

Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Immunomodulatory activity of *Asparagus racemosus* on systemic Th1/Th2 immunity: Implications for immunoadjuvant potential

Manish Gautam^a, Santanu Saha^a, Sarang Bani^{b,1}, A. Kaul^b, Sanjay Mishra^a, Dada Patil^a, N.K. Satti^{c,1}, K.A. Suri^{c,1}, Sunil Gairola^{d,2}, K. Suresh^{d,2}, Suresh Jadhav^{d,2}, G.N. Qazi^{b,1}, Bhushan Patwardhan^{a,*}

^a Bioprospecting Laboratory, Interdisciplinary School of Health Sciences, University of Pune, Pune 411007, Maharashtra, India

^b Cell Biology and Molecular Pharmacology Laboratory, Regional Research Laboratory, Jammu Tawi 180001, India

^c Natural Product Chemistry Division, Regional Research Laboratory, Jammu Tawi 180 001, India

^d Serum Institute of India, 212/2 Hadapsar, Pune 411028, Maharashtra, India

ARTICLE INFO

Article history:

Received 30 March 2008

Received in revised form

18 September 2008

Accepted 23 October 2008

Available online 8 November 2008

Keywords:

Adjuvant

Th1/Th2 immunity

Immunomodulation

Ayurveda

Shatavari

Asparagus racemosus

ABSTRACT

Ethnopharmacological relevance: Roots of *Asparagus racemosus* Willd. (Shatavari in vernacular) are widely used in Ayurveda as Rasayana for immunostimulation, galactogogue as also in treatment of conditions like ulcers and cancer. Various studies have indicated immunomodulatory properties of Shatavari root extracts and formulations.

Aim of the study: To study the effect of standardized *Asparagus racemosus* root aqueous extract (ARE) on systemic Th1/Th2 immunity of SRBC sensitized animals.

Materials and methods: We used HPTLC to quantify steroidal saponins (Shatavarin IV, Immunoside[®]) and flow cytometry to study effects of ARE on Th1/Th2 immunity. SRBC specific antibody titres and DTH responses were also monitored as markers of Th2 and Th1 responses, respectively. We also studied lymphocyte proliferation. Cyclosporin, cyclophosphamide and levamisole were used as controls.

Results: Treatment with ARE (100 mg/(kg b.w. p.o.)) resulted in significant increase of CD3⁺ and CD4/CD8⁺ percentages suggesting its effect on T cell activation. ARE treated animals showed significant up-regulation of Th1 (IL-2, IFN- γ) and Th2 (IL-4) cytokines suggesting its mixed Th1/Th2 adjuvant activity. Consistent to this, ARE also showed higher antibody titres and DTH responses. ARE, in combination with LPS, Con A or SRBC, produced a significant proliferation suggesting effect on activated lymphocytes.

Conclusion: The study suggests mixed Th1/Th2 activity of ARE supports its immunoadjuvant potential.

© 2008 Elsevier Ireland Ltd. All rights reserved.

Abbreviations: AR, *Asparagus racemosus*; ARE, standardized aqueous of *Asparagus racemosus* roots; CD, cluster of differentiation; CP, cyclophosphamide; CPCSEA, committee for the purpose of control and supervision on experiments on animals; Cyc, cyclosporin; DTH, delayed type hypersensitivity; EtOAc, ethyl acetate; FACS, fluorescence-activated cell sorting; MeOH, methanol; IFN, interferon; IL, interleukin; KTA, kinetic turbidimetric test; LPS, lipopolysaccharide from *Escherichia coli*; OECD, organization for economic co-operation and development; RPMI, Roswell Park Memorial Institute; SRBC, sheep red blood cells; WHO, World Health Organization.

* Corresponding author at: Manipal Education, Bangalore, India. Tel.: +91 20 25690174; fax: +91 20 25690174.

E-mail addresses: manish.gautam2003@yahoo.co.in (M. Gautam), santanu.saha@rediffmail.com (S. Saha), sarangbani@rediffmail.com (S. Bani), bt.sanjay@gmail.com (S. Mishra), dadapatil007@hotmail.com (D. Patil), nksatti@rediffmail.com (N.K. Satti), kasuri1@rediffmail.com (K.A. Suri), sunil.gairola@seruminstitute.com (S. Gairola), k.suresh@seruminstitute.com (K. Suresh), ssj@seruminstitute.com (S. Jadhav), qazi.gn@yahoo.com (G.N. Qazi), bhushan@unipune.ernet.in (B. Patwardhan).

¹ Tel.: +91 191 2549051.

² Tel.: +91 20 26993900.

1. Introduction

Asparagus racemosus (AR) Willd. (Asparagaceae) is an important medicinal plant indigenous to South Asian countries. Its medicinal properties are reported in traditional systems of medicine such as Ayurveda, Siddha and Unani (Charak Samhita, 1970). Ayurveda, describes AR as *rasayana* and galactogogue, which is used to treat various diseases such as ulcer, dyspepsia and debility. Chemically, AR roots contain steroidal saponins known as shatavarins I–IV, iso-flavones and alkaloids including asparagamine and racemosol as major compounds (Saxena and Chourasia, 2001). In last decade, a few pharmacological and immunomodulatory activities of AR have been studied. For instance, AR root extract was shown to restore lymphocyte and neutrophils counts in myelosuppressed animals, which was comparable to lithium and glucan (Thatte and Dahanukar, 1988). In addition, AR roots were also reported to modulate macrophage functions resulting in significant reduction in severity of peritoneal adhesions (Rege et al., 1989). Further, AR hydro-alcoholic extract was found to induce lag in tumor development in experimental animals (Seena et al., 1993). The modulatory

effect of AR crude and hydro-alcoholic extracts on TNF-alpha secretion, phagocytosis and neuro-endocrinal secretions is also reported (Bhatnagar et al., 2005; Parihar and Hemnani, 2004; Dalvi et al., 1990; Dhuley, 1997). Previously, we reported immunoadjuvant activity of AR aqueous root extract (ARE) in two different experimental models. In the first model, co-administration with lower immunogenic doses of DPT vaccine resulted in higher anti-pertussis antibody titres and immuno-protection against lethal pertussis challenge (Gautam et al., 2004). While in second, it resulted in myeloprotection and recovery of humoral and cellular immunity in tumor bearing myelosuppressed mice (Diwanay et al., 2004). Overall, these studies project immunostimulant activity of AR, however, its immunological basis still remains unclear.

Modulation of Th1/Th2 immunity is emerging as one of biological targets for such immunostimulants (Romagnani, 2000). Helper T cells (Th) may be subdivided into two cell subsets, termed as Th1 and Th2, according to differences in their corresponding cytokines. Th1 cytokines contribute cell-mediated immunity while Th2 cytokines are responsible for humoral immunity (Warren et al., 1986; Abbas et al., 1996). We have studied possible immunoregulatory effects of ARE on murine Th1/Th2 immunity using SRBC as antigenic stimulus. Flow cytometry was used to monitor immune cell populations. Levamisole is one of the clinically established orally active Th1/Th2 immunomodulators, thus was used as positive control in the entire study (Bozic et al., 2003; Szeto et al., 2000; Argani and Akhtari, 2006). Our study suggests that ARE has cytoprotective, immunorestorative activities with mixed Th1/Th2 response and can be used as vaccine or immuno adjuvant.

2. Materials and methods

2.1. Preparation of extract (ARE)

Asparagus racemosus roots were obtained from Green Pharmacy, Pune, India and were correctly identified and authenticated as *Asparagus racemosus* Willd. (Asparagaceae) by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India (vide NISCAIR/RHMD/Consult/06/734/51). A voucher sample is retained and deposited at Agharkar Research Institute Herbarium, Pune, Maharashtra, India. Powdered roots were extracted as aqueous decoction as per Ayurvedic Pharmacopoeia of India using distilled water (Ayurvedic Pharmacopoeia of India, 2001). The procedure resulted in 30 g of brownish, hygroscopic extract (ARE) obtained from 100 g of powdered roots. It was stored in desiccating conditions till further use. ARE was studied at doses ranging from 6.25 to 200 mg/(kg b.w.) and was administered as oral suspensions using distilled water (0.5 mL).

2.2. Antibodies and chemicals

Fluorescein-isothiocyanate (FITC)-labeled anti-mouse monoclonal antibodies against (CD4⁺, CD19⁺, IFN-gamma); Phycoerytherin (PE)-labeled (CD8, CD3, IL-4, IL-2); FACS lysing and permeablizing solution (B.D. Biosciences, San Jose, CA), LPS (*E. coli*, Sigma, India), Concavalin A (Sigma), Roswell Park Memorial Institute (RPMI) medium 1640 (Sigma), Shatavarin IV and Immunoside was kindly provided by Regional Research Laboratory, Jammu Tawi, India. Unless otherwise specified, all the solvents used were of HPLC grade (Ranbaxy Chemicals, Ltd., India).

2.3. Quality control and chemoprofiling

ARE complied with W.H.O. limits on safety and purity with respect to microbial load, aflatoxins, pesticide residues and heavy

metals (WHO, 1998). Endotoxin levels were estimated using conventionally used kinetic turbidimetric test (KTA). Endotoxins were found below 15 EU/mg, which was within pharmacopoeial limits (USP, 2002).

Shatavarin IV (3-O-[(1-L-rhamnopyranosyl-(1 → 2))-(-D-glucopyranosyl (1 → 4)-O-(-D-glucopyranosyl)]-25(S)-spirostan-3(-ol) and immunoside (3-O-[(1-L-rhamnopyranosyl-(1 → 2))-(-L-rhamnopyranosyl-(1 → 4)-O-(-D-glucopyranosyl)]-25(S)-spirostan-3(-ol) were used as marker compounds and were quantified using a method described earlier (Satti et al., 2006). High performance thin layer chromatography (HPTLC) analysis was carried out using Linomat IV Spotter and densitometer (CS-9301PC, Shimadzu). ARE (2 g) in distilled water (10 mL) was extracted thrice with n-butanol. The resulting n-butanol extract was dried and was reconstituted in methanol (8.46 mg/mL) and was spotted (5 µL/spot) on pre-coated TLC plates (E. Merck-Germany, 60F-254). Marker compounds were dissolved in methanol (0.5 mg/mL) and calibration curves were plotted with linearity observed in the concentration range of 0.5–5 µg/mL. The analysis was performed at room temperature using EtOAc:MeOH:H₂O (75:13.5:10) as mobile phase. The resulting chromatograms were developed by spraying the plate with 1% ceric ammonium sulphate followed by heating at 105 °C for 10 min. The plates were scanned at 450 nm for quantification.

2.4. Animals

All experimental procedures used in present study were in accordance with institutional guidelines for animal research (CPCSEA, 2003). The study protocols were approved by the Institutional Animal Use and Care Committee of Regional Research Laboratory (Now known as Indian Institute of Integrated Medicine), Jammu. Balb/c mice were obtained from healthy animal colony maintained at the Department of Pharmacology, Regional Research Laboratory. Balb/c mice (male, 3–4 weeks old) were randomly distributed in groups as per experimental protocols (*n*=6 or 10). They were kept in an air-conditioned and pathogen-free isolators with temperature of 23 ± 2 °C and humidity of 55.6 ± 10% on a regulated 12-h light and dark cycle. They were given standard laboratory chow (Amrut Mills, Nashik, India) and tap water *ad libitum*. Blood samples were collected through retro-orbital bleeding at specified time points under ether anaesthesia and assayed for cell counts, cytokines and antibody titres.

2.5. Maximum tolerable dose (MTD) determination

The OECD method was used to determine MTD in animals (OECD, 1996). Test material was orally administered in graded doses and animals were monitored for change in weight, general behavior and mortality at 0.5, 2, 6 and 12 hourly intervals after test material administration. Test material was found to be well tolerated up to 2500 mg/kg.

2.6. Antigenic stimulus

Sheep red blood cells (SRBC) suspension in Alsever solution was obtained from animals housed at Regional Research Laboratory, Jammu. Blood cells were always washed three times with pyrogen free sterile normal saline (0.9% NaCl, w/v). Each mouse received 1 × 10⁹ cells in volume of 0.2 mL i.p. for sensitization and challenge at required time schedule. This cell count has been reported to induce optimum immune response in normal and immune suppressed conditions under our assay conditions (Bani et al., 2006).

2.7. Experimental design

Three independent experiments were performed using levamisole (2.5 mg/(kg p.o.)) as positive control.

2.7.1. Experiment 1: effect of ARE on T cell percentages (CD3⁺, CD4⁺ and CD8⁺) and Th cytokines

Immunizations were carried out using SRBC (1×10^9 cells in 0.2 mL saline/i.p.) on day 0 and 7. From day 0 (2 h post-SRBC injection) to 6, ARE at varying doses of 6.25, 12.5, 25, 50, 100 and 200 mg/kg was administered orally once daily in respective groups. Cyclosporin (5 mg/(kg b.w.)) was administered 48 h prior to sensitization as negative control. Blood was collected 48 h post-challenge for estimation of CD3⁺, CD4⁺, CD8⁺ and CD4⁺ (IL-2, IL-4 and IFN- γ) percentages using flow cytometry.

2.7.2. Experiment 2: effect of ARE on in vivo SRBC specific humoral and cellular immune responses

2.7.2.1. Humoral response. Study design is same as of Experiment 1. Except for, ARE was orally administered in doses of (25, 50, 100 and 200 mg/(kg b.w. P) and sera were collected on day 9 for estimation of antibody titres. Cyclophosphamide was used as negative control at 250 mg/(kg b.w. p.o.)/48 h prior to sensitization. The estimation of antibody titres was done using standard haemagglutination test (Nelson and Mildenhall, 1967). Titres were further converted to mean log₂ values for analysis purpose.

2.7.2.2. Cellular response. The method of Doherty was followed to assess SRBC induced DTH response in mice (Doherty, 1981). Cyclosporin was used as negative control at 5 mg/(kg b.w. p.o.) 48 h prior to sensitization. The immunization and treatment schedules were similar to humoral study except for challenge procedure. Animals were challenged with subcutaneous administration of SRBC in the left hind footpad while the right hind paw received saline. On day 9, difference between left and right paw thickness/swelling of foot was measured using digital plethysmometer LE 7500 (Panlab, Barcelona, Spain).

2.7.3. Experiment 3: effect of ARE on in vivo CD3⁺ (total T cell) and CD19⁺ (total B cell) percentages in Naïve (unsensitized) and sensitized animals

SRBC sensitized and/or unsensitized animals were treated either with vehicle, cyclophosphamide (250 mg/(kg p.o.) 48 h prior to sensitization), levamisole (2.5 mg/(kg p.o.)) for 7 days or ARE (100 mg/(kg p.o.)) for 7 days. CD3⁺ (total T cell) and CD19⁺ (total B cell) percentages were determined on 7th day using flow cytometry.

2.8. Splenocyte proliferation assay

ARE was assayed for lymphocyte proliferative responses using Naïve Balb/c mice splenocytes. Spleens were removed under aseptic conditions and homogenized in HEPES-buffered RPMI 1640 medium. Splenocytes were sedimented by centrifugation at 300×3 g for 7 min at 4°C; washed and re-suspended in RPMI 1640 medium supplemented by 10% heat inactivated fetal calf serum, 2-mercaptoethanol (50 μ M), penicillin G (100 U/mL), streptomycin (100 μ g/mL), amphotericin B (0.25 μ g/mL), 1 mM sodium pyruvate and 2 mM L-glutamine (Sigma). Cell suspensions were distributed (5×10^6 viable cells/mL/well) into 96 well flat bottom plates (Costar, Cambridge, MA). *E.coli* polysaccharide (LPS; Sigma) and concanavalin A (Con A; Sigma) was used at 10 μ g/mL as B and T cell mitogens, respectively. The splenocytes were cultures with

different concentrations of ARE (1, 10, 30, 100) μ g/well in presence or absence of mitogens (LPS and/or ConA). Working stocks of mitogen and ARE (1 mg/mL) was prepared using incomplete RPMI medium. After 24 h of incubation at 37°C and 5% CO₂, proliferation of spleen cells was measured by colorimetric reading of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction as described by Mosmann (1983), the plates were read at OD 540 nm to assay proliferative responses to AR and mitogens.

2.9. Estimation of lymphocyte percentages (CD4⁺, CD8⁺, CD3⁺ and CD19⁺)

The analysis of subsets namely CD3⁺ (total T cell), CD19⁺ (total B cell) CD4⁺ (T-helper cells) and CD8⁺ (cytotoxic cells) was performed on peripheral blood (Bani et al., 2005). Briefly, mice were bled at required time schedules and 50 μ L of blood was added to falcon tubes (B.D. Biosciences, San Jose, CA) containing different immuno-labeled monoclonal antibodies. After mixing and incubating at room temperature for 30 min in the dark, FACS lysing solution was added. The samples were incubated for 10 min for room temperature, followed by centrifugation. The cells were washed and enumeration of lymphocytes subsets was done using FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer using Cell Quest Pro software (Becton Dickinson, San Jose, CA). 10,000 events were collected to analyze CD4⁺, CD8⁺ T cells.

2.10. Intracellular cytokine estimation

The detection of cytokines in peripheral blood was performed as per BD Biosciences protocol and reported method (Bani et al., 2005). Briefly, to 80 μ L of peripheral blood CD4⁺ and CD8⁺ monoclonal antibody (mabs) were added. After mixing and incubating at room temperature in the dark, FACS lysing solution was added. The samples were incubated followed by centrifugation at 10,000 rpm for 10 min. Cells were then washed, permeabilized and stained with FITC-coupled CD4⁺ mouse (mab), phycoerythrin (PE) coupled IL-2, IL-4, IL-10 mabs in one set and PE coupled CD8⁺ mabs, FITC coupled IFN- γ mabs in another set. All monoclonal antibodies mentioned here were purchased from B.D. Biosciences. The stained cells were then acquired using FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer. For gating and calculation; cell quest software (Becton Dickinson, San Jose, CA) was used. Gating for lymphocytes using forward/sideward scatter was facilitated by CD4⁺/CD8⁺ staining; 10,000 cells were determined with at least 100 cells in every gate of lymphocyte subpopulations. The resulting numbers are percentages of cytokine expression of those subpopulations.

2.11. Data analysis and statistical considerations

Data is expressed as mean \pm S.E. Percent immunomodulatory activity in normal and immune suppressed animals was derived using earlier reported method: Normal conditions = (test group – sensitized control group/sensitized control group) \times 100. Immune suppressed conditions = $1 - (\text{test group} - \text{sensitized control group}) / (\text{cyclosporin/cyclophosphamide control} - \text{sensitized control}) \times 100$ (Kaul et al., 2003). Statistical significance of differences was assessed by Post-ANOVA (Bonferroni test for multiple comparisons). IFN- γ /IL-4 ratios were evaluated using Mann-Whitney test. $P < 0.05$ was set as the level of significance.

Table 1
Effect of ARE on CD3⁺ CD4⁺ and CD8⁺ percentages in peripheral blood of SRBC sensitized animals.

Group	Dose (mg/kg)	Total T cells	T-helper cells	T-cytotoxic cells	% Modulatory activity		
		CD3 ⁺ (%)	CD4 ⁺ CD8 ⁻ (%)	CD8 ⁺ CD4 ⁻ (%)	CD3	CD4	CD8
Unsensitized control	Saline	35.25 ± 5.27	5.055 ± 0.72	3.272 ± 0.67	NA	NA	NA
SRBC	Cells	53.93 ± 1.51 ^{***}	26.5 ± 0.3 ^{***}	15.7 ± 0.70 ^{***}	NA	NA	NA
Levamisole	2.5	78 ± 0.69 ^{***}	37.1 ± 0.5 ^{***}	22.01 ± 0.5 ^{**}	44.6	40	40.9
Cyclosporin	5	28.7 ± 0.58 ^{***}	12.04 ± 0.12 ^{***}	7.56 ± 0.34 ^{**}	-46	-54	-51.8
ARE	6.25	56.96 ± 0.77	27.14 ± 0.2	15.16 ± 0.39	5.56	2.4	-3.4
ARE	12.5	61.85 ± 0.92 [*]	28.12 ± 0.45 [*]	15.30 ± 0.67	14.6	6.1	-54
ARE	25	62.57 ± 0.52 [*]	29.4 ± 0.4 [*]	15.8 ± 0.45	16.2	10.9	0.63
ARE	50	64.95 ± 0.43 [*]	30.6 ± 0.4 [*]	16.6 ± 0.24 [*]	20.4	15.5	5.73
ARE	100	70.91 ± 0.26 ^{***}	32.8 ± 0.88 ^{***}	18.8 ± 0.33 ^{**}	30	35.1	19.7
ARE	200	64.58 ± 0.2 [*]	31.01 ± 0.4 ^{**}	17.5 ± 0.80 ^{**}	19.7	17	11.5

Values are shown as mean ± standard error. Percent modulatory activity was calculated using formulae given in text under data analysis. Bonferroni test for multiple comparisons generated *P*-values indicating significant differences in T cell percentages due to treatment. *, ^{*} represent comparison vs. SRBC and unsensitized control, respectively. NA = Not applicable; *N* = 10.

^{*} *P* < 0.05 vs. sensitized control.

^{**} *P* < 0.01 vs. sensitized control.

^{***} *P* < 0.001 vs. sensitized control.

3. Results

3.1. Standardization of ARE

HPTLC analysis was done on basis of identification and quantification of two steroidal saponins, Shatavarin IV and immunoside that are reported to be present in ARE. The result suggests assay percentages of Shatvarin IV and immunoside in ARE at 8.53 ± 0.38 and 0.038 ± 0.003, respectively.

3.2. Effect of sensitization protocol on selected immune markers

As a first step to study effect of ARE on Th immunity, effect of sensitization protocol on selected immune markers was established. Animals were injected pyrogen free saline i.p. with and without SRBC on days 0 and 7. Blood samples were drawn after 48 h of second sensitization and were processed for CD3⁺, CD4⁺, CD8⁺ and CD4⁺ (IFN-gamma, IL-2 and IL-4) positive cell percentages. We had previously determined the optimal concentration of SRBC in Balb/c mice, which allows quantification of SRBC specific humoral and cellular immune responses in normal and immune suppressed conditions (Bani et al., 2006). The results suggest that sensitization protocol up regulates Th1 and Th2 cytokine production significantly as compared to unsensitized control (Tables 1 and 2). Such up-regulation effect of SRBC on Th1 and Th2 cytokines is consistent with previous reports (Mashimo and Mita, 1995).

Table 2
Effect of ARE on CD4⁺ positive Th1 (IL-2, IFN-gamma) and Th2 (IL-4) cytokine percentages in SRBC sensitized animals.

Treatment (mg kg ⁻¹)	Dose (mg/kg)	Th1 cytokine CD4 ⁺ IL-2 ⁺	Th1 cytokine CD4 ⁺ IFN-g ⁺	Th2 cytokine CD4 ⁺ IL-4 ⁺	Th1/Th2 ratio Mean ratio IFN-g/IL-4
Unsensitized control	Saline	1.33 ± 0.67	0.936 ± 0.32	1.324 ± 0.31	0.692 ± 0.082
SRBC alone	SRBC	9.2 ± 0.76 ^{***}	6.97 ± 0.3 ^{***}	7.14 ± 0.22 ^{***}	0.976 ± 0.054 [*]
Levamisole	2.5	14.73 ± 0.43 ^{***}	11.73 ± 0.2 ^{***}	10.9 ± 0.06 ^{***}	1.076 ± 0.038 ^{**}
Cyclosporin	5	4.18 ± 0.05 ^{***}	3.93 ± 0.06 ^{***}	4.62 ± 0.29 ^{***}	0.216 ± 0.056 ^{***}
ARE	6.25	7.8 ± 0.36	6.5 ± 0.28	7.1 ± 40.43	0.915 ± 0.078
ARE	12.5	8 ± 0.45	6.67 ± 0.45	7.8 ± 0.58	0.855 ± 0.045 [*]
ARE	25	8.2 ± 0.67	7.05 ± 0.55	8 ± 0.44 [*]	0.881 ± 0.038
ARE	50	9.24 ± 0.34	7.56 ± 0.37	8.8 ± 0.45 ^{**}	0.859 ± 0.029
ARE	100	11.97 ± 0.34 ^{***}	8.12 ± 0.56 [*]	9.8 ± 0.58 ^{***}	0.828 ± 0.038 ^{**}
ARE	200	10.17 ± 0.45 [*]	7.5 ± 0.53	9.0 ± 0.44 ^{**}	0.833 ± 0.075 ^{**}

Values are shown as mean positive percentages in peripheral blood ± standard deviation. Mean IFN-gamma/IL-4 ratios were determined for respective groups. *, ^{*} represent comparison vs. SRBC and unsensitized control, respectively. NA = not applicable; *N* = 10.

^{*} *P* < 0.05 vs. sensitized control.

^{**} *P* < 0.01 vs. sensitized control.

^{***} *P* < 0.001 vs. sensitized control.

3.3. Effect of ARE and levamisole on CD3⁺, CD4⁺ and CD8⁺ T cell percentages

As demonstrated in Table 1, ARE and levamisole up-regulated the population of T cell CD3⁺ and CD4⁺/CD8⁺ subsets percentages as compared to control (*P* < 0.001) suggesting their T cell activating potential. ARE showed a dose dependent increase with optimum effect observed at 100 mg kg⁻¹ (*P* < 0.001). Cyclosporin as expected resulted in significant reduction of CD3⁺, CD4⁺ and CD8⁺ percentages (*P* < 0.001) as compared to control.

3.4. Effect on Th1 and Th2 cytokines

Treatment with ARE resulted in dose dependent increase of Th1 (IL-2, IFN-gamma) and Th2 (IL-4) cytokines, with maximum effect at 100 mg kg⁻¹ as compared to control. Interestingly, the relative immunomodulatory effect of ARE was more apparent on IL-4 as compared to IFN-gamma levels (Table 2). This was further confirmed by estimating mean IFN-g/IL-4 ratios for treatment and control groups. ARE at 100 mg/kg dose resulted in significant increase in ratios (*P* < 0.01) suggesting higher modulatory effect on Th2 cytokine. Levamisole in contrast resulted in significant up-regulation of ratios indicating preferential effect on IFN-gamma level (*P* < 0.01). Cyclosporin treated group showed significant reduction of cytokines indicating immunosuppressive effect (*P* < 0.001).

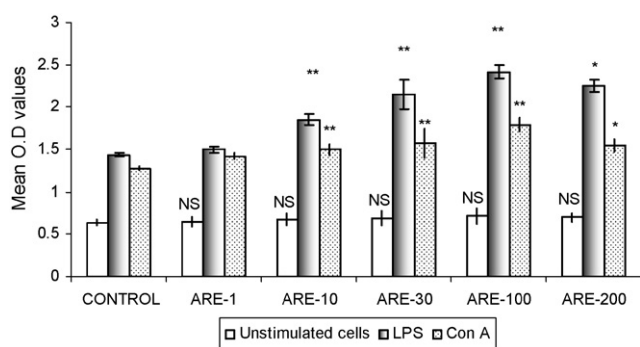


Fig. 1. Effect of ARE on lymphocyte proliferation. Splenocytes (5×10^6 viable cells/mL well) were cultured with different concentrations of ARE in either presence or absence of mitogens (LPS or Con A) stimulus. Histograms represent mean \pm S.D. absorbance units. Bonferroni test for multiple comparisons was used to compute *P*-values. **P*<0.05, ***P*<0.01 vs. paired control.

3.5. Effect of ARE on lymphocyte proliferation

ARE elicited a significant increase in proliferative response in Con A and/or LPS stimulated lymphocytes. The increase in proliferation was concentration dependent with optimum effect observed at 100 μ g/mL. ARE treated splenocytes cultured in absence of mitogens did not show any significant proliferative effect (Fig. 1).

3.6. Effect of ARE on in vivo CD3⁺ (T cell) and CD19⁺ (B cell) percentages in unsensitized and sensitized animals (Fig. 2)

Flow cytometry was used to monitor CD3 and CD19 positive percentages in unsensitized and sensitized animals. In unsensitized conditions, no significant effect of ARE treatment was observed on CD3⁺ and CD19⁺ percentages as compared to control (*P*<0.01). In contrast, levamisole and cyclophosphamide resulted in significant up-regulation and down regulation of CD3⁺ and CD19⁺ percentages, respectively (*P*<0.001). Interestingly, in sensitized conditions, treatment with ARE treatment 100 mg kg⁻¹ resulted in significant up-regulation of CD3⁺ and CD19⁺ positive percentages as compared to control suggesting its effect on activated lymphocytes. Levamisole and cyclophosphamide showed similar trends as observed for unsensitized conditions (Fig. 2).

3.7. Effect of ARE on antigen specific responses

3.7.1. Humoral responses (Table 3)

ARE when administered orally at 25–200 mg/(kg b.w.) doses produced a dose dependent increase in antibody titres. Optimum immunomodulatory activity was observed at 100 mg/(kg b.w.) dose (*P*<0001). In similar conditions, levamisole also resulted in significantly higher anti-body titres as compared to ARE. Pretreatment of animals with CP, 48 h before sensitization resulted in significant reduction of anti-body titres indicative of immune suppression (–38% and *P*<0.001 vs. control).

3.7.2. Cellular responses: SRBC specific DTH responses (Table 3)

As demonstrated in Table 3, ARE modulated cellular immune response in dose dependent manner where optimum activity (55%) was observed at 100 mg/(kg b.w.) Levamisole in similar conditions resulted in higher immunomodulatory activity (122%). This suggests that levamisole has higher modulatory activity on DTH response as compared to ARE. Pretreatment of cyclosporin, 48 h prior to sensitization resulted in significant

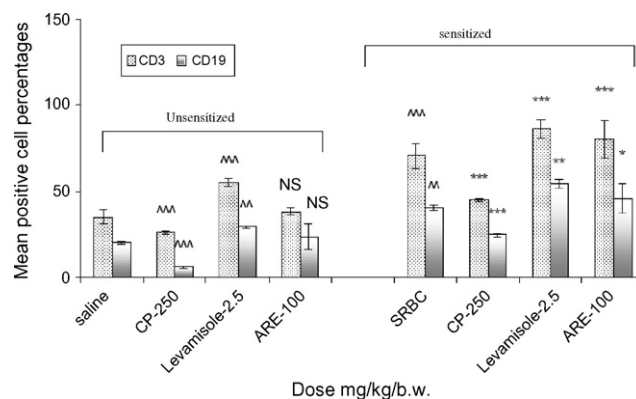


Fig. 2. Effect of ARE on CD3⁺ (T cell) and CD19⁺ (B cell) percentages in unsensitized and SRBC sensitized animals. Data represent group wise mean \pm S.D. positive percentages after treatment with cyclophosphamide (CP) (200 mg/(kg b.w.)) or levamisole (2.5 mg/(kg b.w.)) or ARE (100 mg/(kg b.w.)) in unsensitized and SRBC sensitized animals. Peripheral blood was collected on 7th day for flow cytometry. Bonferroni test for multiple comparisons was used to compute *P*-values. NS, Not significant; ***P*<0.01, ****P*<0.001 vs. saline; **P*<0.05, ***P*<0.01 vs. SRBC.

Table 3
Effect of ARE on SRBC specific humoral and cellular immune responses.

Humoral responses			
Group	Dose (mg/kg)	Antibody titre (mean \pm S.E.)	(%) Modulatory Activity
Sensitized control	SRBC	6.4 \pm 0.24	NA
Levamisole	2.5	8.2 \pm 0.22***	28
Cyclophosphamide	250	3.7 \pm 0.26***	–38
ARE	25	6.6 \pm 0.41	3
ARE	50	7.0 \pm 0.20	9.3
ARE	100	7.6 \pm 0.40***	18.75
ARE	200	7.3 \pm 0.21**	14
Cellular (DTH)			
Group	Dose (mg/kg)	Foot paw thickness (mm) (mean \pm S.E.)	(%) Modulatory activity
Sensitized control	SRBC	0.9 \pm 0.01	NA
Levamisole	2.5	2.0 \pm 0.02***	122.22
Cyclosporin	5	0.5 \pm 0.01***	–44
ARE	25	0.9 \pm 0.11	0
ARE	50	1.1 \pm 0.15**	22.22
ARE	100	1.4 \pm 0.25***	55
ARE	200	1.1 \pm 0.17**	22

All values are shown as mean \pm S.E. Percent modulatory activity was calculated using formulae given under data analysis. *Denotes comparison with respective SRBC controls. *N* = 10; NA: not applicable. **P*<0.05 vs. respective controls.

** *P*<0.01 vs. respective controls.
*** *P*<0.001 vs. respective controls.

down-regulation of DTH response as compared to control (–44%, *P*<0.001).

4. Discussion and conclusions

Modulation of Th1/Th2 immunity is an important parameter to assess therapeutic efficacy of immunomodulators. Based on affinity towards Th1/Th2 subsets, immunomodulators are generally classified as Th1, Th2 or mixed Th1/Th2 agents. *A. racemosus* root aqueous extract is known to exhibit immunopharmacological activities under different biological stimuli. However, its efficacy towards Th1/Th2 immunity has not been investigated. The present study demonstrates that ARE has mixed Th1 and Th2 adjuvant properties.

T cells (CD3 positive cells) carry out specialized functions such as cytokine secretion and B cell help through CD4 (T-helper) and CD8 positive (cytotoxic T cells) cells (Abbas et al., 1996). Several studies have reported significant positive correlations between peripheral CD4/CD8 percentages and host protective cellular or humoral responses in immune compromised conditions such as cancer, AIDS and tuberculosis (Lucy Bird, 2004). In present study, ARE up-regulated CD3 and CD4/CD8 positive percentages in peripheral blood suggesting its immunoadjuvant potential (Table 1). This observation supports our previous study where ARE potentiated cellular and humoral responses in myelosuppressed tumor bearing animals (Diwanay et al., 2004). Further, adjuvant role of ARE in boosting protective immunity against pertussis is also reported (Gautam et al., 2004).

Based on antigenic/adjuvant stimulus, CD4 T cells differentiate into functionally distinct subsets known as Th1 or Th2 that can be identified by monitoring their signature cytokines. ARE was found to up-regulate IL-2, IFN-gamma (Th1) and IL-4 (Th2) cytokines (Table 2). This observation suggests ARE has mixed Th1 and Th2 adjuvant activity. Consistent to this, ARE showed potentiated humoral and DTH responses (Table 3). However, its relative immunomodulatory effect was found higher for Th2 as evidenced by significant reduction in IFN-gamma/IL-4 ratios, a commonly used index of Th1/Th2 immunity (Table 2) (Gamze et al., 2003). Thus, it may be suggested that ARE has mixed Th1/Th2, but Th2 preferential adjuvant activity. However, it will need additional studies for conclusive correlations. Our supposition support earlier studies where Shatavari produced higher anti-body titers, cytoprotection and increased host resistance to tumors in experimental models (Thatte and Dahanukar, 1988; Diwanay et al., 2004; Rao, 1981; Seena et al., 1993; Yun and Lee, 2005). Levamisole as expected showed generalized activation of Th1 and Th2 immunity; with higher Th1 preference (IFN-g/IL-4 ratio) that is consistent with its previous reports (Tables 1–3) (Szeto et al., 2000). Additionally, ARE showed significant proliferative effects on T and B lymphocytes in presence of antigenic stimulus, which further supports its adjuvant role to activated lymphocytes (Shive et al., 2000) (Figs. 1 and 2). Maximum tolerable dose of (2000 mg/(kg b.w.)) with no toxicological consequences suggest ARE is safe to use. It is thus reasonable that bioassay active guided fractionation and in-depth studies on selective activation of transcriptional factors favoring Th1 or Th2 cytokines might provide important information on the molecular mechanisms of such interaction. For instance, ginsenosides, closely related steroidal saponins, present in ginseng mediate immunomodulatory effects through different immune targets (Eui-joon et al., 2006). Present study concludes stimulatory effects of ARE on both Th1 and Th2 immunity.

In summary, our results demonstrate *in vivo* effects of ARE on effector T cell immunity and suggest its use in conditions where broader stimulation of Th1 and Th2 immunity is required (Whelan et al., 2003; Patwardhan and Gautam, 2005). Standardized extracts such as ARE may provide newer adjuvant moieties for safer modulation of host immunity (Patwardhan et al., 2004; Patwardhan, 2000; Jun-ling et al., 2006).

Acknowledgements

Authors are thankful to Regional Research Laboratory (IIIM), Jammu, Council of Scientific and Industrial Research, Government of India for extending the necessary facilities and infrastructure. MG, DP, SM, SG, KS, SSJ and BP are also grateful to Department of Science and Technology, Government of India and Serum Institute of India Ltd for project grant on development of botanical immunomodulators as adjuvants under Drugs and Pharmaceutical Research Program. Thanks to Drs Madhuri

Thakar, Kalpana Joshi and Abhay Jere, for critical comments on manuscript.

References

- Abbas, A.K., Murphy, K.M., Sher, A., 1996. Functional diversity of helper T lymphocytes. *Nature* 383, 787–793.
- Argani, H., Akhtari, E., 2006. Levamisole enhances immune responsiveness of intradermal and intramuscular hepatitis B vaccination in hemodialysis patients. *Journal of Immune Based Therapies and Vaccines* 4, 3.
- Ayurvedic Pharmacopoeia of India, 2001. *Ayurvedic Pharmacopoeia of India*, Govt of India, vol. 1., 1st ed. The Controller of Publications, Delhi, India, pp. 15–16.
- Bani, S., Kaul, A., Khan, B., Ahmad, S.F., Suri, K.A., Satti, N.K., Amina, M., Qazi, G.N., 2005. Immunosuppressive properties of an ethyl acetate fraction from *Euphorbia royleana*. *Journal of Ethnopharmacology* 99, 185–192.
- Bani, S., Gautam, M., Sheikh, F.A., Khan, B., Satti, N.K., Suri, K.A., Qazi, G.N., Patwardhan, B., 2006. Selective Th1 up-regulating activity of *Withania somnifera* aqueous extract in an experimental system using flow cytometry. *Journal of Ethnopharmacology* 107, 107–115.
- Bhatnagar, M., Sisodia, S.S., Bhatnagar, R., 2005. Antiulcer and antioxidant activity of *Asparagus racemosus* WILLD and *Withania somnifera* DUNAL in rats. *Annals of the New York Academy of Sciences* 1056, 261–278.
- Bozic, F., Bilic, V., Valpotic, I., 2003. Levamisole mucosal adjuvant activity for a live attenuated *Escherichia coli* oral vaccine in weaned pigs. *Journal of Veterinary Pharmacology and Therapeutics* 26, 225–231.
- Charak Samhita, 1970. Volume I–V. Chowkhamba Sanskrit Series Orientalia, Varanasi, India, 1970.
- CPCSEA, Government of India, 2003. Committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines for laboratory animal facility. *Indian Journal of Pharmacology* 35, 257–274.
- Dalvi, S.S., Nadkarni, P.M., Gupta, K.C., 1990. Effect of *Asparagus racemosus* on gastric emptying time in normal healthy volunteers. *Journal of Postgraduate Medicine* 36, 91–94.
- Dhuley, J.N., 1997. Effect of some Indian herbs on macrophage functions in ochratoxin A treated mice. *Journal of Ethnopharmacology* 58, 15–20.
- Diwanay, S., Chitre, D., Patwardhan, B., 2004. Immunoprotection by botanical drugs in cancer chemotherapy. *Journal of Ethnopharmacology* 90, 49–55.
- Doherty, N.S., 1981. Selective effects of immunosuppressive agents against delayed hypersensitivity response and humoral response to sheep red blood cells in mice. *Agents and Actions* 11, 237–242.
- Eui-joon, L., Eunjung, K., Jinwoo, L., Samwoong, R., Seonggyu, K., Min-Kyu, S., Byung-il, M., Moo-Chang, H., Si-young, K., Hyunsu, B., 2006. Ginsenoside Rg1 helps mice resist to disseminated candidiasis by Th1 type differentiation of CD4⁺ T cell. *International Immunopharmacology* 6, 1424–1430.
- Gamze, P., Cornelis, W.K., Daisy, P., Jan, D.B., Marcel, B.M.T., 2003. Ultraviolet-B irradiation decreases IFN- γ and increases IL-4 expression in psoriatic lesional skin *in situ* and in cultured dermal T cells derived from these lesions. *Experimental Dermatology* 12, 172–180.
- Gautam, M., Diwanay, S., Gairola, S., Shinde, Y., Patki, P., Patwardhan, B., 2004. Immunoadjuvant potential of *Asparagus racemosus* aqueous extract in experimental system. *Journal of Ethnopharmacology* 91, 251–255.
- Jun-ling, S., Yuan-liang, H., De-yun, W., Bao-kang, Z., Jia-guo, L., 2006. Immunologic enhancement of compound Chinese herbal medicinal ingredients and their efficacy comparison with compound Chinese herbal medicines. *Vaccine* 24, 2343–2348.
- Kaul, A., Bani, S., Zutshi, U., Suri, K., Satti, N.K., Suri, O.P., 2003. Immunopotentiating properties of *Cryptolepis buchanani* root extract. *Phytotherapy Research* 17, 1140–1144.
- Lucy Bird, 2004. HIV: getting to the bottom of CD4 T cell loss. *Nature Reviews Microbiology* 2, 853.
- Mashimo, J., Mita, A., 1995. *In vivo* production of various cytokines in splenocytes of sheep erythrocyte-immunized mice after intravenous administration of bacterial lipid A. *Microbiology and Immunology* 39, 169–175.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65, 56–63.
- Nelson, D.S., Mildenhall, P., 1967. Studies on cytophilic antibodies. The production by mice of macrophage cytophilic antibodies to sheep erythrocytes, relationship to the production of other antibodies and development of delayed type hypersensitivity. *Australian Journal of Experimental Biology and Medical Science* 45, 113–130.
- OECD, 1996. Organization for economic cooperation and development. OECD Guidelines for Testing of Chemicals. Guideline 423, Acute Oral Toxicity–Acute Toxic Class Method, Adopted, March 22.
- Parihar, M.S., Hemnani, T., 2004. Experimental excitotoxicity provokes oxidative damage in mice brain and attenuation by extract of *Asparagus racemosus*. *Journal of Neural Transmission* 111, 1–12.
- Patwardhan, B., 2000. Ayurveda: the designer medicine, review of ethnopharmacology and bioprospecting research. *Indian Drugs* 37, 213–227.
- Patwardhan, B., Gautam, M., 2005. Botanical immunodrugs: scope and opportunities. *Drug Discovery Today* 10, 495–502.
- Patwardhan, B., Vaidya, A.B.D., Chorghade, M., 2004. Ayurveda and natural products drug discovery. *Current Science* 86, 789–799.

- Rao, 1981. Inhibitory action of *Asparagus racemosus* on DMBA-induced mammary carcinogenesis in rats. *International Journal of Cancer* 28, 607–610.
- Rege, N.N., Nazareth, H.M., Isaac, A., Karandikar, S.M., Dahanukar, S.A., 1989. Immunotherapeutic modulation of intraperitoneal adhesions by *Asparagus racemosus*. *Journal of Postgraduate Medicine* 35, 199–203.
- Romagnani, S., 2000. T-cell subsets (Th1 versus Th2). *Annals of Allergy Asthma and Immunology* 85, 9–18.
- Satti, N.K., Suri, K.A., Dutt, P., Suri, O.P., Musrat, A., Qazi, G.N., 2006. Evaluation of *Asparagus racemosus* on the basis of immunomodulating sarasapogenin glycosides by HPTLC. *Journal of Liquid Chromatography and Related Technologies* 29, 119–227.
- Saxena, V.K., Chourasia, S., 2001. A new isoflavone from the roots of *Asparagus racemosus*. *Fitoterapia* 72, 307–309.
- Seena, K., Kuttan, G., Kuttan, R., 1993. Antitumor activity of selected plant extracts. *Amla Research Bulletin* 13, 41–45.
- Shive, C.L., Hofstetter, H., Arredondo, L., Shaw, C., Forsthuber, T.G., 2000. The enhanced antigen-specific production of cytokines induced by pertussis toxin is due to clonal expansion of T cells and not to altered effector functions of long-term memory cells. *European Journal of Immunology* 30, 242–331.
- Szeto, C.C., Gillespie, K.M., Mathieson, P.W., 2000. Levamisole induces interleukin-18 and shifts type 1/type 2-cytokine balance. *Immunology* 2, 217–224.
- Thatte, U.M., Dahanukar, S.A., 1988. Comparative study of immunomodulating activity of Indian Medicinal plants. Lithium carbonate and Glucan. *Methods and Findings in Experimental and Clinical Pharmacology* 10, 639–644.
- The United States Pharmacopeia, 2002. *Bacterial Endotoxins Test*. Rockville, United States, pp. 1889–1893.
- Warren, H.S., Vogel, F.R., Chedid, L.A., 1986. Current status of immunological adjuvants. *Annual Reviews in Immunology* 4, 369–388.
- Whelan, M., Whelan, J., Russell, N., Dalgleish, A., 2003. Cancer immunotherapy: an embarrassment of riches. *Drug Discovery Today* 8, 253–258.
- World Health Organization (WHO), 1998. *Quality control guidelines for medicinal plant materials*, p. 111.
- Yun, A.J., Lee, P.Y., 2005. The link between T helper balance and lymphoproliferative disease. *Medical Hypotheses* 65, 587–590.