



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

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cellular and humoral immune responses to ovalbumin and side effects when mice were administered subcutaneously of 7 days. The mice were immunized subcutaneously in saline containing Quil A (10 µg) or AEPS (25, 50, µg). Splenocyte proliferation, natural killer (NK) cell activity, splenocytes, and serum OVA-specific antibody titers were significantly enhanced by AEPS. Splenocyte proliferation and the serum OVA-specific antibody titers were significantly enhanced by AEPS. AEPS promoted the production of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines. The expression of IL-2, IFN-γ, IL-4 and IL-10 cytokines in splenocytes from the immunized mice ($P < 0.05$, $P < 0.01$ or $P < 0.001$) indicated that AEPS had strong potential to increase Th1/Th2 response, and that AEPS may be for a wide spectrum of prophylactic and therapeutic

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1. Introduction

Vaccination remains the most cost-effective biomedical approach for the control of infectious diseases. The safety of the traditional vaccines based on live attenuated or killed microorganisms is questionable due to the risk of virulence reversion [1]. New generations of vaccines based on purified recombinant proteins, synthetic peptides and plasmid DNA, despite their better tolerability, are unfortunately often much less reactogenic and immunogenic [2]. The majority of these vaccines require association with adjuvants capable of increasing the potency or stimulating the appropriate immune response [3,4]. The benefits flowing from adjuvant incorporation into any vaccine formulation have to be balanced with the risk of adverse reactions induced by these compounds. Unfortunately, strong adjuvant activity is often correlated with increased toxicity. Freund's complete adjuvant

(FCA) remains amongst the most potent of known adjuvants and a particularly powerful stimulant of both cellular and humoral immunities [5]. Unfortunately FCA causes severe reactions and is too toxic for human use. The unique capacity of the extract Quil A from the bark of *Quillaja saponaria* and its purified saponin QS-21 to stimulate both the Th1 immune response and the production of cytotoxic T-lymphocyte against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines [6,7]. However, in addition to pain on injection, severe local reactions and granulomas, toxicity includes severe haemolysis [8–11] making such adjuvants unsuitable for human uses other than for life threatening diseases, such as HIV infection or cancer [12]. Although muramyl dipeptide (MDP) and other derivatives from Gram-negative bacteria, such as lipopolysaccharide (LPS) and monophosphoryl lipid A have also been used as human adjuvants, their toxicities remain the single biggest barrier to the use of such adjuvants for human prophylactic vaccines [13,14]. A major challenge in adjuvant research is to increase adjuvant activity while reducing toxicity [8]. Currently, aluminum compounds

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(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 macrocarpa*, 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 $24 \pm 1^\circ\text{C}$, humidity of $50 \pm 10\%$, 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 AidTM 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 $\mu\text{g}/\text{ml}$ 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 \times 0.32 mm \times 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* amoebocyte 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' 5'-CATTGGGGTTAGGAACACGGA-3'	570
IL-2	5'-CTCTACAGCGGAAGCACAGC-3' 5'-CATCTCTCAGAAAGTCCACCA-3'	381
IFN- γ	5'-TGAACGCTACACACTGCATCTTGG-3' 5'-CGACTCCTTTTCCGCTTCTGAG-3'	459
IL-4	5'-ATGGGTCTCAACCCAGCTAGT-3' 5'-GCTCTTAGGCTTCCAGGAAGTC-3'	399
IL-10	5'-CCAGTTTACCTGGTAGAAGTGATG-3' 5'-TGCTAGGTCTCGAGTCCAGCAGACTCAA-3'	324
T-bet	5'-AACCAGTATCTGTTCACAGC-3' 5'-TGTCGCCACTGGAAGGATAG-3'	436
GATA-3	5'-GAAGGCATCCAGACCCGAAC-3' 5'-ACCATGGCGGTGACCATGC-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 μ l containing 0.5 μ l (10 μ M) of each cytokine- or transcription factor-specific primers (Table 1), 2 μ l of 10 \times PCR buffer, 1.2 μ l of MgCl₂ (25 mmol/l), 0.5 μ l of dNTP (10 mM), 1 μ l of transcribed cDNA, and 0.25 μ l 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 ImageMaster VDS Software (Pharmacia 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 \pm 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

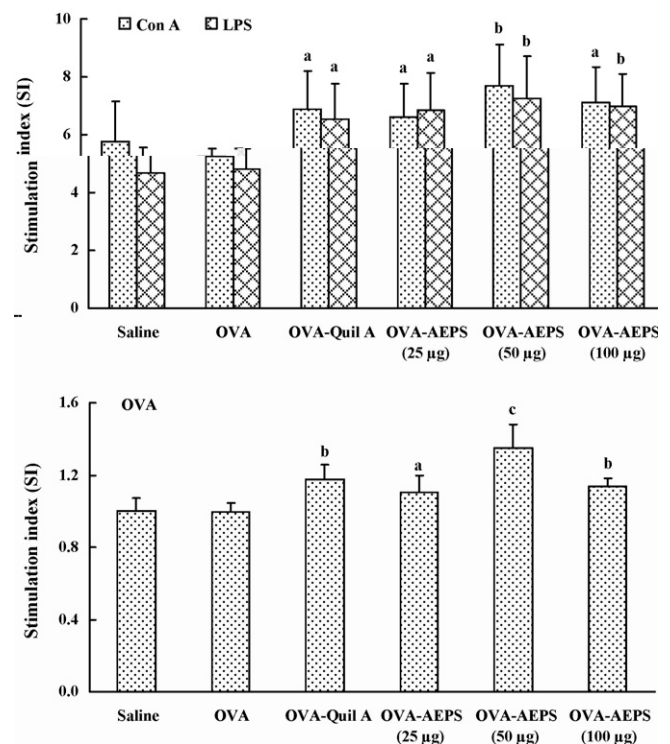


Fig. 1. Effect of AEPS on mitogen- and OVA-stimulated splenocyte proliferation in the mice immunized with OVA. Significant differences with OVA alone group were designated as ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$.

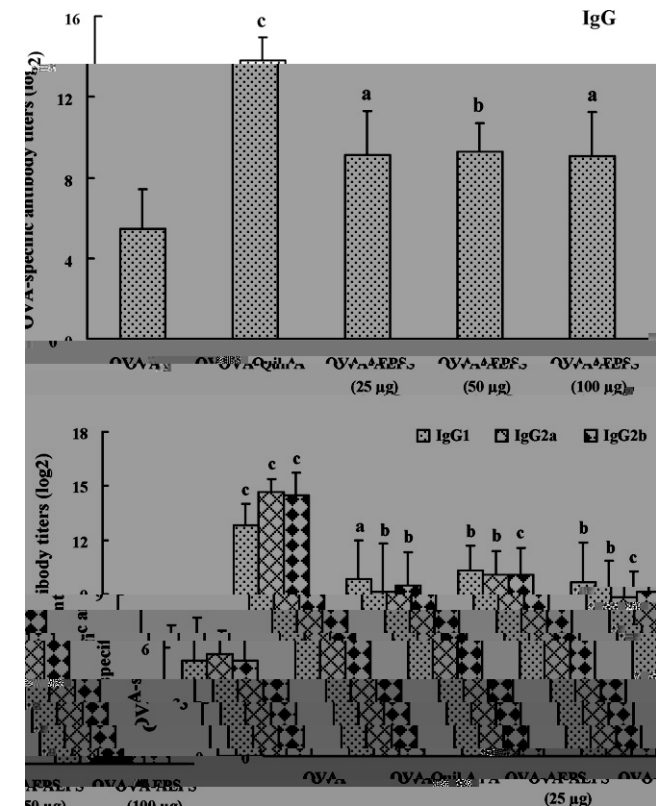


Fig. 2. Effect of AEPS on OVA-specific IgG, IgG1, IgG2a, and IgG2b antibodies in OVA-immunized mice. Significant differences with OVA alone groups were designated as ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$.

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.

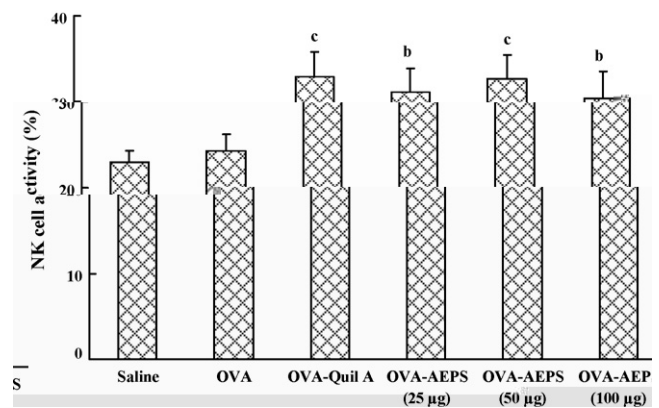


Fig. 3. Effect of AEPS on NK cell activity in mice immunized with OVA. Significant differences with OVA alone group were designated as ^b $P < 0.01$ and ^c $P < 0.001$.

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

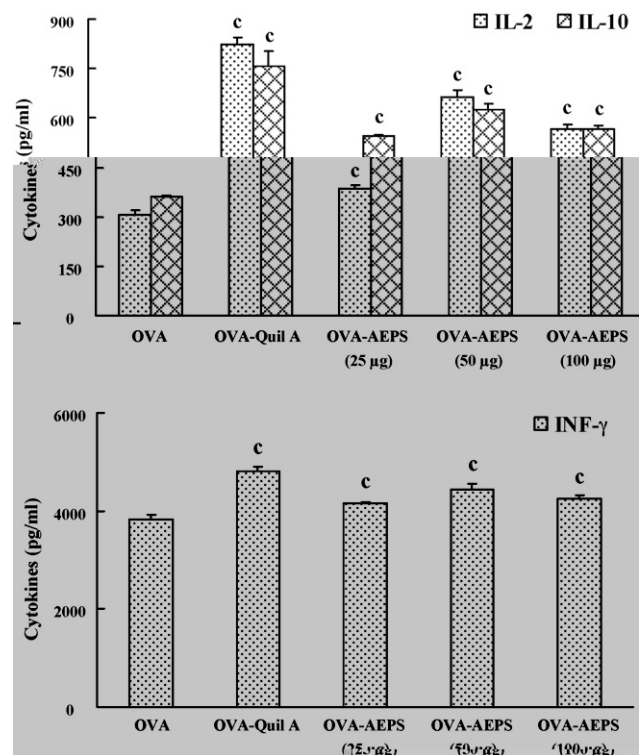


Fig. 4. Effects of AEPS on cytokine production in splenocytes from the OVA-immunized mice. Significant differences with OVA alone group were designated as ^c $P < 0.001$.

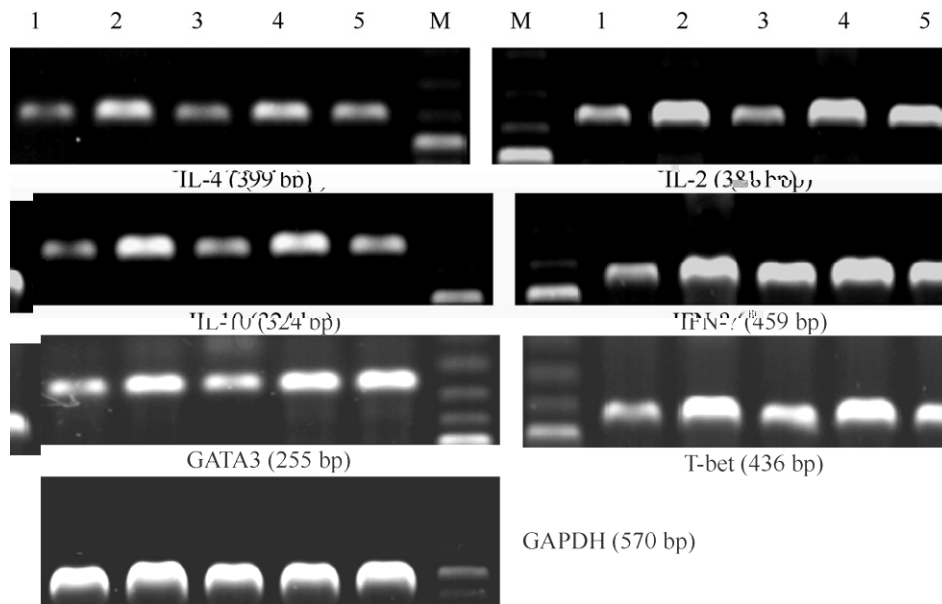


Fig. 5. Effect of AEPS on the mRNA expression of GAPDH, cytokines and transcription factors in splenocytes from the OVA-immunized mice. Lane M, DNA marker; lane 1, OVA; lane 2, OVA–Quil A; lane 3, OVA–AEPS (25 μ g); lane 4, OVA–AEPS (50 μ g); lane 5, OVA–AEPS (100 μ g).

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 pharma-

cological 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 μ g)	(50 μ g)	(100 μ g)
IL-4	0.28 \pm 0.02	0.48 \pm 0.04 ^b	0.29 \pm 0.06	0.46 \pm 0.05 ^b	0.43 \pm 0.08 ^a
IL-10	0.34 \pm 0.02	0.70 \pm 0.02 ^c	0.43 \pm 0.02 ^b	0.66 \pm 0.01 ^c	0.47 \pm 0.03 ^c
GATA-3	0.40 \pm 0.03	0.66 \pm 0.04 ^c	0.46 \pm 0.02	0.55 \pm 0.01 ^b	0.58 \pm 0.05 ^b
IL-2	0.57 \pm 0.01	0.92 \pm 0.01 ^c	0.58 \pm 0.01	1.00 \pm 0.01 ^c	0.81 \pm 0.00 ^c
IFN- γ	0.52 \pm 0.01	0.91 \pm 0.01 ^c	0.87 \pm 0.01 ^c	1.05 \pm 0.02 ^c	0.92 \pm 0.05 ^c
T-bet	0.47 \pm 0.04	0.94 \pm 0.01 ^c	0.63 \pm 0.07 ^a	0.87 \pm 0.04 ^c	0.73 \pm 0.01 ^c

^a Significant differences with OVA alone group were designated as $P < 0.05$.

^b $P < 0.01$.

^c $P < 0.001$.

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