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Full Length Research Paper

Adjuvant effect of saponins isolated from the sepal of *Physalis alkekengi* L. var. *francheti (Mast.) Makino* fruit on the immune responses to ovalbumin in mice

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Saponins (designated SP) were isolated and purified from the sepals of the mature fruit of *Physalis alkekengi L. var. francheti (Mast.) Makino* which is a traditional Chinese medicine herb. Immunologic enhancement of the SP was evaluated in this paper. Male ICR mice were immunized subcutaneously with ovalbumin (OVA) alone or with OVA containing SP in doses of 0.63, 1.25, 2.50 mg/kg body weight (BW), or QuilA in doses of 0.5, 1.0 mg/kg BW 2 times at 14-day interval. The mice were sacrificed two weeks after the last immunization to analyze humoral and cellular immunity. Delayed-type hypersensitivity (DTH) response was further detected. The results showed that SP (1.25 mg/kg BW) significantly enhanced OVA-specific antibody titers (IgG, IgG1, IgG2b) in serum, the concanavalin (ConA), lipopolysaccharide (LPS), OVA-induced splenocyte proliferation, proportions of $CD4^+$ and $CD8^+$ T lymphocytes in spleen, delayed-type hypersensitivity (DTH) response when compared with OVA-injected mice ($P<0.05$ or $P<0.01$). More importantly, haemolytic activity assay indicated SP had lower haemolytic effect than QuilA. Therefore, SP evoked both humoral immunity and cellular immunity, and can be considered as a promising adjuvant eliciting both Th1 and Th2 responses to improve the efficacy of vaccine.

Key words: Herbal medicine, saponin, humoral response, cellular response, adjuvant.

INTRODUCTION

Adjuvants are substances that when co-administered with antigen, can act generally to accelerate, prolong, or enhance the quality of specific immune responses to antigens (Hunter, 2002; Guy, 2007). So far, alum salts are the only immunologic adjuvants approved by the US Food and Drug Administration (FDA), since they have a good safety recorder and induce strong humoral responses. Unfortunately, alum adjuvants still have some side effects, these include: failure to induce cell-mediated immunity which is necessary for intracellular infection; increase IgE antibody responses, allergenicity and potential neurotoxicity (Aguilar and Rodríguez, 2007; Guy and Burdin, 2005; Ebensen and Guzmán, 2008; Reed et al., 2009).

Saponins are chemically a heterogeneous group of sterol glycosides and triterpene glycosides. Previous studies have shown that saponins from some herbal plants can elicit the faster and stronger humoral and cellular immune responses and the production of

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Abbreviations: OVA, Ovalbumin; **SP,** Saponin purified from the sepal of *Physalis alkekengi L. var. francheti (Mast.) Makino* fruit; **OVA/QuilA (0.5, 1.0),** ICR mice immunized with OVA (5 mg/kg body weight) and QuilA 0.5, 1.0 mg/kg body weight respectively; **OVA/SP (0.63, 1.25, 2.50),** ICR mice immunized with OVA (5 mg/kg body weight) and SP 0.63, 1.25, 2.50 mg/kg body weight respectively; **IFT,** increase in footpad thickness.

cytotoxic T-lymphocytes (CTL), which are more effective than alum (Sun et al., 2009b; Yang et al., 2005). The most commonly used saponins as adjuvants are Quil A and QS-21 derived from the bark of *Quillajia saponaria*. Although it has been reported that Quil A and QS-21 can induce the stronger humoral and cell immune responses, their high toxicity and undesirable haemolytic effect is unsuitable for most human uses (Liu et al., 2011; Marciani et al., 2003; Ragupathi et al., 2010; Song and Hu, 2009; Sun et al., 2009a; Xie et al., 2008b).

Physalis alkekengi L. var. francheti (Mast.) Makino (*P. alkekengi*) is widely distributed in Europe and Asia including Russia, China, Japan, etc. It has been reported that *P. alkekengi* has numerous ethnopharmacological properties including anti-inflammatory, anti-cold, anti-cough and anti-fungal activities (Basey and Woolley, 1973; Vessal et al., 1991; Vessal et al., 1996; Zhou and Wang, 1997). However, no specific studies on the immunologic enhancement of the saponins, which is isolated from the sepals of *P. alkekengi* fruit, have been carried out. We therefore specifically focused on evaluating the adjuvant effect of this saponins. In this paper, immunologic enhancement of the saponins (designated SP below) isolated from the sepal of *P. alkekengi* fruit was evaluated. The results showed SP can significantly enhance humoral and cellular responses and could be used as an adjuvant.

MATERIALS AND METHODS

Experimental animals

Five week-old male ICR mice (Grade II, body weight 20±2 g) used for experiment were breed at the School of Public Health, Jilin University, China (certificate no. SCXK-(JI)2007-0003, Changchun, China).

The mice were acclimatized for a period of 2 to 3 days before using it for experiment. Before and during the experiment the mice were fed with standard laboratory diet, given tap water and maintained under a constant 12 h light and dark cycle at 21 to 23°C.

Materials and chemicals

The mature fruits of *P. alkekengi* were collected in Shuangyang district, Changchun city, Jilin province in September 2007. It was identified by Prof. Hongxing Xiao (School of Life Science, Northeast Normal University, Changchun, China). Ovalbumin (OVA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalina(ConA), lipopolysaccharide (LPS), ureahydrogen peroxide Addition compound and aluminum hydroxide gel (Alum) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Goat anti-mouse IgG1-HRP and IgG2b-HRP were from Southern Biotechnology Associate lnc (Birmingham, AL, USA); goat anti-mouse IgG-HRP was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co.LTD (China). RPMI-1640 tissue culture medium was from Gibco (USA); Fetal calf serum (FCS) was provided by Hangzhou Sijiqing Corp. of China. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (L3T4, cloneH129.19) and phycoerythrin (PE)-conjugated rat anti-mouse CD8 (LY-2, clone 53-6.7) monoclonal antibody were from BD Biosciences Pharmingen, CA, USA. All other chemicals were of grade AR.

Extraction, isolation and identification of SP

The sepals were from the mature fruits of *P. alkekengi.* Powdered sepals (200 g) were extracted with 70% EtOH (1.5 L) under reflux three times for 2 h at 60°C at each time. The supernatant recovered by filtering from the whole extract was concentrated by evaporation at 45°C under reduced pressure. The concentrated supernatant was extracted with water-saturated *n*-BuOH until *n*-BuOH layer became coloreless. The *n*-BuOH solution was concentrated by evaporation at 45°C under reduced pressure. The concentrated extract was washed with ether three times and dried in vacuum. Crude saponins (3.40 g) was obtained, and subjected to D101 resin column chromatography, then eluted with 60% EtOH to obtain saponins (designed SP) after being washed with water. The solution was sterilized by 0.22 μm millipore filter for all animal experiments. The endotoxin level in SP solution was less than 0.5 EU (endotoxin unit)/ml.

Phytochemical test

The standard methods of Trease and Evans (Trease and Evans, 1989) were used to identify the compositions of SP.

Haemolytic activity

Red blood cell suspension (0.6 ml of 0.5%) from New Zealand rabbit was mixed with 0.6 ml diluents of SP or QuilA in saline solution (0.89% w/v NaCl, pyrogen free) with the various concentrations. Mixtures were incubated at 37°C for 30 min and centrifugated at 70 g for 10 min. Free haemogloblin in the supernates was measured at 412 nm (Xie et al., 2008a). Saline and distilled water were included as minimal and maximal haemolytic controls. The concentration inducing 50% of the maximum haemolysis was considered the HD50. Each experiment included triplicates at each concentration. Four independent experiments were performed for the analysis of the HD50.

Immunization, collection of blood samples and spleen lymphocytes

In order to detect the immunologic enhancement of SP on the mice, the male ICR mice (5 mice per group) were immunized subcutaneously with OVA (5 mg/kg) alone, or with OVA (5 mg/kg) containing QuilA (0.5, 1.0 mg/kg) (OVA/QuilA 0.5, 1.0), or SP (0.63, 1.25, 2.50 mg/kg) (OVA/SP 0.63, 1.25, 2.50) twice at 14-day interval. Mice were treated with saline as a control group. Before the first immunization, the mice from groups OVA/QuilA, OVA/SP were immunized with QuilA , and SP alone with respective doses for two successive days. Other groups were injected saline. OVA was dissolved in saline for injection. The mice were sacrificed two weeks after the second immunization. The mice serum and the spleen lymphocytes were collected to detect humoral and cellular immune responses.

Splenocyte proliferation assay

Splenocytes were aseptically prepared from immunized ICR mice in Hank's balanced salt solution by mincing with a pair of scissors and filtering through a fourply gauze. After the erythrocytes were lysed with red blood cytolysate (Tris-NH_4Cl), splenocytes were collected by centrifugation (206 × g at 4°C for 2 min). Splenocytes were later washed twice in Hank's balanced salt solution and re-suspended in complete medium RPMI1640, which was supplemented with 100 IU/ml penicillin, 100 mg/L streptomycin and 10% FCS.

Table 1. Haemolytic activities of SP. Haemolytic percents of saline and distilled water were included as minimal abd maximal haemolytic controls. The values are presented as mean ± SD (n=3).

Group	Absorbance value	Haemolytic percent (%)
Saline	0.11 ± 0.01	0.00 ± 0.03
Distilled water	1.98 ± 0.02	100 ± 0.01
SP 500 µg/ml	0.18 ± 0.01	3.50 ± 0.01
SP 250 µg/ml	0.14 ± 0.01	1.49 ± 0.03
SP 125 µg/ml	0.12 ± 0.01	0.53 ± 0.02
SP 50 µg/ml	0.11 ± 0.01	0.27 ± 0.01

The splenocytes were seeded into 3 wells of a 96-well flat-bottom microtiter plate (Nunc) at 1×10^6 cells in 100 µl complete medium. Thereafter ConA, LPS, OVA (final concentration 5, 10, 10 mg/L respectively), or medium were added to a final volume of 200 µl. After 68 h incubation at 37°C in 5% CO_2, the splenocytes were incubated with 10 µl of MTT solution (5 g/L) for 4 h. The supernatant was removed carefully. 200 µl of a DMSO working solution (192 µl with 8 µl 1 mol/L HCl) was added to each well, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min. The stimulation index (SI) was calculated by the following formula:

SI = The absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures (Sun, 2006; Yang et al., 2008).

Measurement of OVA-specific antibody

OVA-specific antibodies (IgG, IgG1 and IgG2b) in serum of the immunized mice were evaluated by an indirect ELISA according to the methods of Yang et al. (2005) and Xie et al. (2008b). Briefly, microtiter plates (Nunc) were coated with 100 µl OVA (50 mg/L) in 0.05 mol/L carbonate-bicarbonate buffer pH 9.6 for 24 h at 4°C. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20, and blocked with PBS containing 5% skim milk powder at 37°C for 1 h. After washing with PBS containing 0.05% (v/v) Tween 20 three times, 100 µl of a series of diluted serum from immunized mice or PBS containing 5% skim milk powder as control were added to the triplicate wells. The plates were then incubated for 1 h at 37°C, followed by washing with PBS containing 0.05% (v/v) Tween 20. Aliquots of 100 µl of goat anti-mouse IgG-HRP, IgG1-HRP and IgG2b-HRP (diluted 1:5000 with PBS containing 5% skim milk powder respectively) were added to each plate. The plates were further incubated for 1 h at 37°C. Substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well after washing with PBST, and the plate was incubated for 15 min at room temperature. Reaction was terminated by adding 50 µl of 2 mol/L H_2SO_4 to each well, and optical density (OD) was detected at 450/630 nm with ELISA reader (Model 680, Bio-Rad, USA). Antibody titer was expressed by Log_2 value of the highest dilution of serum.

Flow cytometric assays

The method for flow cytomeric assay was conducted following procedures by Zaharoff et al. (2007). Lymphoid cells (1×10^6) prepared from the spleen of immunized mice were incubated with either FITC- conjugated rat anti-mouse CD4 mAbs or PE-conjugated rat anti-mouse CD8 mAbs for 1 h at 4°C, and collected by centrifugation at 380 × *g* for 15 min. Lymphoid cells were washed with PBS and centrifuged at 380 × *g* for 5 min, re-suspended with 200 µl PBS for immediate flow cytometric analysis. The percentage of positively stained cells, determined over 10,000 events, was analyzed by a FACScan cytofluorimeter (EPICS XL, Beckman Coulter, USA).

Delayed-type hypersensitivity (DTH) assay

The male ICR mice (5 mice per group) were immunized subcutaneously with OVA (5 mg/kg) alone, or with OVA (5 mg/kg) containing QuilA (0.5, 1.0 mg/kg), or SP (0.63, 1.25, 2.50 mg/kg). One week after the immunization, the mice were injected on one footpad with 20 µl of OVA (1 g/L), and 20 µl of saline was injected on the contralateral footpad. Increase in footpad thickness (IFT) was measured by a dial caliper 48 h later (Ajdary et al., 2007; Fang et al., 2005; Wang et al., 2006).

SATISTICAL ANALYSIS

All results were expressed as mean ± standard deviation (SD). Data were analyzed by standard *t*-test. P values less than 0.05 were considered statistically significant.

RESULTS

Phytochemical screening

Phytochemical analysis of SP gave a positive reaction for each of the following secondary metabolites: saponins, carbohydrates, sterols and flavonoids. Saponin was the main component of SP and it made up 72.6% of SP.

Haemolytic activities

To compare the haemolytic activities between SP and QuilA, the HD50 was investigated. The haemolytic activity of SP on red blood cells was shown in Table 1. Results indicated the HD50 value of QuilA was 17.11±0.30 µg/ml. However, haemolytic activity of SP at concentration of 500 µg/ml was 3.50±0.01 µg/ml. The data revealed that SP had lower toxicity than QuilA.

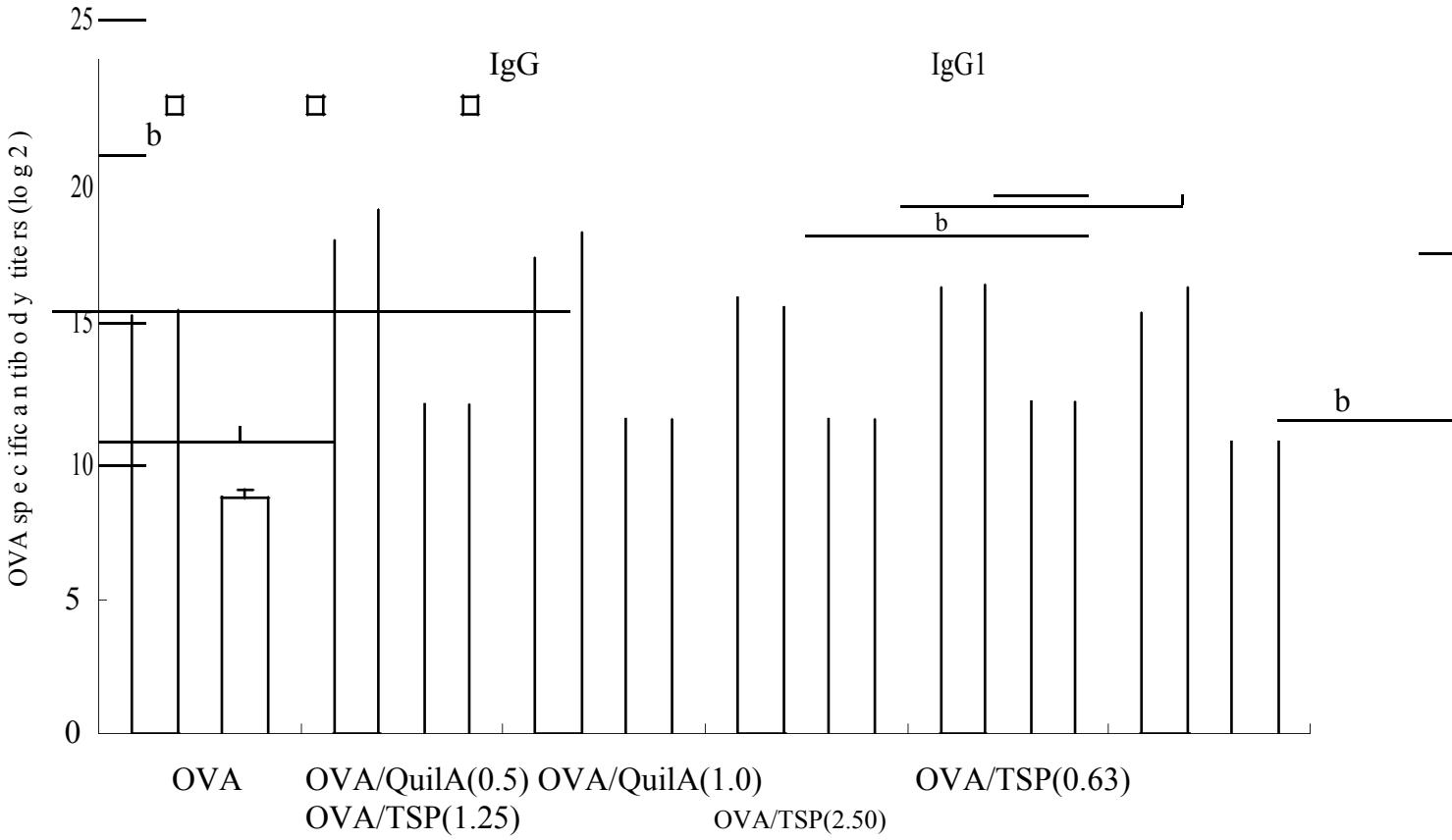

Figure 1. Effect of SP on OVA-specific IgG, IgG1, IgG2b antibody titers in OVA-immunized mice. The values are present as mean ± SD. [a]P <0.05, [b]P< 0.01 *vs* group OVA.

OVA- specific antibody response evoked by SP

Anti-sera of immunized mice were collected two weeks after the last immunization to analyze OVA-specific antibody (IgG, IgG1 and IgG2b) titers by indirect ELISA. Data (Figure 1) demonstrated that IgG in mice immunized with OVA/SP (1.25) and OVA/QuilA (0.5, 1.0) were increased significantly when compared with OVA-injected alone (group OVA) (P<0.01), there were no significant differences when groups OVA/SP (0.63, 2.5) were compared with group OVA. Meanwhile, IgG1 isotype in group OVA/SP (1.25, 2.5) and OVA/QuilA (0.5, 1.0) were enhanced greatly when compared with group OVA (P<0.05, P<0.01), no great difference was observed between group OVA/SP (0.63) and group OVA. More importantly, there were significantly higher level IgG2b titers in groups OVA/SP (0.63, 1.25, 2.50) and OVA/QuilA (0.5, 1.0) when compared with group OVA (P<0.01).The results indicated clearly that SP (1.25) evoked a stronger antibody response in OVA-immunized mice.

Cellular immune response evoked by SP

Effect of SP on splenocyte proliferation

The effects of SP on ConA, LPS, and OVA-stimulated splenocyte proliferation in immunized mice are shown in Figure 2. ConA, LPS, and OVA -stimulated splenocyte proliferation in the mice immunized with OVA/SP (0.63, 1.25, 2.50) or OVA/QuilA (0.5, 1.0) were notably higher than these in group OVA-injected alone (P<0.01). The results showed that SP greatly promoted the ConA, LPS, and OVA-stimulated splenocyte proliferation.

Effect of SP on $CD4^+$ and $CD8^+$ spleen T lymphocytes subsets

As seen in Figure 3, the percentage of $CD4^+$ T lymphocytes were increased markedly in the spleens of mice immunized with OVA/SP (1.25) or OVA/QuilA (0.5, 1.0) as compared with group OVA (P<0.01); however, no significant difference was observed when groups OVA/SP(0.63, 2.50) were compared with group OVA.

More importantly, SP (0.63, 1.25, 2.50) and QuilA (0.5, 1.0) significantly augmented the proportion of $CD8^+$ T lymphocytes in OVA-immunized mice (P<0.05, P<0.01). These results suggested that SP increased the proportions of both $CD4^+$ and $CD8^+$ T lymphocytes, that secret cytokines to mediate immunologic enhancement.

Effect of SP on DTH response

The effects of SP on DTH response are shown in

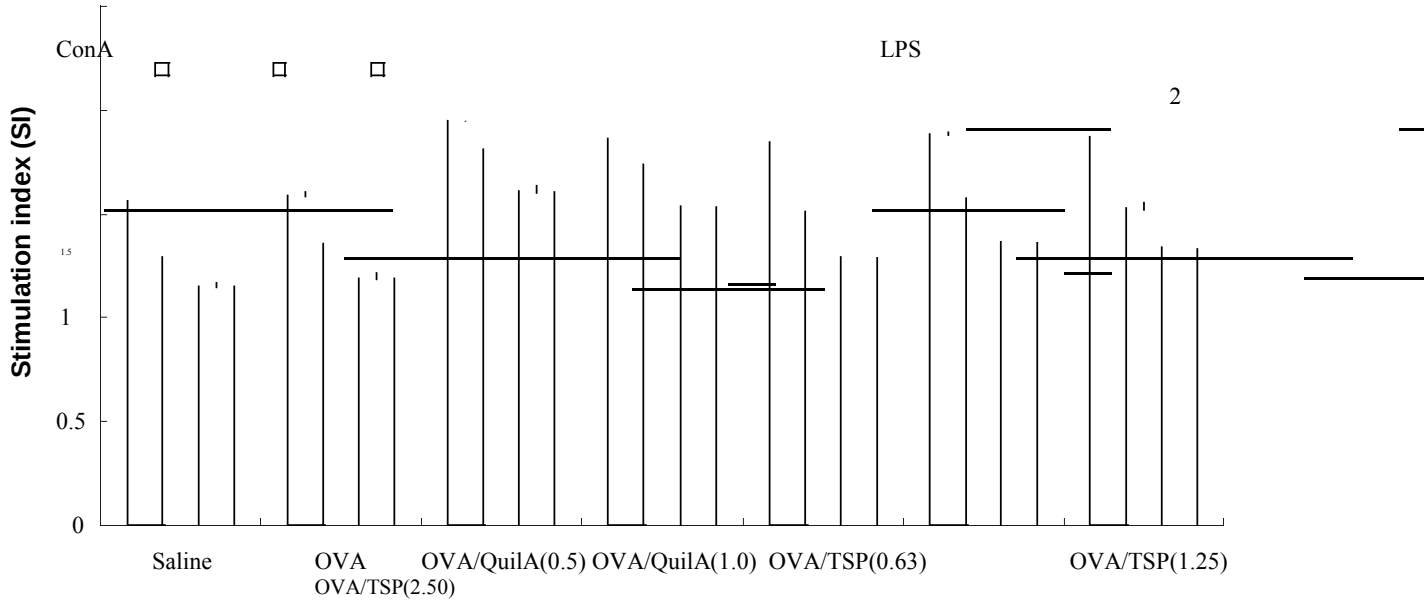

Figure 2. Effect of SP on splenocyte proliferation assay. The values were present as mean ± SD. $^{b}P<0.01$ vs group OVA.

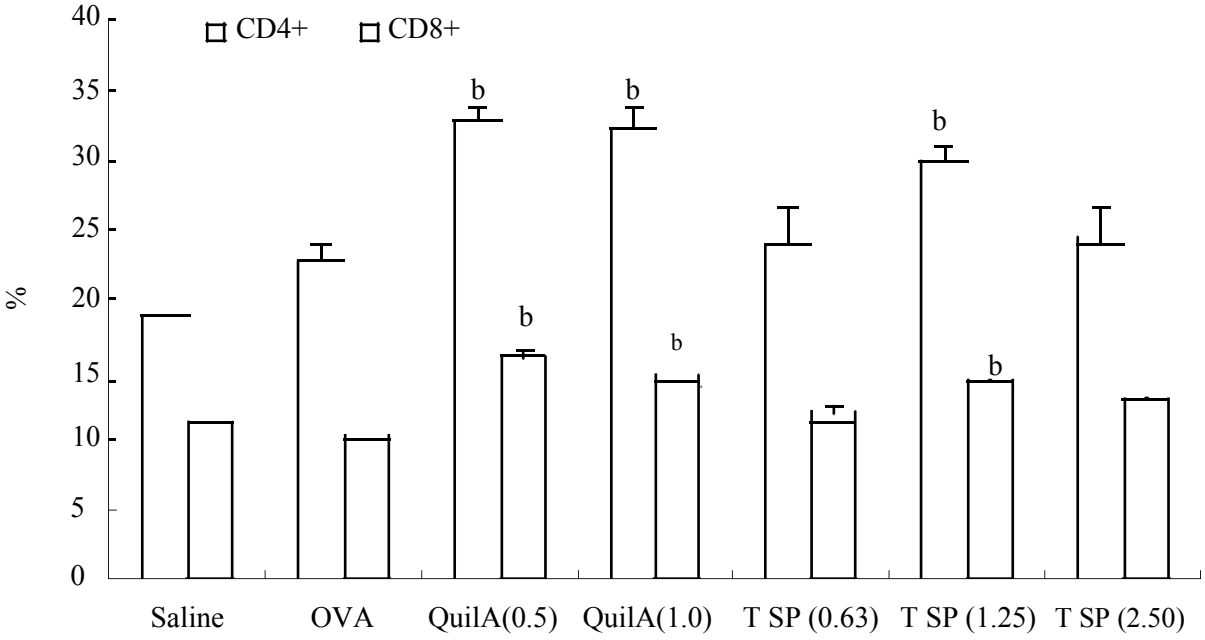

Figure 3. Effect of SP on $CD4^{+}$ and $CD8^{+}$ T cell populations of spleen in OVA-immunized mice. The values are present as mean ± SD. $^{a}P<0.05$, $^{b}P<0.01$ vs group OVA.

Table 2. DTH response in the immunized mice from group OVA/SP (1.25) and OVA/QuilA (0.5, 1.0) were significantly higher when compared with either group OVA ($P<0.01$). However, no significant differences were observed between the mice injected with OVA/SP (0.63, 2.50) and OVA alone. The results confirmed SP in dose of 1.25 mg/kg evoked the stronger DTH response.

DISCUSSION

New technology provides the new generation of vaccines such as subunit vaccines, synthetic peptide or plasmid DNA. Although they carry no risk of inadvertent infection when compared with live attenuated and whole inactivated organisms, they are poor immunogenic and

Table 2. Effect of SP on DTH response in OVA-immunized mice. The values are presented as mean ± SD. IFT stands for Increase in Footpad Thickness. [a]P<0.01 *vs* group OVA. n=5.

Groups	IFT (mm)
OVA	0.26±0.04
QuilA0.5	0.46±0.03 [a]
QuilA1.0	0.43±0.02 [a]
SP0.63	0.27±0.03
TSP1.25	0.39±0.02 [a]
TSP2.50	0.31±0.03

some of them can have high production costs (McCluskie and Weeratna, 2001; Perrie et al., 2008; Vandepapelière et al., 2008; Yang et al., 2007). Hence, there is an urgent need for the development of novel or improved vaccine adjuvants which may be helpful in overcoming the limitation of alum and Quil A.

In the present study, the immunologic enhancement of SP isolated from the sepal of *P. alkekengi* fruit was investigated. To detect the adjuvant effect of SP, male ICR mice were immunized subcutaneously with OVA alone or OVA containing either SP (0.63, 1.25, 2.50 mg/kg) or QuilA (0.5, 1.0 mg/kg), and the OVA-specific antibody responses and cellular reponses evoked by SP have been discussed in this paper. It was found that the adjuvant potential of SP in dose of 1.25 mg/kg in OVA-immunized mice was as effective as that of QuilA. Although there are many cell types participating in combating infection, Th lymphocytes critically determine the outcome of infection and can be subdivided into two cell subsets, termed as Th1 and Th2.

Accordingly, adjuvants can be classified according to their capacity to stimulate Th1 and Th2 responses. Th1 responses are required for protective immunity against intracellular infectious agents such as viruses, certain bacteria or protozoa; Th2 responses are effective for protection against most bacteria as well as some viral infections (Cox and Coutler, 1997). In general, Th1 responses mediating T cell immunity are associated with serum antibody responses in which the IgG2a, IgG2b and IgG3 isotypes predominate, while in Th2 responsese associated with humoral immunity the predominant isotypes are IgG1 and IgA (Marciani, 2003; Chiarella et al., 2007; Guy, 2007). Analysis of antibody showed clearly that SP (in dose of 1.25 mg/kg) not only substantially enhanced OVA-specific IgG level in OVA-immunized mice (P<0.01), but also increased OVA-specific IgG1 and IgG2b antibody titers (P<0.05 or P<0.01) (Figure 1). The finding demonstrated SP at suitable dose was effective on Th1 and Th2 cells as associated sensitively with an enhancement of IgG2b and IgG1 levels respectively. Lymphocyte proliferation is the indicator reflecting the state of cellular immunity, as it is well known that ConA and LPS stimulate T cells and B cells preration, respectively (Zhang et al., 2009).

Therefore, the capacity of eliciting T- and B-lymolifphocyte immunity was assessed by the stimulation of lymphocyte proliferation response. The proliferation assay indicated that SP (0.63, 1.25, 2.50) and QuilA (0.5, 1.0) could significantly promote the ConA, LPS, and OVA-induced splenocyte proliferation when compared with OVA group (P<0.01) (Figure 2). The results indicated that SP could significantly increase the activation potential of T and B cells.

T cell population with respect to both functional capabilities and cell surface phenotypes are divided into helper T cells (Th), which promote cell-meidated (Th1) and antibody responses (Th2), and cytotoxic T cells (Tc or CTL), which kill antigen-bearing target cells. It is well known that Th cells and Cytotoxic T lymphocytes (CTLs) responses are associated with the enhancement of $CD4^+$ and $CD8^+$ T lymphocytes, respectively. To further investigate the adjuvant effect of SP, $CD4^+$ and $CD8^+$ spleen T lymphocytes subsets from immunized ICR mice were analyzed by flow cytometric assay. It was shown (Figure 3) that the number of both $CD4^+$ and $CD8^+$ T lymphocytes were enhanced in mice immunized with OVA/SP in suitable dose of 1.25 mg/kg and QuilA (0.5, 1.0) (P<0.01). The enhanced percentages of both $CD4^+$ and $CD8^+$ spleen T lymphocytes indicated that both Th and CTLs were activated greatly by SP.

It is well known that DTH response is important in host defense against parasites and bacterial that can live and proliferate intracellularly. T_{DTH} cells generally appear to be a Th1 subpopulation although sometimes Tc cells are also involved. IFN-γ and IL-2 produced by Th1 are the primary mediators of host defenses associated with contribute to DTH response and activation of phagocytes (Seder and Paul, 1994). Therefore, DTH response was further measured as an *vivo* assay of cell-mediated immune function. Treatment of SP enhanced DTH reaction, which is reflected from the increased footpad thickness compared to OVA group (P<0.01) (Table 2), suggesting that Th1 immunity was evoked remarkably by SP.

General alum adjuvant only greatly induced Th2 immune response. Indeed, it was poor at eliciting Th1 immune response, and very poorly activated $CD8^+$ T lymphocytes (Yang et al., 2007; Sun et al., 2009b; Giudice et al., 2002). Although, it has been extensively proved that QuilA can stimulate Th1 and Th2 immune responses, its high toxicity and haemolytic activity are the main restriction from human use (Liu et al., 2002; Sun et al., 2009a). Fortunately, the data from haemolytic activities indicated clearly that SP had a slight haemolytic activity, with 3.50% haemolysis at concentration of 500 μg/ml (Table 1). However, the HD50 value of QuilA was 17.11 μg/ml. It implicated the lower haemolytic activity for SP than QuilA.

In general, it is believed that an effective means of protection is a vaccine which activates both the cell and humoral immune response. Accordingly, adjuvant

should induce humoral and cellular immune response. Although the exact underlying mechanism of SP is not yet known, we observed that immunologic enhancement evoked by SP was through the induction of humoral and cellular immunity (antibodies, Th1, Th2, and TCLs) in ICR mice immunized with OVA; additionally, SP has lower haemolytic activity than QuilA. Thus, SP may have enormous potential for use in vaccine against both pathogens and cancer, and may be used in a wide spectrum of prophylactic and therapeutic vaccines.

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