affecting mouse immune function of crude extracts from the *Actinintia eriantha* Benth root. J Fujian Normal Univ (Nat Sci Ed) 1987;3(2):108–10.
[34] Xu HS, Wu YW, Sun HX, Yao L. Antitumor and immunomodulatory activity of polysaccharide from the roots of *Actinidia eriantha*. J Ethnopharmacol; submitted for publication.
[35] Larsen ST, Nielsen GD. The adjuvant effect of di-(2-ethylhexyl) phthalate is mediated through a PPARα-independent mechanism. Toxicol Lett 2007;170(3):223–8.
[36] Sun HX, Xie Yong, Ye YP. Advances in saponin-based adjuvants. Vaccine 2009;27(12):1787–96.
[37] Xie Y, Ye YP, Sun HX, Li D. Contribution of the glycidic moieties to the haemolytic and adjuvant activity of platycodigenin-type saponins from the root of *Platycodon grandiflorum*. Vaccine 2008;26(27):3450–8.
[38] Xie Y, Sun HX, Duo L. Platycodin D is a potent adjuvant of specific cellular and humoral immune responses against recombinant hepatitis B antigen. Vaccine 2009;27(5):757–64.
[39] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28: 350–6.
[40] Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem 1962;4:330–4.
[41] Mawhinney TP, Feather MS, Barbero GJ, Martinez JR. The rapid, quantitative determination of neutral sugars (as aldononitrile acetates) and amino sugars (as O-methyloxime acetates) in glycoproteins by gas–liquid chromatography. Anal Biochem 1980;101:112–7.
[42] Sun HX, Ye YP, Pan HJ, Pan YJ. Adjuvant effect of *Panax notoginseng* saponins on the immune responses to ovalbumin in mice. Vaccine 2004;22:3882–9.
[43] Tu J, Sun HX, Ye YP. Immunomodulatory and antitumor activity of triterpenoid fractions from the rhizomes of *Astilbe chinensis*. J Ethnopharmacol 2008;119(2):266–71.
[44] Barr TA, Carlring J, Heath AW. Co-stimulatory agonists as immunological adjuvants. Vaccine 2006;24:3399–407.
[45] Marciani DJ. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov Today 2003;8:934–43.
[46] Edelman R. Vaccine adjuvants. Rev Infect Dis 1980;2(3):370–83.
[47] Aguilar JC, Rodríguez EG. Vaccine adjuvants revisited. Vaccine 2007;25: 3752–62.
[48] Sun HX. Adjuvant effect of *Achyranthes bidentata* saponins on specific antibody and cellular response to ovalbumin in mice. Vaccine 2006;24(17):3432–9.
[49] Yang ZG, Sun HX, Fang WH. Haemolytic activities and adjuvant effect of *Astragalus membranaceus* saponins on the immune responses to ovalbumin in mice. Vaccine 2005;23(44):5196–203.
[50] Sun HX. Haemolytic activities and adjuvant effect of *Bupleurum chinense* saponins on the immune responses to ovalbumin in mice. Vaccine 2006;24(9):1324–31.
[51] Sun HX, Pan HJ. Immunological adjuvant effect of *Glycyrrhiza uralensis* saponins on the immune responses to ovalbumin in mice. Vaccine 2006;24(11):1914–20.
[52] Xie Y, Pan HJ, Sun HX, Li D. A promising balanced Th1 and Th2 directing immunological adjuvant, saponins from the root of *Platycodon grandiflorum*. Vaccine 2008;26(31):3937–45.
[53] Sun HX, Zheng QF. Haemolytic activities and adjuvant effect of *Gynostemma pentaphyllum* saponins on the immune responses to ovalbumin in mice. Phytother Res 2005;19(10):895–900.
[54] Gupta RK, Siber GR. Adjuvants for humans vaccines: current status, problems and future prospects. Vaccine 1995;13:1263–6.
[55] Audibert FM, Lise LD. Adjuvants: current status, clinical perspectives and future prospects. Immunol Today 1993;14:281–4.
[56] Sedlik C. Th1 and Th2 subsets of T lymphocytes: characteristics, physiological role and regulation. Bull Inst Pasteur 1996;94:173–200.
[57] Livingston PO, Adluri S, Helling F, Yao TJ, Kensil CR, Newman MJ, et al. Phase I trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine 1994;12(14):1275–80.
[58] Cher DJ, Mosmann TR. Two types of murine helper T-cell clones. II. Delayed-type hypersensitivity is mediated by Th1 clones. J Immunol 1987;138:3688–94.
[59] Mosmann TR, Sad S. The expanding universe of T-cells subsets: Th1, Th2 and more. Immunol Today 1996;17:138–46.
[60] Mahon BP, Katrak K, Nomoto A, Macadam AJ, Minor PD, Milles KHG. Poliovirus-specific $CD4^+$ Th1 clones with both cytotoxic and helper activity mediate protective humoral immunity against a lethal poliovirus infection in transgenic mice expressing the human poliovirus receptor. J Exp Med 1995;181:1285–92.
[61] Coffman RL, Seymour BWP, Lebman DA, Hiraki DD, Christiansen JA, Shrader B, et al. The role of helper T-cell products in mouse B-cell differentiation and isotype regulation. Immunol Rev 1988;102:5–28.
[62] Constant SL, Bottomly K. Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. Annu Rev Immunol 1997;15:297–322.
[63] McKee AS, Munks MW, Marrack P. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 2007;27(5):687–90.
[64] Finkelman FD, Holmes J, Katona IM, Urban Jr JF, Beckmann MP, Park LS, et al. Lymphokine control of in vivo immunoglobulin isotype selection. Annu Rev Immunol 1990;8:303–33.
[65] Germann T, Bongartz M, Dlugonska H, Hess H, Schmitt E, Kolbe L, et al. Interleukin-12 profoundly up-regulates the synthesis of antigen-specific complement-fixing IgG2a, IgG2b and IgG3 antibody subclasses in vivo. Eur J Immunol 1995;25(3):823–9.
[66] Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 1997;89(4):587–96.
[67] Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000;100(6):655–69.
[68] Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. J Immunol Methods 2003;278(1/2):157–69.
[69] Kos FJ, Engleman EG. Immune regulation: a critical link between NK cells and CTLs. Immunol Today 1996;17:174–6.
[70] Medzhitov R, Janeway Jr CA. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 1997;9:4–9.
[71] Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 1994;12:337–65.
[72] Moretta L, Bottino C, Cantoni C, Mingari MC, Moretta A. Human natural killer cell function and receptors. Curr Opin Pharmacol 2001;1:387–91.
[73] Rappuoli R. Bridging the knowledge gaps in vaccine design. Nat Biotechnol 2007;25:1361–6.
[74] Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol 2008;8:713–25.
[75] Zhang J, Sun R, Wei HM, Tian ZG. Antitumor effects of recombinant human prolactin in human adenocarcinoma-bearing SCID mice with human NK cell xenograft. Int Immunopharmacol 2005;5:417–25.