Research article

Shatavarin-IV saponin adjuvant elicits IgG and IgG2b responses against *Staphylococcus aureus* bacterin in a murine model

Arivukarasu Palanisamy [a], Rinku Sharma [a,*], Prithvi Pal Singh [b], Upendra Sharma [b], Rajendra Damu Patil [c], Gorakh Mal [a], Birbal Singh [a]

[a] *Disease Investigation Laboratory, ICAR-Indian Veterinary Research Institute, Regional Station, Palampur 176061, Himachal Pradesh, India*
[b] *Chemical Technology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur 176 062, Himachal Pradesh, India*
[c] *Department of Veterinary Pathology, DGCN COVAS, CSK HPKV, Palampur 176 062, Himachal Pradesh, India*

A R T I C L E   I N F O

A B S T R A C T

Asparagus adscendens Roxb. also known as "safed musli" or "shatavari" is a medicinal plant commonly found in South Asian countries. Shatavari is effective for the treatment of gastric ulcers, renal stones, bronchitis, diabetes, diabetic neuropathy, irritable bowel syndrome, alcohol withdrawal and has reported immunostimulatory effects. In this study, the adjuvant potential of Shatavarin-IV saponin against *Staphylococcus aureus* bacterin in mice was investigated. Shatavarin-IV was evaluated for its toxicity and immunomodulatory potential against *S. aureus* bacterin in mice. Cellular and humoral immune responses were assessed. Shatavarin-IV was isolated from the fruit extract of *Asparagus adscendens*. The confirmation of the isolated molecule as Shatavarin-IV was done *via* TLC-based comparison with the standard molecule. Further, the structure was confirmed by using extensive spectroscopic analyses and comparing the observed data with literature reports. It was found safe up to the dose of 0.1 mg in the mice model. Shatavarin-IV adjuvant elicited IgG and IgG2b responses at the dose of 40 μg against *S. aureus* bacterin. However, the cell-mediated immune response was lesser as compared with the commercial Quil-A saponin . We demonstrated that Shatavarin-IV saponin adjuvant produced an optimum humoral immune response against *S. aureus* bacterin. These results highlight the potential of Shatavarin-IV as an adjuvant in a combination adjuvant in vaccine formulations for induction of potent immune response.

1. Introduction

Infectious diseases are the major etiological factor for most of the morbidity and mortality in animal populations. The vaccines can effectively be used to control and eradicate the most contagious diseases. Modern vaccines contain inactivated microbes or highly purified antigens and are poorly immunogenic as compared with live vaccines. Currently, the use of adjuvants in contemporary commercial vaccines is increased and many new adjuvants have been explored and formulated in vaccines to strengthen the immune response. Although a variety of adjuvants have been developed over the last 90 years, only a few adjuvants like aluminum and calcium salts are commonly used in human vaccines [1]. The adjuvant effect of saponins was found in the 1930s in France [2,3], and it has been commonly used in veterinary vaccines, especially against foot and mouth disease [4–6].

* Corresponding author. ICAR-Indian Veterinary Research Institute, Regional Station, Palampur 176 061, Himachal Pradesh, India. ,
E-mail address: rinku.sharma@icar.gov.in (R. Sharma).

Abbreviations

HD50	50% of maximum haemolysis
CMI	cell-mediated immune
CTL	cytotoxic T lymphocytes
IAEC	Institute Animal Ethics Committee
IFN-γ	interferon-gamma
IL	interleukin
MSA	mannitol salt agar
NMR	nuclear magnetic resonance
PMN	polymorphonuclear
RE	reticuloendothelial
S-IV	shatavarin-IV
Th1	T helper cell-1
Th2	T helper cell-2
TLC	thin-layer chromatography

Saponins are steroidal or triterpenoid glycosides widely present in wild or cultivated plants, aquatic animals, and some bacteria [7, 8]. Apart from their immunomodulatory effect, they show some pharmacological and biological actions such as immunomodulatory, anti-inflammatory, antitumor, antiviral, hypoglycemic, hypocholesterolemic, and antifungal [9–11]. In addition to their stimulatory effects on a particular immunity, saponins also have certain non-specific immune responses, such as inflammation [12,13] and proliferation of monocytes [14,15]. Novel saponin-based combination adjuvants were used to stimulate balanced immune responses, and it includes AS01, which contains monophosphoryl lipid A (MPL), and QS-21 saponin (purified saponin from *Quillaja saponaria*) [16]. Saponins like QS-21 can enhance the activation and cross-presentation of dendritic cells through stimulation of NLRP3 inflammasome [17]. Quil-A saponin is used as an adjuvant in veterinary vaccines. It is also being studied for utilization as an adjuvant in vaccine formulations against HIV in guinea pigs and humans [18–20], *Toxoplasma gondii* [21], visceral leishmaniasis in mice [22–24], malaria in Aotus monkeys and mice [25], cancer in humans [26], and respiratory syncytial virus [27]. However, its strong haemolytic activity and aqueous phase instability restrict its usage as an adjuvant for human vaccines [28–31].

Therefore, many saponins from various natural sources have been screened and shown to possess adjuvant activities during the last decade. *Asparagus adscendens* Roxb. also known as "safed musli" or "shatavari" from class Liliaceae is a medicinal plant commonly found in South Asian countries. The medicinal properties of this indigenous plant have been reported in traditional medicine systems such as Siddha, Ayurveda, and Unani [32]. Shatavari is effective for the treatment of gastric ulcers, renal stones, bronchitis, diabetes, diabetic neuropathy, irritable bowel syndrome, and alcohol withdrawal. Phytochemical studies on the *A. adscendens* roots showed the presence of steroids (Shatavarin I-IV), isoflavones and alkaloid components (asparagamine and racemosol) [33]. The root extract of *A. adscendens* exhibited an antidiabetic effect [34] and cured cognitive disorders and brain aging in animal models related to Alzheimer's disease [35]. Shatavari exerts immunomodulatory effect by stimulating the RE system and PMN cells; induces lag in tumor development; prevents leucopenia induced by cyclophosphamide; inhibits ochratoxin A-induced suppression of IL-1, TNF-α; and antiaging and macrophage chemotaxis [36]. Shatavarin has been shown to produce an immunomodulatory effect by stimulating immune cell proliferation, IgG, and IL-12 production against human peripheral blood lymphocytes in *vitro* system [37].

The root aqueous extract of *Asparagus racemosus* resulted in significant upregulation of Th1 (IL-2, IFN-γ) and Th2 (IL-4) cytokines suggesting its immunoadjuvant activity in sheep RBC-sensitized mice [38]. A recent study on saponins-enriched fraction isolated from *Asparagus adscendens* fruits showed improved immunomodulatory response against ovalbumin antigen in mice [31]. However, earlier, the adjuvant activity of Shatavarin-IV saponin along with a vaccine against an infectious organism has not been reported. Therefore, the present study was planned with the objective to evaluate the adjuvant potential of Shatavarin-IV saponin (isolated from the fruits of *Asparagus adscendens*) alongside *Staphylococcus aureus* bacterin in the mouse model.

2. Materials and methods

2.1. Extraction, isolation, and characterization of Shatavarin-IV saponin

Fruits of *Asparagus adscendens* [31] were collected, processed, and used for isolation of Shatavarin-IV saponin. The isolation procedure was carried out as per the earlier described protocol [39]. Dried-powdered fruit (120 g) of *A. adscendens* was first defatted in the Soxhlet apparatus with *n*-hexane and then sequentially extracted with methanol and *n*-butanol solvents. The n-butanol fraction was subjected to column chromatography for the isolation of Shatavarin-IV. A total of 7.5 g of saponin fraction was packed for Column Chromatography (CC) using silica (60–120 mesh size) and eluted with different proportions of MeOH/CHCl$_3$ (00:10–10:00) to give five major fractions (A to E). Fraction D (4.95 g) was again subjected to CC on silica (230–400 mesh size) and eluted with MeOH/CHCl$_3$ (00:10–10:00) to give 6 sub-fractions (D1 to D6). Fraction D4 (1.03 g) was further packed for reverse-phase (Rp) column chromatography and eluted with increased polarity by using solvents MeOH/H$_2$O (00:10–10:00) resulting in the isolation of pure Shatavarin-IV (S-IV) saponin (102 mg). The isolated molecule was characterized by thin-layer chromatography (TLC) [40,41], nuclear

magnetic spectroscopy (NMR), and electrospray ionization-mass spectrometry (ESI-MS).

2.2. Haemolytic assay

A blood sample was collected from a healthy sheep in Alsever's solution (Sigma-Aldrich) to obtain RBCs. It was centrifuged at 2000 rpm for 5 min and washed with sterile saline solution (0.89% w/v NaCl) three times to remove the buffy coat. Pelleted RBCs were mixed with saline (0.89% w/v NaCl, pyrogen-free) to make 0.5% RBC suspension. The working stock of RBC suspension was mixed with a series of Shatavarin-IV saponin at varying concentrations (5, 6, 6.5, 7, 7.5, 10, 12.5, 15 μg) and incubated at 37 °C for 30 min. After centrifugation (90 g for 10 min), free hemoglobin (Hb) present in the supernatant was measured by a spectrophotometer at 412 nm [35]. Saline and distilled water were taken as a negative and positive control, respectively. Experiments were carried out with standard protocol and each concentration was taken in a triplicate fashion. The concentration induce 50% percent hemolysis was indicated as HD_{50} [31].

2.3. Staphylococcus aureus identification and bacterin preparation

Staphylococcus aureus strain (*S. aureus* Anm38, procured from NCVTCC, Hisar, Haryana, India) isolated from a bovine mastitis case was cultured on mannitol salt agar at 37 °C in a 5% CO2 enriched environment for 24 h. Cultured *S. aureus* bacteria were identified by Gram's staining method and mannitol fermentation test. After purity checking, colonies were transferred into 200 ml sterile BHI broth and kept at 37 °C for 24 h. Then, 1 ml of 37% formaldehyde was added for bacterial inactivation and incubated overnight at 37 °C. After that, the broth was centrifuged at 5000 rpm for 30 min, and the supernatant was discarded. Following this, the bacterial pellet was suspended in sterile 100 ml PBS containing 0.2 ml formalin, and the suspension was incubated overnight at 37 °C. The viability of the bacteria was then estimated by inoculating the inactivated bacterial suspension in the culture media and kept for overnight incubation at 37 °C. The concentration of *S. aureus* was estimated by using a spectrophotometer and adjusted to the OD value of 0.01 (1 $\times 10^6$ cells/ml) by diluting bacterial pellets with phosphate-buffered saline at 600 nm. The prepared bacterin was dispensed into sterile glass vaccine vials and kept at refrigeration temperature [42,43].

2.4. Mice

Six to eight weeks old Swiss albino mice (approx. 20–28 g) of both sexes were procured from Central Research Institute, Kasauli (HP). Animals were housed in propylene cages and acclimatized for 7 days under standard environmental conditions of 12 h light and 12 h dark cycle, 25 ± 2 °C, and fed with *ad libitum* amount of feed and water before experimenting.

Experimental protocols were followed as per the norms and guidelines framed by the Institute Animal Ethics Committee (IAEC) (No: PLP-IAEC-17), Control and Supervision of Experiments on Animals Committee (CPCSEA), and Organization for Economic Corporation and Development (OECD).

2.5. Toxicity assay

For the acute toxicity study, three single doses of Shatavarin-IV saponin (0.1, 0.2, and 0.5 mg) were administered subcutaneously (skin fold over the flank region) in mice (6 animals in each group). In line with OECD guidelines, a total of 14 days (OCED-425) experimental study was conducted [44]. Animals were continuously monitored till the end of the study period. Vital organs such as the liver, kidneys, and spleen (to check organ to body weight index), anticoagulant mixed blood sample, and serum samples were collected on the 7th and 14th day (post-injection) to check the toxic dose and *in vivo* effects of Shatavarin-IV saponin.

2.6. Immunization

The mice were categorized into six groups with 12 animals in each. Animals were immunized subcutaneously with inactivated *S. aureus* (Isa) alone or with adjuvants Quil-A (10 μg) or Shatavarin-IV (10, 20, 40 μg) on day 0. In addition, the PBS-treated animals were included as a control group. A booster dose was given on day 14 after the primary immunization. Sera and tissue samples (mesenteric lymph node and spleen) were collected at two and four weeks after booster immunization to evaluate antibody response, cytokine expressions, and lymphocyte proliferation (Table 1).

Table 1
Details of samples used for mRNA isolation.

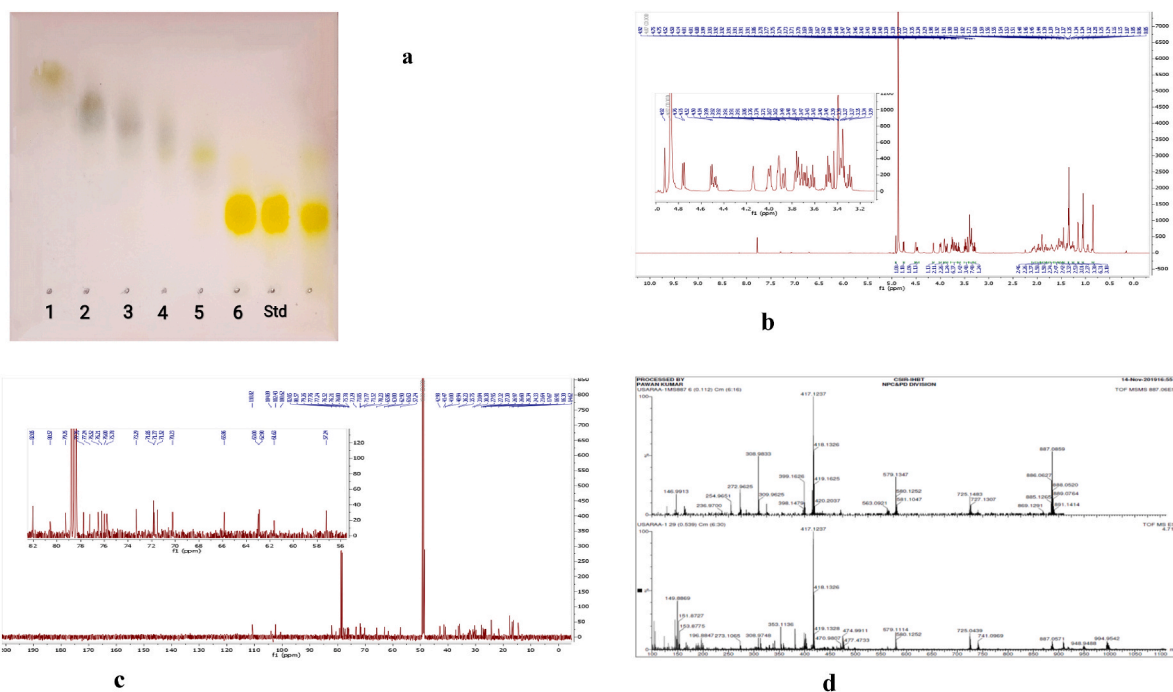| dpi	Control	ISa	Quil-A (10 μg)	S-IV (10 μg)	S-IV (20 μg)	S-IV (40 μg)
7	LN (2)	LN (3)	LN (3)	LN (3)	LN (3)	LN (3)
	S (4)	S (6)	S (6)	S (6)	S (5)	S (5)
14	LN (2)	LN (2)	LN (2)	LN (3)	LN (2)	LN (3)
	S (3)	S (5)	S (5)	S (5)	S (5)	S (5)

Dpi - days post immunization; LN - Lymph node (mesenteric); S – Spleen, values in parenthesis indicate the number of samples processed from each group.

2.7. Measurement of S. aureus specific IgM, IgG, and IgG2b antibodies

S. aureus-specific IgM, total IgG, and IgG2b antibody subtypes were estimated by indirect ELISA. Briefly, a 96-well microtiter plate was coated with 1×10^7 cells of sonicated *S. aureus* antigen in 100 µl of 50 mM carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Antigen-coated plates were washed thrice with PBST (pH 7.4) containing 0.05% Tween 20 (wash buffer) and then blocked with 5% FBS/PBS at 37 °C for 2 h. Followed by three washings, 100 µl of diluted serum samples (1:50) or FBS/PBS as control were added to the wells in triplicate fashion and incubated for 30 min at 37 °C. Aliquots of 100 µl of diluted (1:2000) HRP-conjugated goat anti-mouse IgM antibody (Invitrogen-UG287703) was added and allowed to incubate for 1 h. Similarly, 100 µl of diluted (1: 40,000) HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) and IgG2b (1:2000) antibodies (Invitrogen-UJ294970) were added. Later, wells were washed five times, and 100 µl of tetramethylbenzidine (TMB) (Sigma-Aldrich, USA) was added individually. After 30 min incubation, the enzyme reaction was stopped by 50 µl of 1 N H_2SO_4, and optical density (OD) was read on a plate reader at a wavelength of 450 nm.

2.8. Effect of Shatavarin-IV on mRNA expression of cytokines

RNA extraction was carried out from spleen and mesenteric lymph node samples of immunized mice by using RNeasy Mini Kit (Qiagen, Germany) as per the manufacturer's protocol. The purity and concentration of extracted RNA were measured at the absorbance of 260 nm in an optical nanodrop spectrophotometer (Thermo Scientific, USA). The range of the A260/A280 ratio from 1.8 to 2.1 is an indication of highly purified RNA (Sambrook and Russell, 2001). Quantitative real-time PCR (qRT-PCR) was carried out by using the Verso SYBR Green 1-Step qRT-PCR ROX kit in Step One Plus Real-time PCR system (Applied Biosystems, USA). $1x$ reaction was set up by using 10 ng of extracted RNA in a triplicate fashion for each gene. GAPDH (housekeeping gene) gene was used as an internal control. The total volume of 12.5 µl containing 0.13 µl of Verso Enzyme mix (Reverse transcriptase), 0.6 µl RT Enhancer, 6.25 µl of 2x 1-Step qPCR SYBR ROX Mix, 0.88 µl of forward and reverse primers (1 mM), 1 µl of RNA template (10 ng) and 2.75 µl of NFW was used to set reverse transcription and cDNA amplification reactions. Reverse transcription was carried out at 50 °C for 15 min followed by a thermo-start activation step at 95 °C for 15 min. PCR was performed for 40 cycles with the following program of denaturation at 95 °C for 15 s, annealing at 60 °C (GAPDH, IL-12, IFN-γ), 59 °C for IL-5, 60.5 °C for IL-2 and 61 °C for IL-4 at 30 s and extension at 72 °C for 30 s ΔΔCt method was employed to calculate the relative fold-change in gene expression among various treatment groups compared to the control.

Fig. 1. a. TLC of 1-5 (collected fractions), 6 (isolated Shatavarin-IV compound) along with commercial standard (std). Solvent system used: 70% MeOH: H_2O, visualization agent used (p-anisaldehyde-H_2SO_4 stain). **b.**[1]H Spectra of Shatavarin-IV. **c.**[13]C Spectra of Shatavarin-IV. **d.**ESI-MS of Shatavarin-IV.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was conducted for the mean values (three individual tests) of treatment and control groups through JMP software (9.0). Tukey's test was used to compare the results of treatment and control groups, and statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Total percent yield of Shatavarin-IV saponin

In the current study, Shatavarin-IV saponin was isolated from the fruit extract of *Asparagus adscendens* Roxb. collected from the North-western Himalayan region. The saponins-enriched fraction of 7.5 g was extracted and Shatavarin-IV pure saponin of around 102 mg with >95% purity was isolated from 120 g of dried fruit sample. The total yields of saponins-enriched fraction and Shatavarin-IV pure saponin were 6.25% and 0.085%, respectively.

Table 2
^{1}H NMR and ^{13}C NMR data (600 and 150 MHz) of isolated Shatavarin-IV in CD$_3$OD. δ in ppm, J in Hz.

Aglycone				Glycone			
Position	1H	13C	DEPT	Position	1H	13C	DEPT
1	1.24–1.32 *m*, 1.44–1.60 *m*	27.32	CH2	6′	3.60–3.78 *m*, 3.86–3.93 *m*	61.63	CH2
2	1.44–1.48 *m*, 1.51–1.56 *m*	26.34	CH2	**2′-O- α-L-Rhamnose**			
3	4.14 *m*	75.78	CH	1″	4.92 s	102.43	CH
4	1.44–1.51 *m*, 1.53–1.60 *m*	30.38	CH2	2″	3.86–3.93 *m*	71.77	CH
5	1.90–12.93 *m*	37.14	CH	3″	3.60–3.78 *m*	71.85	CH
6	1.44–1.60 *m*, 1.68–1.85 *m*	26.97	CH2	4″	3.46–3.50 *m*	73.29	CH
7	1.68–1.73 *m*, 1.77–1.85 *m*	26.60	CH2	5″	3.99–4.01 *m*	70.23	CH
8	1.68–1.76 *m*	36.23	CH	6″	1.35 d, ($J = 7.1$ Hz)	17.67	CH3
9	1.24–1.32 *m*	40.94	CH	**4′-O- β-D-Glucose**			
10	–	35.75	C	1‴	4.76 d, ($J = 7.7$ Hz)	104.09	CH
11	1.44–1.48 *m*, 1.53–1.60 *m*	21.64	CH2	2‴	3.28–3.31 *m*	76.0	CH
12	1.44–1.60 *m*, 1.77–1.85 *m*	41.0	CH2	3‴	3.46–3.50 *m*	77.24	CH
13	–	41.47	C	4‴	3.28–3.40 *m*	71.52	CH
14	1.24–1.32 *m*	57.24	CH	5‴	3.34–3.40 *m*	77.76	CH
15	1.44–1.60 *m*, 2.05–2.11 *m*	32.31	CH2	6‴	3.60–3.78 *m*, 3.86–3.93 *m*	62.90	CH2
16	4.46–4.48 *m*	82.05	CH				
17	1.77–1.85 *m*	63.0	CH				
18	0.85 s	16.91	CH3				
19	1.05 s	24.33	CH3				
20	1.90–1.93 *m*	42.98	CH				
21	1.05–1.07 *m*	14.62	CH3				
22	–	110.8	C				
23	1.44–1.60 *m*, 1.90–1.93 *m*	31.04	CH2				
24	1.44–1.60 *m*, 2.05–2.11 *m*	27.30	CH2				
25	1.95–2.01 *m*	27.95	CH				
26	3.28–3.40 *m*, 3.99–4.01 *m*	65.86	CH2				
27	1.15 d, ($J = 6.9$ Hz)	16.30	CH3				
Glycone							
3-O- β-D-Glucose							
1′	4.51 d, ($J = 7.8$ Hz)	100.62	CH				
2′	3.60–3.78 *m*	79.26	CH				
3′	3.60–3.78 *m*	76.21	CH				
4′	3.60–3.78 *m*	80.57	CH				
m = Multiplet (Overlapped signals)							

3.2. Characterization of saponins

3.2.1. Thin layer chromatography (TLC) detection of Shatavarin-IV

Saponin-loaded reverse phase TLC sheet was run in a 70% methanol + 30% water solvent system and stained with *p*-anisaldehyde-H_2SO_4 staining reagent. Saponin fraction produced a greenish-yellow spot and purified Shatavarin-IV compound exhibited a dark yellow spot against the pink background on aluminum plates coated with silica gel 60 (Fig. 1). Eluted S-IV saponin was identified by comparing observed Rf values of standard commercial S-IV saponin.

3.2.2. Nuclear magnetic resonance (NMR) and spectroscopic analysis of Shatavarin-IV

The mass of the isolated molecule was observed as 887.0859 $(M + H)^+$, which confirms its molecular formula is $C_{45}H_{74}O_{17}$. Furthermore, the MS-MS mass fragmentation of the molecule showed the presence of two glucose and one rhamnose moiety in the glycone portion with sarsasapogenin aglycon moiety, i.e., 887 $(M + H)^+$, 725 $(M + H\text{-Glu.})^+$, 579 $(M + H\text{-Glu.-Rha.})^+$ and 417 $(M + H\text{-Glu.-Rha. -Glu.})^+$. The isolated molecule was further characterized by NMR analysis.

The presence of four methyl signals in the 1H spectrum, δ_H 0.85 s (3H), 1.05–1.07 m (6H), 1.15, d ($J = 6.9$ Hz, 3H), and a downfield quaternary carbon at δ_C 110.8 indicating the aglycon moiety as sarsasapogenin, which was also confirmed by mass fragmentation. Besides, the presence of three anomeric protons at δ_H 4.92 s (1H), 4.76 d ($J = 7.7$ Hz, 1H), 4.51 d ($J = 7.8$ Hz, 1H) showed the HSQC correlation with three anomeric carbons at δ_C 102.4104.0 and 100.6, indicated the presence of two β-D-glucopyranosyl and one α-L-rhamnopyranosyl in the glycone moiety. Thus, from the NMR analysis and comparison of the observed data with the literature-reported data (Hayes et al., 2006), the isolated compound was identified as Shatavarin-IV. The 1H NMR and 13C NMR data of Shatavarin-IV saponin have been given in Table 2. The spectral data of Shatavarin-IV, NMR (^{1}H, ^{13}C), ESI-MS, and structure have been shown in Fig. 1.

3.3. Haemolytic activity assay

This study investigated the hemolytic activity of isolated Shatavarin-IV and commercial Quil-A saponins at varying concentrations in 0.5% of sheep RBCs, and values were expressed as HD_{50}. The obtained HD_{50} values of Shatavarin-IV and Quil-A saponins were 5.880 $\pm$ 1.050 μg/ml and 16.725 $\pm$ 2.210 μg/ml, respectively (Fig. 2). The results exhibited the higher haemolytic nature of Shatavarin-IV saponin at a lower dose (5.880 $\pm$ 1.050 μg/ml) itself as compared with Quil-A saponins.

Being a triterpenoid saponin, the haemolytic activity of Quil-A was mainly due to their increased number of monosaccharides and acyl residues [45]. The branched sugar chain of Quil-A possesses around 8–10 monosaccharide sugar moieties with aldehyde and acyl domains in their aglycone portion [46]. Different from Quil-A, Shatavarin-IV saponin has three unbranched sugars (two glucose and one rhamnose) moiety attached with the C-3 portion of the aglycone (sarsasapogenin) and the absence of acyl domain in its molecule [47]. The number of sugar molecules in the Shatavarin-IV is comparatively less than in Quil-A. However, Shatavarin-IV exhibited more haemolytic activity at a very lower concentration due to their core steroidal nucleus. It promotes the binding of saponin with the phospholipid membrane of RBC and initiates haemolysis by membrane perturbation mechanism. In general, steroidal saponins with monodesmosidic sugar moiety are more haemolytic than triterpenoids with bidesmosidic ones [48]. Shatavarin-IV saponin possesses the above-mentioned properties (steroidal saponin with monodesmosidic sugar), and this fact could account for the high haemolytic nature of Shatavarin-IV.

3.4. Effect of Shatavarin-IV on body weight and organ to body weight index

Shatavarin-IV saponin exhibited no mortality or adverse effects in the 14-day acute toxicity study when given by subcutaneously as a single dose of three graded concentrations (0.1, 0.2, and 0.5 mg). The absence of mortality or adverse events during the 14 days of the
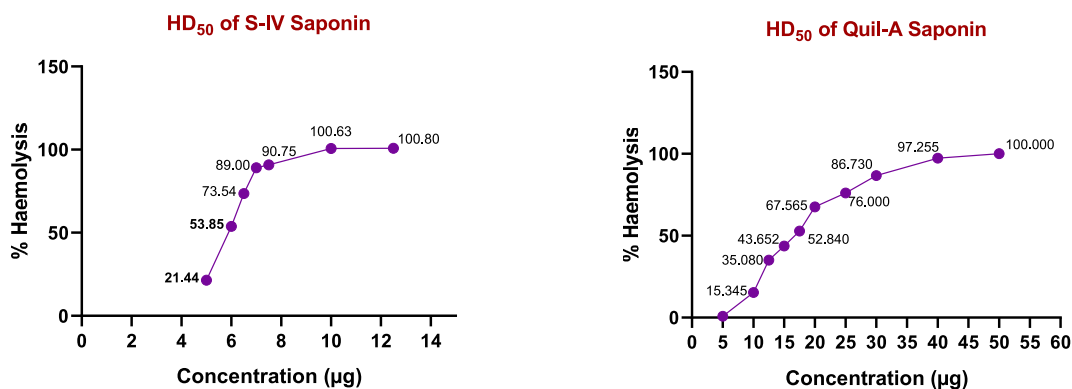

Fig. 2. Haemolytic activity of Shatavarin-IV and Quil-A saponin on sheep RBCs. * Haemolysis percent of saline and distilled water were included as minimal (0%) and maximal (100%) haemolysis controls, respectively. All values represent the mean $\pm$ standard error (n = 3).

observation period indicated its primary safety. Changes in body weight and food/water consumption have generally been regarded as a preliminary indication of an adverse effect of a drug [49]. Change in body weight was measured on 3rd-day post-injection and the level of body weight gain was significantly raised in all three Shatavarin-IV dosage groups throughout the study period (Fig. 3). At 72 h, the mice treated with 0.1 mg dose of S-IV showed higher weight gain (4.82 ± 1.74%) as compared with other treatment (2.36 ± 0.84% in 0.2 mg group and 3.70 ± 1.13% in 0.5 mg group) and control (5.01 ± 1.20%) groups. Even at the highest dose, the animals showed a substantial body weight gain at 72 h post-injection, indicating no detrimental impact of Shatavarin-IV to suppress the animals' feed and water intake.

Another measure used to determine the physiological and pathological condition in toxicological research is the organ-to-body weight index. The liver, kidneys, spleen, lungs, and heart are the primary organs that show immediate pathological reactions to any toxicant [50]. In the present study, the animal's organ-to-body weight index was calculated and given in Fig. 4. Organ-to-body weight index showed no significant variation in the weight of the liver and kidney (both at 7 and 14 dpi), whereas mild changes in the weight of spleen were observed in 0.2 and 0.5 mg Shatavarin-IV treated animal groups at 7 dpi. However, reduction in spleen weight at 0.2 and 0.5 mg Shatavarin-IV treated groups did not show any pathological changes, and a significant difference was not observed at 14 dpi. During necropsy inspection, no abnormal macroscopic lesions were observed in the vital organs such as the liver, spleen, and kidneys of any of the treated animals in this study. The overall effect of Shatavarin-IV saponin showed a positive response on animal body weight and the absence of gross pathological lesions.

3.5. Effect of Shatavarin-IV on blood and serum profile

The haematological profile showed notable changes in blood parameters like Hemoglobin (Hb), Red Blood Cells (RBCs), White Blood Cells (WBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), thrombocytes, granulocytes, monocytes, and lymphocytes in all groups of Shatavarin-IV treated animals (Table 3a and b). At 7 dpi, a significant ($P < 0.05$) decrease in Hb and RBC levels was observed in S-IV (0.2 mg) treated animals. Significant ($P < 0.01$ and $P < 0.05$) reduction of MCHC, thrombocyte counts, and elevated MCV levels were observed in all groups of Shatavarin-IV treated animals. On the other hand, WBC and lymphocyte population levels were significantly ($P < 0.05$) increased in S-IV (0.1 mg) group and reduced in S-IV (0.5 mg) group. Both S-IV (0.2 mg and 0.5 mg) treated animals showed a significant ($P < 0.05$) decrease in MCH levels.

The biochemical and haematological parameters are some important measures for assessing the toxicity of plant extracts in animals. Haematological parameter evaluation can be used to determine the extent to which foreign substances, including plant extracts, have a deleterious impact on the blood components of animals [51]. In the present study, the haematological parameters at 7 dpi showed a significant ($P < 0.05$) decrease in Hb and RBC levels at the dose of 0.2 mg S-IV treated animals as compared to the control group. Reduction of RBCs and Hb level is indicative of an anaemic condition due to various conditions such as blood loss, *in vivo* haemolysis by intoxication, and some nutritional disorders (iron, folic acid, Vit B12 deficiency). Thrombocyte level in the blood plays a vital role in the coagulation mechanism, and its level was significantly reduced in all three groups of S-IV treated animals at 7 dpi whereas, at 14 dpi, platelet count was decreased in the lower doses (0.1 and 0.2 mg) of S-IV. A significant decrease in mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) was observed at both intervals of this toxicity study. At 7
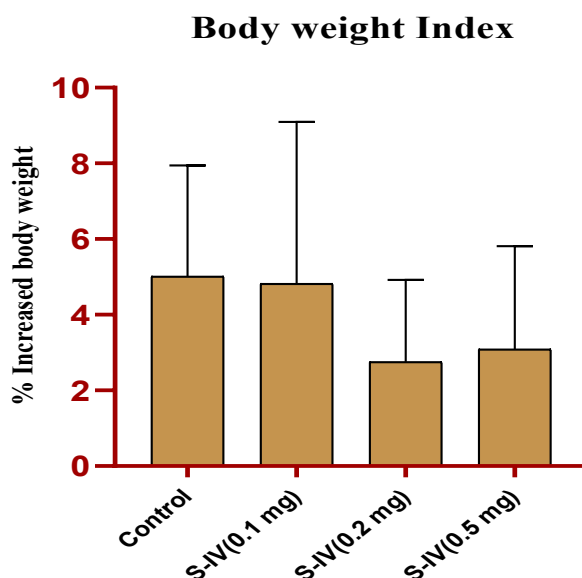
Body weight Index

Fig. 3. The bar graph shows the effect of Shatavarin-IV (S-IV) saponin on body weight of mice (72 h post injection). All values represent the mean $\pm$ standard error (n = 6). Significant difference between groups were designated as **$P < 0.05$.
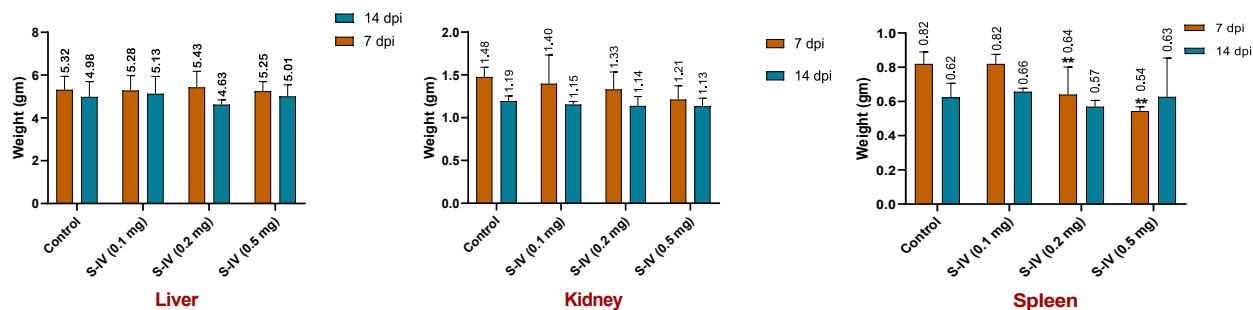
Fig. 4. The bar graph shows the effect of Shatavarin-IV (S-IV) saponin on organ to body weight index (liver, kidney, spleen). All values represent the mean ± standard error (n = 3). Significant difference between groups were designated as **P < 0.05.

Table 3a
Effect of Shatavarin-IV (S-IV) saponin on blood parameters in Swiss albino mice (7-days post injection).

Organs (gm)	Control	S-IV (0.1 mg)	S-IV (0.2 mg)	S-IV (0.5 mg)
Hb (g/dl)	14.80 ± 0.42	12.77 ± 0.23	10.03 ± 1.24*	12.56 ± 0.07
RBC (10^6/μl)	9.34 ± 0.23	9.52 ± 0.20	7.25 ± 0.91*	9.07 ± 0.05
WBC (10^3/μl)	9.18 ± 0.28	14.91 ± 1.32*	9.67 ± 3.37	4.30 ± 0.20*
MCV (fl)	46.32 ± 1.09	55.82 ± 0.94*	61.70 ± 3.67*	56.00 ± 1.00*
MCH (pg)	15.39 ± 0.21	14.63 ± 0.19	13.80 ± 0.30*	13.83 ± 0.13*
MCHC (g/dl)	35.64 ± 1.187	25.27 ± 0.27*	22.60 ± 1.36*	24.73 ± 0.17*
Platelet count (10^3/μl)	895 ± 2.89	534 ± 12.50*	277.66 ± 58.08**	228 ± 71.00**
Granulocytes (10^3/μl)	1.00 ± 0.06	1.81 ± 0.05	1.50 ± 0.45	0.90 ± 0.10
Monocyte (10^3/μl)	0.13 ± 0.02	0.28 ± 0.01	0.30 ± 0.10	0.17 ± 0.03
Lymphocyte (10^3/μl)	5.55 ± 0.03	14.14 ± 0.32*	7.86 ± 2.82	3.23 ± 0.07*

Table 3b
Effect of Shatavarin-IV (S-IV) saponin on blood parameters in Swiss albino mice (14-days post injection).

| Organs (gm)	Control	S-IV (0.1 mg)	S-IV (0.2 mg)	S-IV (0.5 mg)
Hb (g/dl)	15.77 ± 0.07	14.97 ± 0.27*	15.48 ± 0.19	15.78 ± 0.10
RBC (10^6/μl)	11.39 ± 0.22	11.37 ± 0.79	12.27 ± 0.32	11.44 ± 0.22
WBC (10^3/μl)	14.07 ± .41	12.02 ± 0.33*	8.27 ± 0.41**	14.36 ± 0.38
MCV (fl)	45.21 ± 0.63	56.03 ± 0.17**	55.66 ± 0.30**	53.68 ± 0.12*
MCH (pg)	15.82 ± 0.11	14.23 ± 0.12*	13.52 ± 0.11*	13.30 ± 0.06**
MCHC (g/dl)	35.88 ± 0.69	25.25 ± 0.14*	23.82 ± 0.14*	25.10 ± 0.21*
Platelet count (10^3/μl)	691.67 ± 15.98	357.67 ± 14.50*	478.33 ± 8.19*	971 ± 9.82
Granulocytes (10^3/μl)	2.27 ± 0.09	2.0 ± 0.00	1.45 ± 0.07*	4.55 ± 0.25**
Monocyte (10^3/μl)	0.16 ± 0.04	0.48 ± 0.02*	0.33 ± 0.02*	0.73 ± 0.03**
Lymphocyte (10^3/μl)	8.93 ± 0.41	9.33 ± 0.33	5.19 ± 0.24**	9.67 ± 0.17
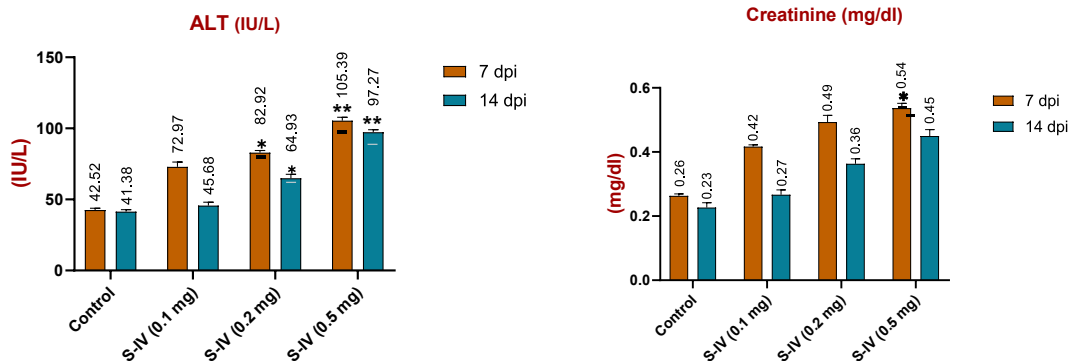
Fig. 5. Bar graph shows the serum ALT and creatinine levels from different treatment groups of Shatavarin-IV saponin. All values represent the mean ± standard error (n = 3). Significant difference between groups were designated as **P < 0.05.

dpi, the WBC level was significantly reduced in the S-IV (0.5 mg) treated group. It indicates the decreased immune potential of the body against foreign pathogens. The lymphocytic population level was significantly reduced at both intervals at the dose of S-IV (0.5 mg).

The biochemical analysis of liver and kidney function enzyme profiles in Shatavarin-IV treated animals has been given in Fig. 5. The serum ALT levels were significantly (P < 0.01) elevated in S-IV (0.2 mg and 0.5 mg) at both 7 and 14 dpi. At 7 dpi, a significant increase in serum creatinine level was observed in S-IV (0.5 mg) treated animals, whereas at 14 dpi, no significant changes were observed in all groups of S-IV treated animals. Measurement of liver and kidney function tests is important to assess the health status of animals in toxicological studies. Analysis of enzyme levels such as ALT and AST is extensively used in the evaluation of liver injury by drugs or some other hepatotoxin [52]. The results of the current toxicological investigation revealed a significant (P < 0.01) increase in serum ALT levels in the S-IV (0.2 mg and 0.5 mg) treatment groups at both 7 and 14 dpi. ALT enzyme is released into circulation through necrosis or membrane damage of hepatocytes, and it can be measured in the serum [53]. Serum creatinine and urea are important markers to assess renal health status [54]. In this study, serum creatinine level was elevated only at the higher dose of S-IV (0.5 mg) at 7 dpi. At 14 dpi, creatinine level was normal in all the treated animals as compared to the control group and it showed that Shatavarin-IV could produce mild repairable tubular damage at the dose of 0.5 mg. Based on the results of mortality pattern, haematology, and serum biochemistry findings, it was concluded that Shatavarin-IV saponin isolated from the fruits of *A. adscendens* Roxb. was found to be well accepted by the laboratory animals and didn't induce any *in vivo* acute toxicity up to the dose of 0.1 mg in a mouse model. Hence, 1/10, 1/5, and 2/5th doses of 100 μg Shatavarin-IV (10 μg, 20 μg, and 40 μg, respectively) saponin were selected to study their adjuvant potential with inactivated *S. aureus* bacterin in a mouse model.

3.6. Effect of Shatavarin-IV on S. aureus specific IgM, IgG, and IgG2b antibodies

The sera *S. aureus*-specific IgM, IgG, and IgG2b antibody responses were measured after one and two weeks post-booster immunization using indirect ELISA. One week after the booster, a significant increase in antibody responses was observed in Quil-A, and all three groups of Shatavarin-IV treated mice as compared with the control group (***P < 0.001, **P < 0.01, or *P < 0.05). Animals that received vaccine preparation with Quil-A elicited a statistically significant increase in IgM antibody levels compared with all three doses of Shatavarin-IV treated animals. However, IgG and IgG2b levels in the group immunized with the preparation containing Shatavarin-IV (40 μg) adjuvant were significantly enhanced as compared with Quil-A saponin (Fig. 6). Immune responses in vaccinated mice were found to be dose-dependent in Shatavarin-IV treated groups. Two weeks after the booster the IgG and IgG2b levels were substantially higher, however, the IgM levels were significantly reduced as compared to one week after the booster in all the treatment groups. After two weeks of booster immunization, both IgG and IgG2b levels in the groups of S-IV (20 μg and 40 μg) were considerably higher than Quil- A (10 μg) treated group. Treatment groups of S-IV (40 μg) and Quil-A (10 μg) were comparatively equal to each other for total IgG response two weeks after the booster.

A versatile adjuvant that can trigger the appropriate form of immune response to provide optimum protection against each type of infection is highly coveted in the vaccine industry. Oda and co-workers [45] stated that the adjuvant activity of saponin molecules is related to the balance between the hydrophobic and hydrophilic (sugar chains) components. This balanced amphipathic nature can promote better stimulation of Th2 immune responses against various antigens. In Quil A, the most abundant saponins (QS-17, QS-18, and QS-21) have branched sugar chains, 8–10 monosaccharide compounds, a distinct acyl domain, and an aldehyde group [55], whereas Shatavarin-IV has 3 unbranched sugars (2 glucose and 1 rhamnose) at the C-3 portion of the aglycone [47]. In this study, Shatavarin-IV saponin could significantly enhance the *S. aureus* specific IgM, total IgG, and IgG2b isotype antibody responses. The level of antibody responses was comparatively higher in adjuvants treated group than in bacterin alone treated groups. The aldehyde component in the Quil-A saponin can enhance the binding and activation of Th1 cells. The lipophilic acylated side chain attached to the sugar molecule confers significant activation of cytotoxic T lymphocytes (CTL) against exogenous antigens through stimulation of Th1 cells [56]. On other hand, the presence of balanced core triterpene or steroidal (hydrophobic) nucleus and sugar chain (hydrophilic)
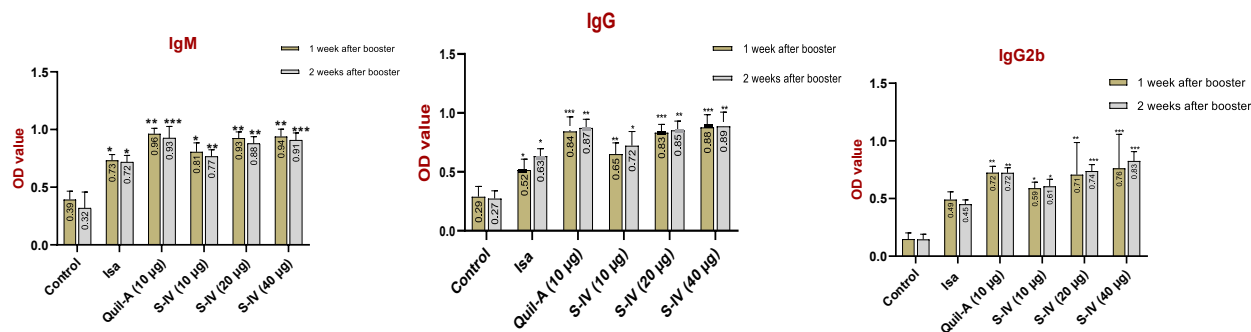

Fig. 6. Effect of Shatavarin-IV saponin on serum *S. aureus* specific IgM, IgG, IgG2b antibody titres in immunized mice. Six groups (12 mice/group) of mice were immunized subcutaneously with inactivated *S. aureus* alone or with Quil-A (10 μg) or Shatavarin-IV (10, 20, 40 μg) on day 0 and 14. Sera were collected on one and two weeks after booster immunization and *S. aureus* specific IgM, IgG, IgG2b antibodies in the sera were measured by an indirect ELISA. The values are presented as means ± SE (n = 6). Significant differences with control groups were designated as *P < 0.05, **P < 0.01, and ***P < 0.001.

can enhance the stimulation of humoral immune (Th2) response [56]. Oda and co-workers (2003) [45] found decreased immu-noadjuvant action in platicodigenin saponin due to the presence of more sugar residues. The amount of sugar residues were comparatively lesser in Shatavarin-IV saponin (3 unbranched sugars) than in Quil-A (8–10 monosaccharides). So the reduced level of sugar molecules and their balanced nature with a core nucleus in S-IV saponin could bias the immune response towards Th2-mediated immunity. Similarly, aldehyde and lipophilic acyl chain components in the Quil-A saponin showed increased Th1 responses. This demonstrated that Shatavarin-IV saponins were shown to possess adjuvant activity by activating the humoral immune response against *S. aureus*.

3.7. Effect of Shatavarin-IV on mRNA expression of different cytokines in splenocytes and lymph node

The fold change of Th1 and macrophage (IL-2, IFN-γ, IL-12), Th2 (IL-4, IL-5) mRNA cytokines levels were evaluated from spleen and mesenteric lymph node samples of immunized and control mice. GAPDH gene measurements have been used to equalize the expression data of the target genes. The findings revealed that Shatavarin-IV saponin significantly increased Th2 cytokines (IL-4 and IL-5) activities at one and two weeks after booster immunization. Among Shatavarin-IV treated groups, a dose of 40 μg produced better mRNA expressions of IL-4 and IL-5 cytokines at both one and two weeks after booster immunization. Enhancement of Th1 and macrophage cytokines (IL-2, IFN-γ, and IL-12) were observed in mice immunized with Quil-A saponins. Cytokine expression study revealed the adjuvant activity of Shatavarin-IV saponins was certainly shifted towards humoral immune responses than cellular im-mune activity.

In the current study, the fold shifts of IL-2, IFN-γ, IL-4, IL-5, and IL-12 mRNA cytokines levels were evaluated from spleen and lymph node samples of immunized and control mice to elucidate the efficacy of Th1, Th2, and macrophage immune response against *S. aureus*. Th1 cytokines provoke CMI response by activating macrophages, NK cells, and differentiation of naive T cells into Th1 lineages [49,57]. The results showed that both Quil-A and Shatavarin-IV could significantly increase the mRNA expression of IL-2 and IFN-γ cytokines (Figs. 7 and 8). On the other hand, the maximum response was observed in Quil-A treated group than in Shatavarin-IV saponins one week after the booster dose. Dose-dependent sequential rise of mRNA cytokine expressions was observed in Shatavarin-IV treated groups, and higher cytokines expression was noticed in S-IV at 40 μg. Two weeks after booster vaccination, the level of IL-2 and IFN-γ cytokine expression was decreased in all groups of treated animals except in Shatavarin-IV (40 μg). Oda and co-workers (2003) [45] stated that the presence of the aldehyde group and the length of the glycemic chain in the saponin core structure can enhance adjuvant activity. Quil-A saponins have an aldehyde group and longer sugar chain as compared with Shatavarin-IV saponin. The low efficacy of Shatavarin-IV from Quil-A saponin was possibly due to their lack of aldehyde group in its chemical structure. These factors account for the reduced mRNA expressions of IL-2 and IFN-γ cytokines expression levels. However, mildly elevated IL-2 and IFN-γ cytokine expressions were observed in Shatavarin-IV treated groups, indicating that S-IV saponin induced marginal CMI response which was comparatively lesser than Quil-A saponin.

Th2-mediated humoral response of immunized animals was evaluated by mRNA cytokine expression of IL-4 and IL-5 in spleen and lymph node samples after one and two weeks of booster immunization. At one and two weeks after the booster dose, both Quil-A and Shatavarin-IV (40 μg) treated groups had a stronger humoral response than other treatment groups (Figs. 7 and 8). However, at both, time points, the expression level of cytokines (IL-4 and IL-5) in the Shatavarin-IV (40 μg) treated group was significantly lower than Quil-A, with limited variation between these two groups. The levels of IL-4 and IL-5 cytokine expression in all the classes of treated animals were decreased two weeks after the booster dose. Marciani (2003) [58] stated that activation of Th2-mediated immune response was related to the balance between hydrophilic and hydrophobic components of saponins. In this study, Quil-A (10 μg) and Shatavarin-IV (40 μg) saponin produced a better humoral immune response. It indicated the balanced nature of the Shatavarin-IV saponin steroidal nucleus and sugar chains in its chemical structure.

IL-12 is a heterodimeric pro-inflammatory cytokine and is produced by the macrophages, dendritic cells, and to some extent by B lymphocytes. It stimulates the growth of T lymphocytes and enhances the differentiation of T cells into the Th1 lineage [56]. This pro-inflammatory cytokine stimulates cell-mediated immunity through the activation of NK cells and CD8^{+} T lymphocytes [59]. The findings of IL-12 cytokine level expression in immunized mice showed a higher response in the Quil-A treated group than in Shatavarin-IV treated animals (Figs. 7 and 8). Shatavarin-IV at the dose of 40 μg showed a good response, but it was comparatively lesser than Quil-A at both one and two weeks after booster immunization. Two weeks after the booster dose, the fold change of IL-12 cytokine expression was decreased in all groups of treated animals except in Shatavarin-IV (40 μg). The inability of Shatavarin-IV saponin to induce better IL-12 cytokine expression was possibly due to the presence of a short sugar chain compared to Quil-A and low IFN-γ cytokine expression level by Th1 cells. However, the IL-12 cytokine expression level of S-IV (40 μg) was better than in the Isa group. It depicted the fact that Shatavarin-IV saponin possesses minimum CMI immune response through stimulation of macrophage function.

4. Conclusions

The present study highlights the potential of Shatavarin-IV as an adjuvant in vaccine formulations. According to the results, Shatavarin-IV saponin at the dose of 40 μg possesses better adjuvant activity against *S. aureus* bacterin by eliciting desirable humoral responses. Whereas the CMI response was lesser as compared with the commercial Quil-A saponin . However, this study has its limitations such as the haemolytic activity of Shatavarin-IV saponin at higher dose and lack of aldehyde group in its chemical structure (reduced mRNA expressions of IL-2 and IFN-γ cytokines). In the future, a vaccine formulation with a combination adjuvant, using a lower dose of Shatavarin-IV saponin and CMI stimulating adjuvant (like QS-21, MF59, or complete Freund's adjuvant) needs to be
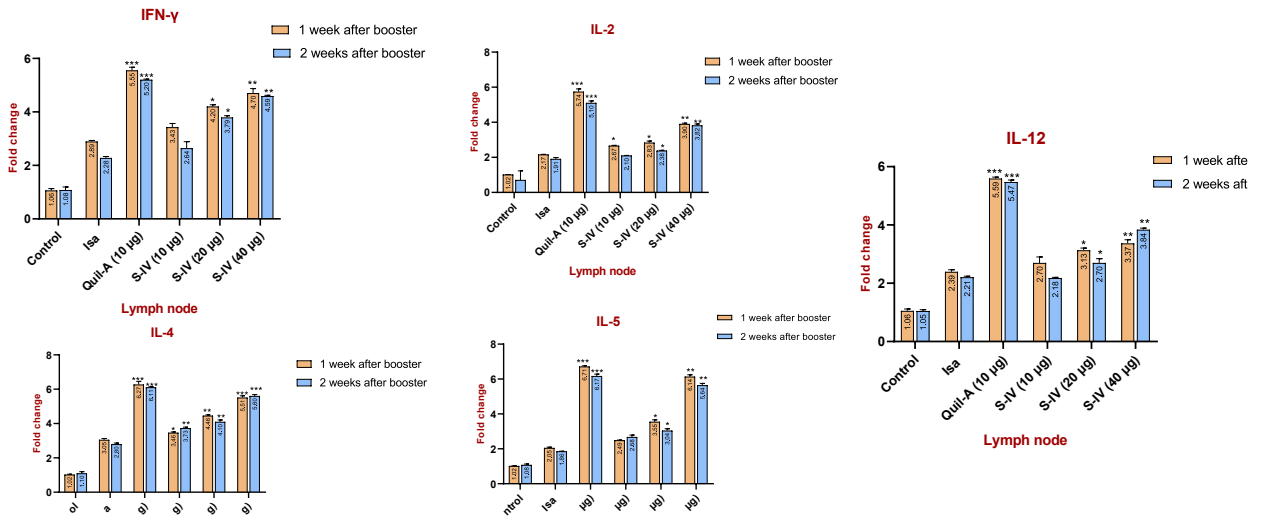
Fig. 7. Effect of Shatavarin-IV on different cytokines mRNA expression in lymph node of immunized mice. Six groups (12 mice/group) of mice were immunized subcutaneously with inactivated *S. aureus* alone or with Quil-A (10 µg) or Shatavarin-IV (10, 20, 40 µg) on day 0 and 14. The values are presented as means ± SE. Significant differences with control groups were designated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
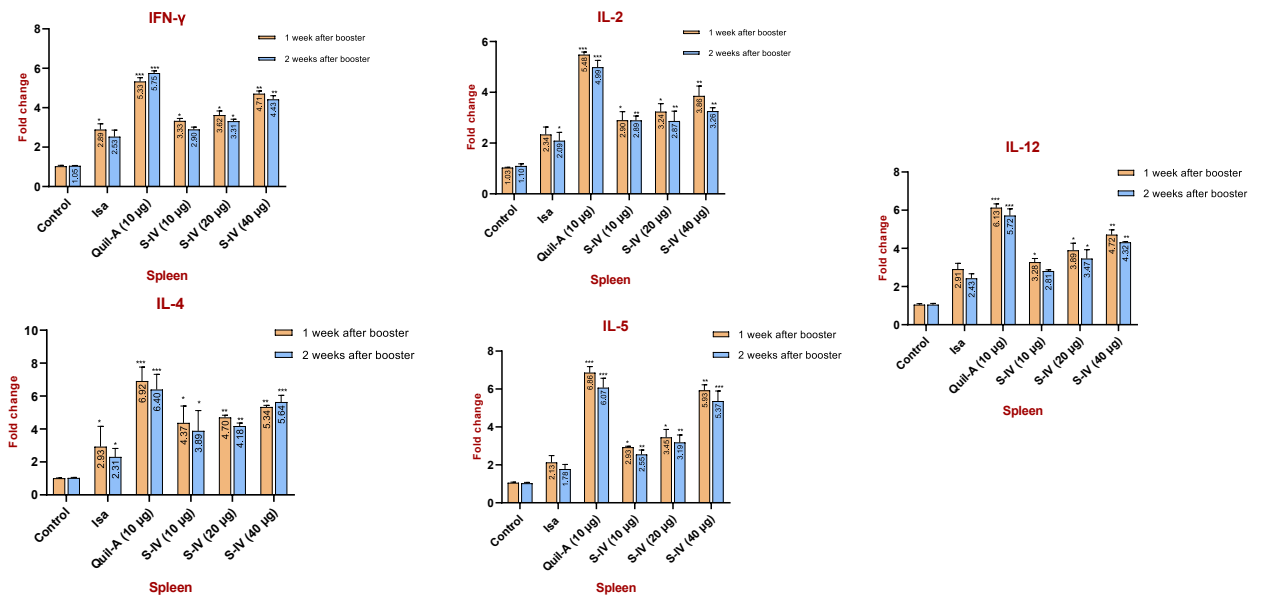

Fig. 8. Effect of Shatavarin-IV on different cytokines mRNA expression in the spleen of immunized mice. Six groups (12 mice/group) of mice were immunized subcutaneously with inactivated *S. aureus* alone or with Quil-A (10 µg) or Shatavarin-IV (10, 20, 40 µg) on day 0 and 14. The values are presented as means ± SE. Significant differences with control groups were designated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

tested for induction of potent immune response.

Authors contributions

Rinku Sharma: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Arivukarasu Palanisamy: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Prithvi Pal Singh, Upendra Sharma: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Rajendra Damu Patil: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Gorakh Mal, Birbal Singh: Contributed reagents, materials, analysis tools or data.

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Data availability statement

The data used to support the findings of this study are available from the corresponding author by request.

Statement regarding prior or duplicate publication

The prior or duplicate publication or submission elsewhere of any part of the work has not been done.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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