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Adjuvant effect of *Asparagus racemosus* Willd. derived saponins in antibody production, allergic response and pro-inflammatory cytokine modulation



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ABSTRACT

The study manifests the immunoadjuvant potential of saponin rich fraction from *Asparagus racemosus* in terms of cellular and humoral immune response that can be exploited against microbial infections. *Asparagus racemosus* (AR) has been attributed as an adaptogen and rasayana in traditional medication systems for enhancing the host defence mechanism. Spectrophotometric and HPTLC analysis ensured the presence of saponins. The saponin rich fractions were tested for immunoadjuvant property in ovalbumin immunised mice for the humoral response, quantified in terms of prolonged antibody production upto a duration of 56 days. Proinflammatory cytokines (IL-6 and TNF) were estimated for the cellular immune response in LPS stimulated primary murine macrophages. The safety evaluation in terms of cytotoxicity and allergic response has also been evaluated through *in-vitro* (MTT) and *in-vivo* (IgE) respectively. ARS significantly inhibited the pro-inflammatory cytokines, in LPS stimulated murine macrophages with no intrinsic cytotoxicity. The significant increase in IgG production infers the utility of ARS for prolonged humoral response. Further, the antigen specific response of IL-12 at early stage and IgE titres also suggests the generation of cellular immune response and low allergic reaction respectively, as compared to conventional adjuvants. IL-6 and TNF fluctuations in LPS stimulated and non-stimulated macrophages along with IgG and IL-12 also confirmed the Th1/Th2 modulating effect of ARS. The study indicates potential effect of ARS as an adjuvant for the stimulation of cellular immune response in addition to generating a sustained adaptive response without any adverse effects paving way for further validation with pathogenic organisms.

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1. Introduction

Asparagus racemosus Willd. (AR) commonly known as Satawar, Satamuli, Satavari belongs to family Liliaceae and is found at South Asian nations and has been attributed as adaptogen in traditional medication systems. In Ayurveda, it is mentioned as *rasayana*, acknowledged for promoting physical and mental health, for increasing longevity through the enhancement of host defence mechanisms. *A. racemosus* has been actively known for augmenting the female reproductive health. It accounts for potent pharmacological effects like galactagogue, anti-tussive, anti-plasmodial, anti-leishmanial, anti-bacterial, hepato-protective, diuretic, anti-ulcer, anti-diarrheal, antenatal tonic,

cardioprotective, anti-cancerous, anti-epileptic and anti-depressant [1,2]. Immunostimulation forms an essential essence of this plant and thus it is used in many herbal formulations. The wide spectrum of efficiency enhances its probability in immunomodulatory activity and its role in adjuvant properties too. The compounds illustrated in this plant are of keen interest in research areas and the saponin moiety may also account for potent adjuvant activity. Roots of *A. racemosus* Willd. have been reported with the major active components as steroidal saponins (Shatavarins I–IV), Isoflavones, Asparagamine, Racemosol, Polysaccharides, mucilage, vitamins A, B1, B2, C, E, Mg, P, Ca, Fe, and folic acid. The saponins isolated are of novel importance in field of vaccine and immunostimulation. Recent contemporary vaccines made from purified antigens, recombinant proteins and DNA account for low immunogenicity due to the lack of innate immune response imparting the depletion of immune response as compared to the traditional vaccines. This depicts the urgent need of novel

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adjuvants which not only elicit better antibody response and cellular immunity but also have no or minimal side effects [3–5].

Saponins are naturally occurring surface active steroid or triterpenoid glycosides. These biologically active and structurally diverse compounds have been attributed to wide biological effects in human and animal systems. Medicinal plant based saponins not only minimize the possibility of the adverse effects but also establish a strong background for the efficacious adjuvants. Quil A and further purified QS-21 isolated from bark of *Quillaja saponaria* have been extensively used in veterinary vaccines and HIV-1 clinical trials, respectively. It has been found that saponins stimulate both cellular and humoral response and are required in the minimal concentrations for the adjuvant activity [6]. Another example is tetracyclic triterpenoid saponins extract from the roots of *Panax notoginseng* (Sanqi), generally called as “*Panax notoginseng* saponins” (PNS) as an active ingredient of Xuesaitong Injection (XST), which is one of the remarkably used medicines for treating acute cerebral infarction in China [7]. Previously, there have been reports on the aqueous extract of the plant in response to the lower immunogenic dose of DTP vaccine and SRBC, but exclusive studies on saponin are missing. Further, there is a need to escalate the findings into the direction of microbial antigen related studies [8,9].

In our present study, the saponin rich fraction was quantified and confirmed for the presence of saponin by haemolytic analysis, spectrophotometric quantification and High Performance Thin Layer Chromatography (HPTLC). Further, saponin rich fraction isolated from *Asparagus racemosus* (ARS), has been preliminarily assayed for their immuno-adjuvant activity. Cellular and humoral responses in terms of cytokine production against LPS and antibody regulation against ovalbumin, respectively, have been analysed along with the evaluation of allergic response in terms of IgE production.

2. Material and methods

2.1. Plant material

Asparagus racemosus (root) was collected from National field Gene Bank of CSIR-CIMAP.

2.2. Isolation of saponin rich fraction

Crude saponins which are chemically steroidal and triterpene glycosides were isolated as previously described by [10] with minor modifications. Briefly, the plant material was defatted with hexane followed by extraction in methanol. The methanolic extracts were concentrated under reduced pressure and suspended in water pre-saturated with *n*-butanol and allowed to stand in a separating funnel. The *n*-butanol partition was then separated and diethyl ether was gradually added to precipitate the saponin rich fraction.

2.3. Spectrophotometric analysis

The quantification of the saponin rich fractions were carried out by spectrophotometric determination as described by [11] with minor alterations in the standard used (Quil A). Briefly, two reagents, i.e., (A) *p*-anisaldehyde in ethyl acetate (0.5:99.5) and (B) H₂SO₄: Ethyl acetate in equal parts were mixed in equal proportions. The saponin rich fractions and the standard were dissolved in ethyl acetate and the above mixture was mixed with (A) and (B) in the ratio of 2:1:1. The mixture was stirred and incubated at 60 °C for 10 min in a water bath. The solutions were cooled at room temperature for 10 min and the absorbance was

recorded at 430 nm. The reference curve was obtained using diosgenin.

2.4. HPTLC analysis

The extracts were subjected to TLC analysis (silica gel 60 F₂₅₄ Plate) using the solvent system (*n*-butanol: water: acetic acid: 4.5:1:0.5, v/v/v) followed by UV detection at 254 nm or by spraying with vanillin reagent for visualization of the targeted compounds. The quantification of the saponins rich fraction in the *n*-butanol extract was carried out through HPTLC analysis utilizing the above mentioned solvent systems following the previously published protocol [12].

2.5. Haemolytic assay

Blood was collected in Alsever's solution from healthy mice to obtain the RBCs. Collected blood was washed three times with sterile saline solution (0.89% w/v NaCl) by centrifugation at 2000 rpm for 5 min. The cell suspension was prepared by diluting the pellet (0.5 ml) in saline (100 ml). Cell suspension (0.5 ml) was mixed with varying concentrations of the samples ranging from 5 to 1000 µg/ml in similar volume of saline [13]. The mixtures were incubated for 30 min at 37 °C and centrifuged at 90 × g for 10 min. The free hemoglobin in the supernatants was measured spectrophotometrically at 412 nm in volume of 200 µl. Saline and distilled water were considered as minimal and maximal haemolytic controls respectively. The haemolytic percentage resulting from the saline control was subtracted from all groups. The results were expressed as 50% hemolysis (HC₅₀) which was calculated through regression analysis. The test was performed in triplicates for each sample to minimize experimental error.

2.6. In-vitro studies

2.6.1. Antibacterial studies

Human pathogens, *Staphylococcus aureus* (SA96), *Pseudomonas aeruginosa* (PA), *Klebsiella pneumoniae* (KP), *Streptococcus mutans* (SM), *Escherichia coli* (EC), Methicillin resistant *Staphylococcus aureus* (MRSA 4696 and 1745) authenticated and maintained at regular intervals were considered. The antibacterial activity of the saponin rich fractions was determined through the broth micro-dilution assay using 96 'U'-bottom micro-titre plates as per CLSI guidelines [14]. Saponins were serially diluted two fold (4000–15.625 µg/ml) in MHB and inoculated with 10.0 µl of overnight grown cultures of the test organism matched with 0.5 McFarland standard. The inoculated plates were then incubated at 37 °C for 16–24 h and the growth was recorded spectrophotometrically at 600 nm using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The minimum inhibitory concentration (MIC) value was determined from the turbidimetric data as the lowest concentration of derivatives showing growth inhibition equal to or greater than 80% as compared to the control. Experimental observations were performed in triplicates to rule out any error during the procedure. Two antibiotics, oxacillin and norfloxacin were used as the positive control.

2.6.2. Animals

Eight week old male and female Swiss albino mice (18–22 g) from a colony maintained at the *in vivo* Testing Facility, Central Institute of Medicinal and Aromatic Plants, India were considered for the experiments. The mice were housed and acclimatized under standardized conditions (25 ± 2 °C), 12 h light/12 h dark cycle and fed with animal pellets and water *ad libitum*. The protocol (CIMAP/IAEC 2016-19/05) was duly approved by the Institutional Animal Ethics Committee (IAEC).

2.6.3. Peritoneal macrophage culture and treatment

Murine peritoneal macrophages were isolated from healthy mice under aseptic conditions [15]. Briefly 1% peptone was injected intraperitoneally prior to isolation. 18 h after the injection, the intraperitoneal exudate corpuscles were collected by lavage with phosphate buffered saline containing penicillin (100 IU/ml) and streptomycin (100 µg/ml). The collection was washed thrice through centrifugation (300 × g) and resuspended in RPMI 1640. The cells (2 × 10⁶/ml) were suspended in MEM (Sigma, USA) containing 10% fetal calf serum (Gibco, USA), 50 IU of penicillin, 50 µg streptomycin, and 2 mM glutamine per ml and incubated at 37 °C for 4 h under 5% CO₂. The non-adherent cells were then removed by washing five times in 500 µl of RPMI 1640 leaving approximately 1 × 10⁶ cells/ml. The cells were stimulated by lipopolysaccharide (*E. coli* 050: B5, Sigma, USA) at 1 µg/ml of media followed by various treatments. The stock of saponin rich fraction was made in DMSO at a concentration of 10 mg/ml. The stock was further diluted to achieve final concentrations of 100, 10, 1 µg/ml. Similarly Quil A, the standard immunopotentiating agent was also considered at three concentrations of 100, 10, 1 µg/ml. Treatments of QuilA and ARS were same in another set of non stimulated cells. The cells were allowed to incubate for 16–18 h after which the supernatant was collected for cytokine quantification, while the plate with adherent cells was subjected to MTT assay.

2.6.4. Cytokine expression analysis

2.6.4.1. Expression of TNF-α, IL-6 from lipo-polysaccharide (LPS) stimulated and non stimulated peritoneal macrophages. Peritoneal macrophages were cultured at 37 °C with 5% CO₂ under humidified condition for 16–18 h with LPS (*E. coli* 050: B5; Sigma Chemical Co., USA) at 1 µg/ml and another set having non stimulated cells with media alone. IL-6 and TNF levels in culture supernatants were estimated by Enzyme Immune Assay (EIA) using commercial kits for mouse IL-6 and TNF (BD Biosciences, USA).

2.6.5. Cell viability through MTT assay

The effect of the ARS, saponin and QuilA on cell viability was determined by using the above mentioned isolated peritoneal macrophages stimulated with LPS and culture method. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution at 0.1 mg/ml was added to each well having peritoneal macrophages and incubated at 37 °C for 4 h. Dark blue formazan crystals formed were solubilised by adding 100 µl DMSO to each well and the absorbance was measured at 570 nm [16].

2.7. In-vivo studies

2.7.1. Immunization

Healthy swiss albino mice (20 ± 2 g) were divided into 7 groups, each consisting of five animals. The combinations were individually homogenized under cold and septic conditions and observed for stability, ensured through absence of phase separation for 48 h. The subcutaneous injections were made in the scruff region in volumes of 100 µl each. Animals were immunized with OVA at 100 µg dissolved in saline and in combination of OVA with saponin rich fraction at three doses 10, 50, 100 µg, while for standard adjuvant, OVA with Quil A at 10 and 20 µg and FCA along with OVA in ratio of 1:1 on Day1. A booster dose was given two times at Day14 and Day 21. Serum was collected at Day 24, 42 and 56 for measurement of OVA specific antibody.

2.7.2. Scoring of local inflammation at injection site

The scoring of the local inflammation resulting from the injection of the antigen along with the adjuvants was done based on the nodule formation, the thickness of which was measured

using a vernier calliper. Since the external thickness was prone to variation, scoring (0–3) was done considering the range of 2–3 mm, 5–6 mm and more than 6 mm respectively. The observations were recorded seven days after the third booster (day 21) i.e., on day 28.

2.7.3. Measurement of OVA- specific IgG

OVA-specific IgG was determined by direct ELISA. The microtitre plate wells were coated with 100 µl OVA (80 pg/ml) diluted in coating buffer (carbonate-bicarbonate buffer pH 9.5) and incubated overnight at 4 °C. The coated plates were washed thrice with PBS (pH 7.0) containing 0.05% Tween 20 (wash buffer) and blocked for 15 min with Pierce Superblocker containing TBS undiluted with 0.05% Tween 20. After three washings, 100 µl of sera diluted in assay diluents in ratio of 1:100 was added and plates were incubated for two hours at RT. After five washes, anti-Mouse IgG (whole molecule)–Peroxidase antibody was added after dilution of 1:40,000 in assay diluents. After incubation of 1 h, the wells were washed seven times and 100 µl of tetramethylbenzidine (TMB) and hydrogen peroxide was added and an incubation of 30 min was allowed. Further enzyme reaction was terminated by the addition of stop solution containing 1 M H₂SO₄ in a volume of 50 µl. The absorbance was measured at 450 nm with a 570 nm reference. The data were expressed as the mean OD value of the samples minus the mean OD value of the controls. The sets of the sera samples have been subjected to within and between group comparisons.

2.7.4. Quantification of IgG2a, IL-12 and IgE

IL-12 and IgG2a titres in serum were estimated by Enzyme Immune Assay (EIA) using commercial kits for mouse IL-12 p40 and IgG2a (BD Biosciences, USA). Similarly, IgE was quantified through mouse IgE quantification kit (Bethyl Laboratories, USA).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the mean values obtained for the treatment and control through Graph Pad Prism. Tukey's test was used to compare the treatment and control and statistical significance was set at P ≤ 0.05.

3. Results and discussion

3.1. Haemolytic assay, spectrophotometric quantification and HPTLC analysis of the ARS

The spectrophotometric analysis of the ARS confirmed the presence of saponin at a concentration of 20.45 µg/ml when compared to the standard saponin (30.43 µg/ml) (Table 1). The HPTLC analysis of the ARS fraction confirmed the presence of saponins in the crude *n*-butanol extracts at the retention of 0.20 (Supplemental Fig. 1), resulting in the maximum recovery of saponin rich fraction (9.08 ± 1.6 mg/gm DW).

Table 1

Comparative analysis of 50% hemolysis and spectrophotometric quantification (µg/ml) between saponin rich fraction of *Asparagus racemosus* with Quil A and Saponin.

	Spectrophotometric quantification (µg/ml)	50% Hemolysis (µg/ml)
Saponin	30.43	70.51
Quil A	24.78	118.97
ARS	20.45	35.49

Saponin and Quil A were considered as standards in comparison to saponin rich fraction (ARS).

Saponins trigger cell membrane scrambling, an effect partially due to entry of extracellular Ca^{2+} and ceramide formation leading to hemolysis [17] and this hemolytic activity by saponins has been one of the methods to characterize saponins. The haemolytic activity of ARS on mice red blood cells was found to be higher in terms of concentration in ARS. ARS concentration causing 50% hemolysis was 35.49 $\mu\text{g}/\text{ml}$ indicating the presence of saponins (Table 1). We performed this assay to confirm the presence of saponins at various steps of its isolation. For a comparative purpose, we observed that the saponin rich fraction from *Asparagus* quantitatively contained saponin, which was further supported by the observations on hemolysis. Since ARS is not pure saponin, other components like salts could be present in the ARS which might be altering the osmotic balance and causing the hemolysis under *in-vitro* condition, but would be getting absorbed harmlessly under *in-vivo* condition.

3.2. Antimicrobial activity

The isolated rich fraction of saponin was also explored for antimicrobial activity. Three strains each of Gram positive (Gm +ve) and four Gram negative (Gm -ve) strains were considered to ascertain if there is any antimicrobial activity. This was to test the hypothesis that saponin if used as an adjuvant for attenuated bacteria should not have an inhibitory effect on the pathogen itself. It was observed that the activity (MIC) was not very significant, which ranged from 500 to >3000 $\mu\text{g}/\text{ml}$ as ascertained through microbroth dilution assay. (Supplementary data).

3.3. Effect of ARS on LPS stimulated and non stimulated proliferation and pro-inflammatory response

The effect of saponin rich fractions of AR on LPS treated macrophages was significantly noticed in lowering the titres of the cytokine. The inhibition of two cytokines, IL-6 and TNF was noticed in a dose dependent manner (Fig. 1). The highest concentration of ARS 100 $\mu\text{g}/\text{ml}$ inhibited IL-6 most significantly while in case of the TNF all the three concentrations significantly inhibited the titres ($P < 0.001$). In the case of IL-6, the control group of Quil A could only inhibit the cytokine level up to a significant level at a concentration of 100 $\mu\text{g}/\text{ml}$, while ARS produced a dose dependent significant lowering in both 100 and 10 $\mu\text{g}/\text{ml}$. The level of IL-6 was quite comparably low in ARS (1135.71 pg/ml at 50 $\mu\text{g}/\text{ml}$) as compared to the Quil A (1463.89 pg/ml at 50 $\mu\text{g}/\text{ml}$) as deduced through regression analysis (Fig. 1a). Additionally, for the pro-inflammatory cytokine TNF, Quil A and ARS significantly ($P < 0.001$) depleted the titres at all the three concentrations and did not show any difference between them in terms of inhibition at 50 $\mu\text{g}/\text{ml}$ as deduced through regression analysis (Fig. 1b). ARS showed a significant elevation in TNF titre at all concentrations while in IL-6, though the titres were high as compared to the cells (control) but only highest dose of 100 $\mu\text{g}/\text{ml}$ depicted the significant rise in IL-6. ARS triggers the pro-inflammatory cytokines IL-6 and TNF, that are involved in innate response which have an important role towards the protection of host from infectious diseases. These observations indicate the triggering of the strong innate immune mechanism

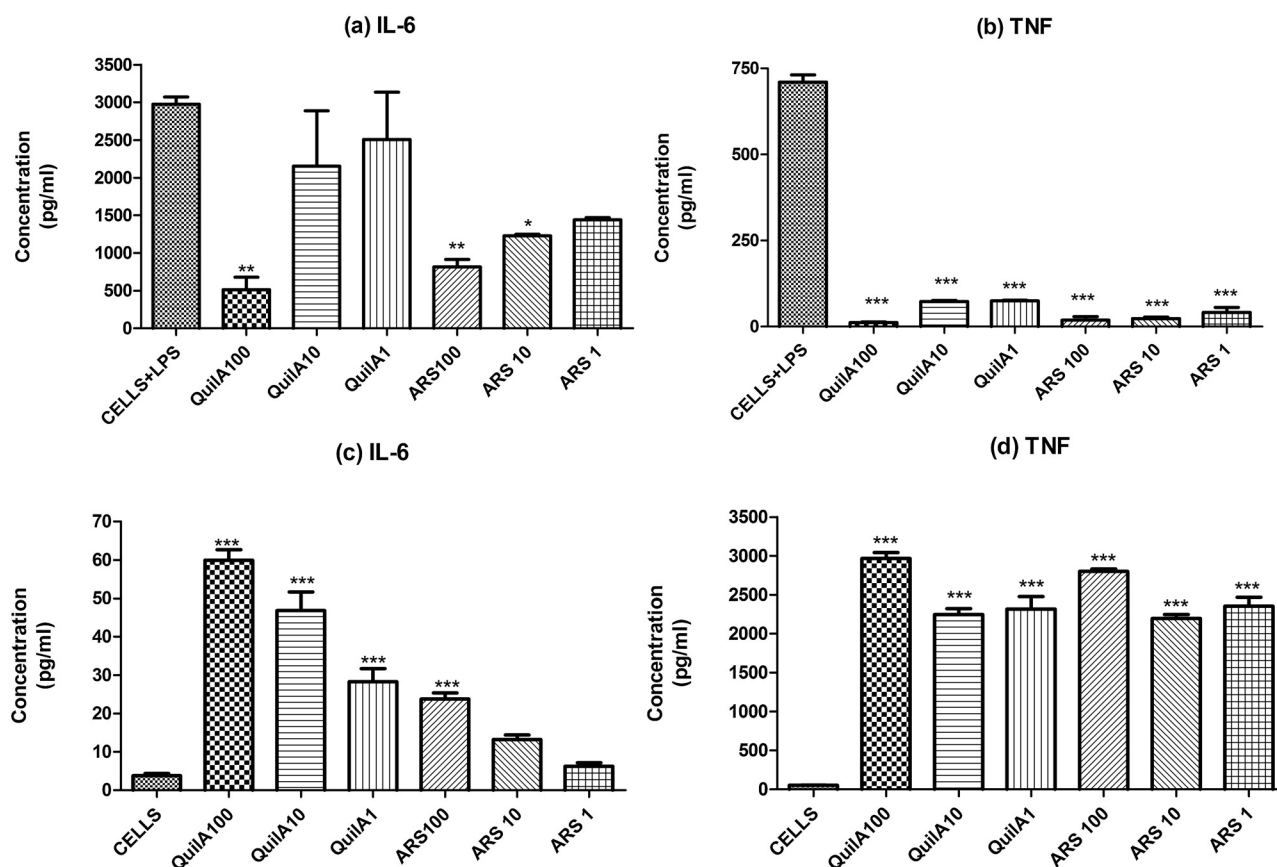


Fig. 1. Effect of ARS on pro-inflammatory cytokines (IL-6 & TNF) regulation on LPS stimulated (a)&(b) and non stimulated (c) & (d) murine macrophages. LPS treatment was given at 1 $\mu\text{g}/\text{ml}$, followed by the treatment at decremental concentrations of 100, 10 and 1 $\mu\text{g}/\text{ml}$. The values are represented as the means and standard errors of triplicates. *- $p < 0.5$, **- $p < 0.01$ & ***- $p < 0.001$ Control vs. Treatment Controls: a & b – Cells +LPS c & d – Cells only

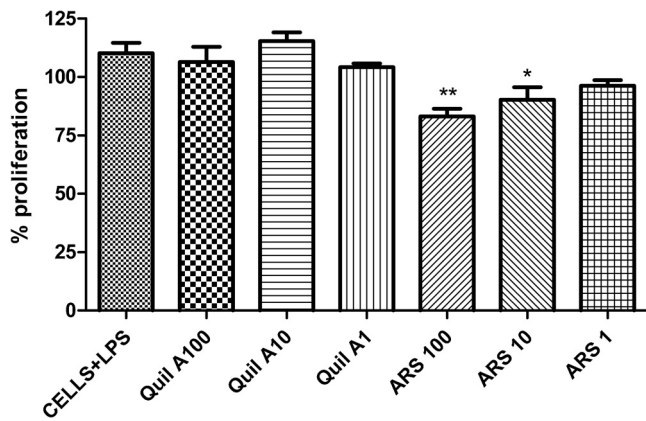


Fig. 2. Cytotoxicity of ARS and QuilA assayed on murine peritoneal macrophages. Cells were treated with ARS or QuilA for 18 h at doses ranging 100, 10 and 1 μ g/ml, respectively. MTT (0.1 mg/ml) was added and cells were incubated for 4 h and the absorbance was measured at 570 nm. The cytotoxicity was calculated by comparing the samples to the controls. Percentage values were represented as means $\pm$ SD in each group.
* $p < 0.05$, ** $p < 0.01$ & *** $p < 0.001$ Cells + LPS vs. treatment (QuilA and ARS)

and regulatory effect on the cellular immunity. Further, the MTT assay (Fig. 2) indicates that ARS has a dose dependant cytotoxicity on macrophages while no such effect was observed in the absence of LPS.

3.4. Scoring of local inflammation at site of injection

The nodule formation at the site of the administration indicated local inflammation in the two control groups Quil A and FCA (Supplementary data), while the animals of test treatments of ARS and control animals of ovalbumin alone did not show any nodule formation.

3.5. Effect of ARS on OVA-specific antibody response

The OVA specific antibody response was estimated on day 24, 42 and 56. The positive control of QuilA at 10 and 20 μ g and FCA along with ovalbumin showed a positive IgG elevation throughout the period. The test treatment of ARS at three doses 100, 50 and

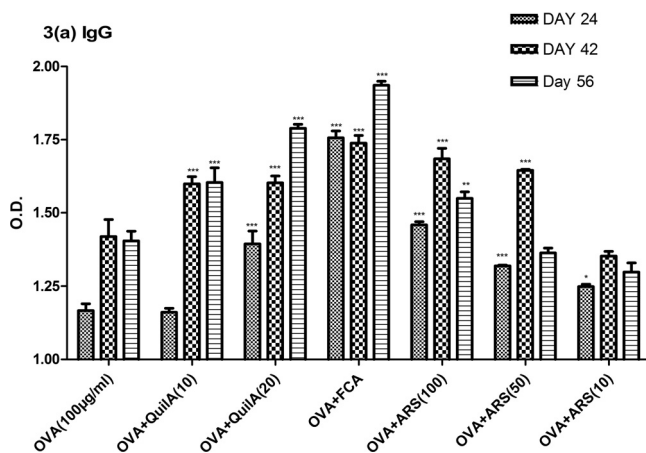


Fig. 3. Serum OVA-specific (a) IgG, (b) IgG2a, (c) IL-12, and (d) IgE titers. Mice (n = 5 per group) were s.c. immunized with OVA antigen alone and containing QuilA (10 and 20 μ g), FCA and ARS (100, 50 and 10 μ g) on day 1 followed by boosters on day 14 and 21. Sera were collected and were assayed for the quantitative estimation of IgG2a, IL-12 and IgE.
* $p < 0.05$, ** $p < 0.01$ & *** $p < 0.001$
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, OVA vs. treatment (QuilA and ARS)

3(b) IgG2a

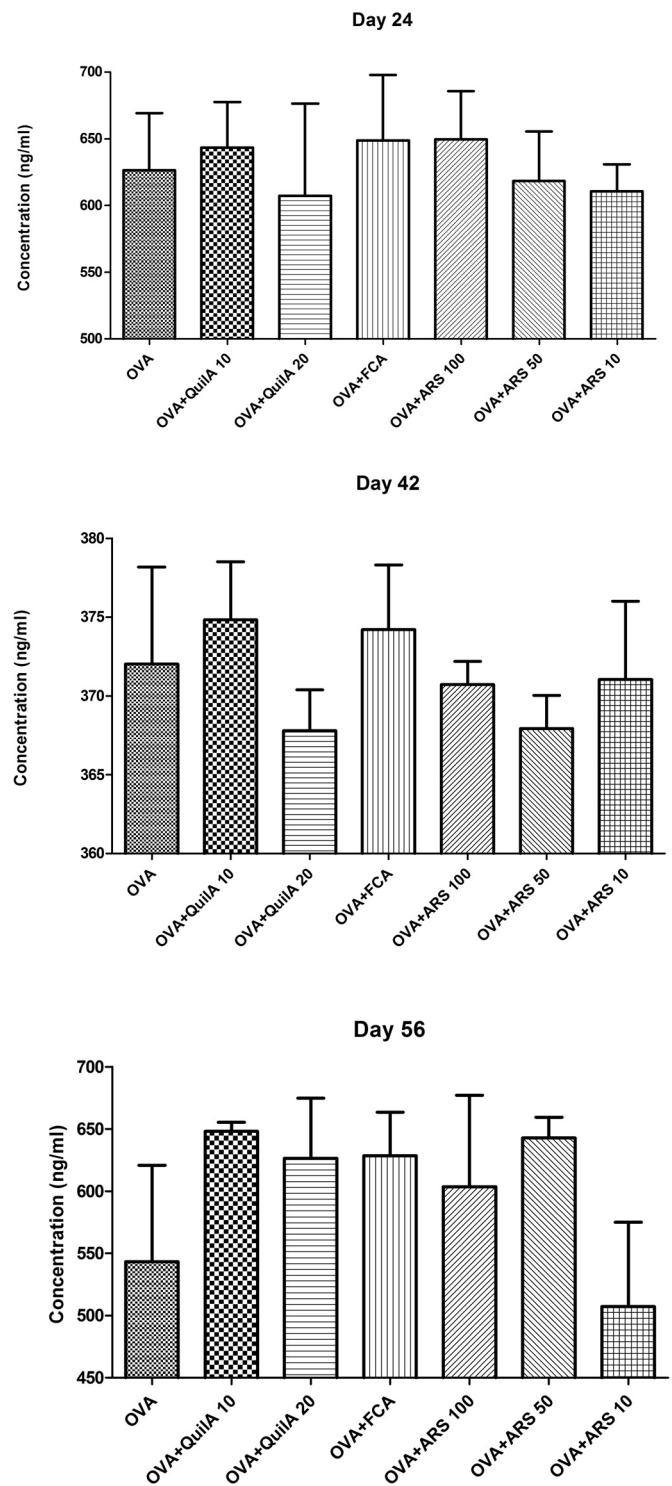


Fig. 3. (Continued)

10 μ g also elevated the IgG titre as compared to the ovalbumin alone animals on Day 24 (Fig. 3a).

The OVA specific IgG titre was significantly increased in a dose dependant manner by ARS. The highest dose of ARS (100 μ g) was comparable to that of FCA and Quil A (20 μ g). However, on day 42, this pattern was maintained with Quil A exhibiting significant rise of the titre at lowest dose also (10 μ g) while the ARS at the minimal

3(c) IL-12

3(d) IgE

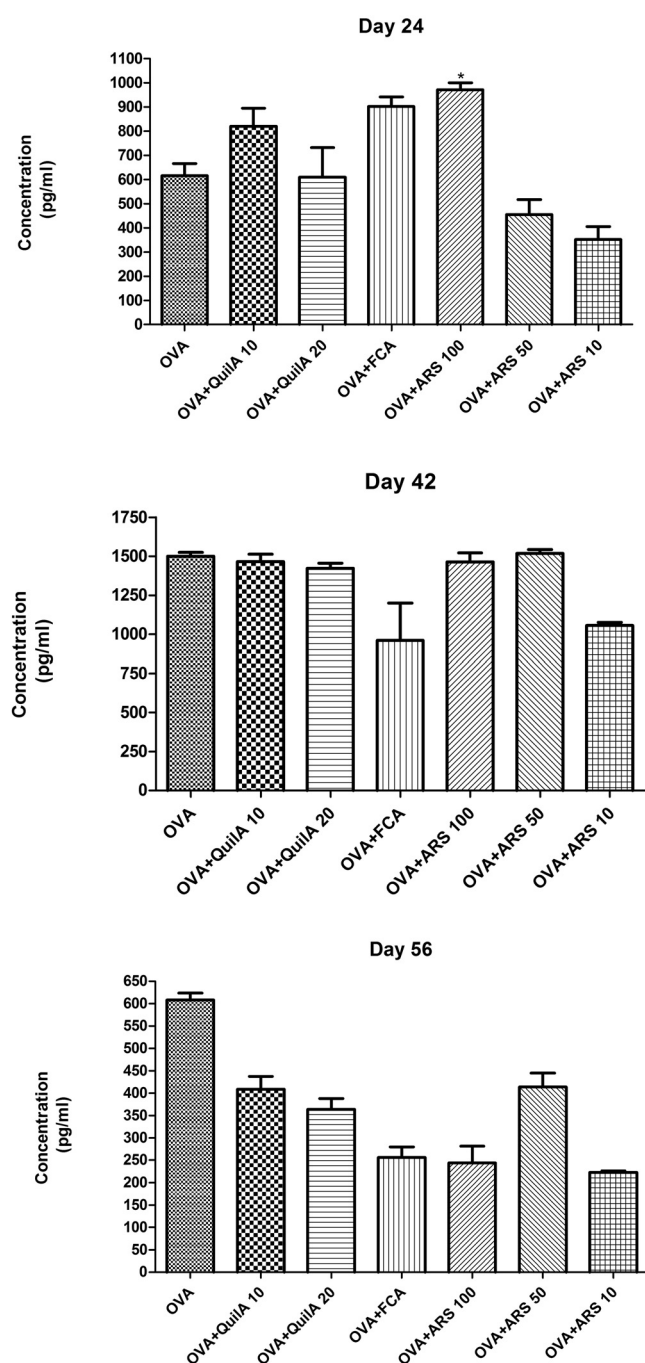


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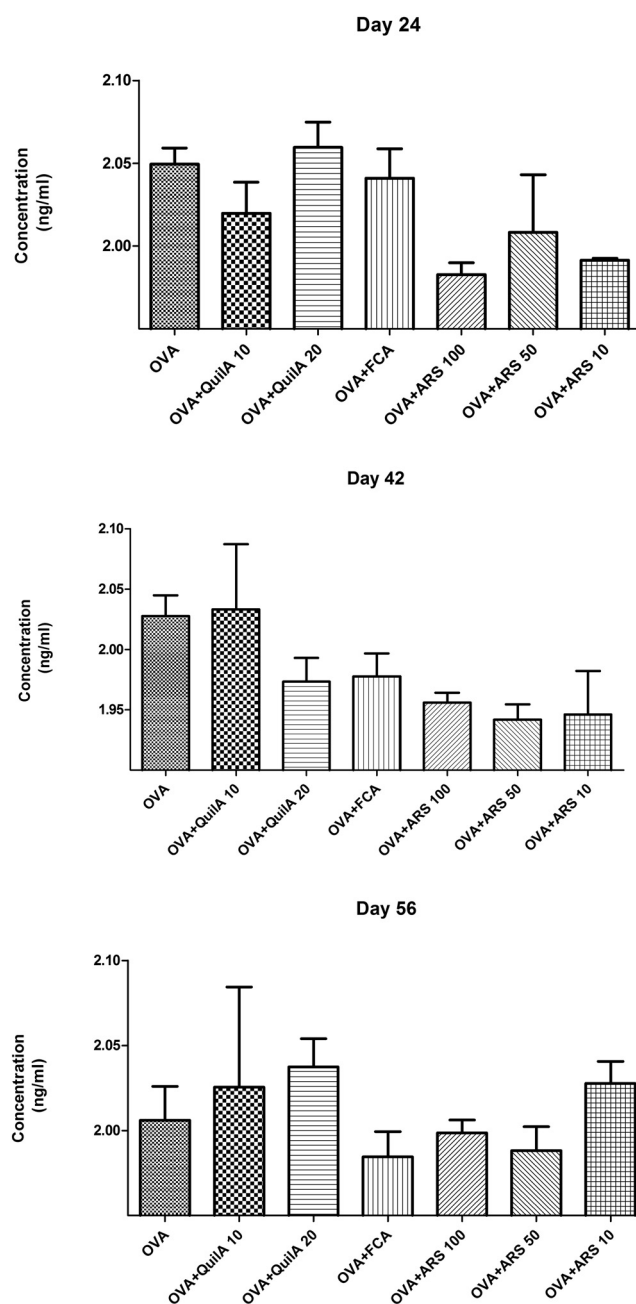


Fig. 3. (Continued)

dose (10 μ g) lost its significance. However, on day 56, the maintenance of the titre was significant only in the positive controls along with the highest concentration group of ARS. This observation indicates that the ARS has the potential to stimulate the humoral immune response.

3.6. Effect of ARS on IgG2a, IL-12 and IgE production

Through antibody-dependent phagocytosis and antibody-dependent cell-mediated cytotoxicity, both IgG2a and IgG2b are

considerably competent for eliminating microbes [18,19]. The IgG2a in our studies did not exhibit any significant difference between the groups or across the duration and were maintained between 400 and 600 ng/ml (Fig. 3b). IL-12 is a cytokine that promotes the differentiation of CD4⁺ T cells into T helper 1 (Th1) cells that produce IFN- γ and aid in cell-mediated immunity [20]. In our studies, the immunizations were made on day 0, 14 and 21. The IL-12 titre on day 24 was observed to be in a dose dependent manner (Fig. 3c) and our observation indicated that ARS is able to stimulate the production of IL-12 as early as day 24. In parallel, the positive controls of Quil A and FCA could stimulate its production only at a later stage of 42 days. Further, since ARS is a mixture of saponins the results should not be directly compared with the

positive controls (pure molecules) but ARS shows a potential to stimulate the humoral and innate immune system. ARS promotes IL-12 mediated response accounting for enhanced cellular immunity which accounts for macrophage stimulation and their functional regulation.

Novel adjuvants for vaccine development seek lower toxicity and decrease in IgE production representing allergenicity. In our experiment, IgE production did not exceed the control groups indicating the absence of allergenic response (Fig. 3d). Apart from the parameters representing the B cell activation response, the macroscopic observations on the site of injection revealed significant observable sizes of the local lesions which were maximum in FCA followed by Quil A, which was nonexistent in ARS, indicating a non-existent site reaction. (Supplementary material).

4. Conclusion

Our observations confirm the immunopotentiating effect of the saponin rich fraction from *Asparagus racemosus* in terms of cellular and humoral immune response, devoid of any allergic reaction. Further studies using this rich fraction could be taken up to ascertain its role as an adjuvant for an efficient and prolonged immune stimulation considering bacterial pathogens of veterinary and human interest as antigen candidates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biopha.2016.11.087>.

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Table 1. Antimicrobial activity against standard human pathogens

	Sa 96	Pa	Kp	Sm	Ec	MRSA(4696)	MRSA(1745)
Saponin	3000	500	>3000	>3000	>3000	>3000	>3000
Quil A	3000	500	>3000	>3000	>3000	>3000	>3000
ARS	500	1000	1000	500	1000	1000	>3000

Sa 96: *Staphylococcus aureus* ATCC 29213, Pa: *Pseudomonas aeruginosa*, Kp: *Klebsiella pneumonia*, Sm: *Streptococcus mutans*, Ec: *Escherichia coli*, MRSA: Methicillin resistant *Staphylococcus aureus* (4696 and 1745)

Table 2: Scoring of nodule formation at injection site

Mice	QuilA 10		QuilA 20	FCA	AR 100	AR10	AR10
	OVA	OVA	OVA	OVA	OVA	OVA	OVA
1	0	0	0	2	0	0	0
2	0	0	1	3	0	0	0
3	0	1	1	2	0	0	0
4	0	0	1	2	0	0	0
5	0	1	1	3	0	0	0
Average	0.00	0.40	0.80	2.40	0.00	0.00	0.00

0: no nodule formation – 0.9-1.2mm, 1: 2-3mm nodule, 2: 5-6mm, 3: >6mm

At the site of sub cutaneous injection, nodule formation of 2-3 mm was generally observed in all the animals which subside within few hours. However, in some of the groups these nodules increase in size owing to the local inflammatory reactions. The animals administered with FCA had persistent nodules till the end of the experiment followed by the group administered with Quil A at 20µg/ml and 10µg/ml respectively. Interestingly, these were totally absent in the groups administered with ARS.