6. Pontes, M. T., Aguiar, R. and Pires, H. O., A nearshore wave energy atlas for Portugal. *J. Offshore Mech. Eng.*, 2005, **127**, 249–255.
7. Rusu, E. and Guedes Soares, C., Numerical modelling to estimate the spatial distribution of the wave energy in the Portuguese nearshore. *Renew. Energy*, 2009, **34**, 1501–1516.
8. Cornett, A. M., Inventory of Canada's offshore wave energy resources. In Proceedings of the 25th International Conference on Offshore Mechanics and Arctic Engineering, Hamburg, Germany, 2006.
9. Wilson, J. and Beyene, A., California wave energy resource evaluation. *J. Coastal Res.*, 2007, **23**, 679–690.
10. Beels, C., Henriques, J., De Rouck, J., Pontes, M., De Backer, G. and Verhaeghe, H., Wave energy resource in the North Sea. In Proceedings of the 7th European Wave and Tidal Energy Conference, Porto, Portugal, 11–13 September 2007.
11. Wan Nik, W. B., Sulaiman, O. O., Rosliza, R., Prawoto, Y. and Muzathik, A. M., Wave energy resource assessment and review of the technologies. *Int. J. Energy Environ.*, 2011, **2**, 1101–1112.
12. Cornett, A. M., A global wave energy resource assessment. In 18th International Offshore and Polar Engineering Conference (ISOPE-2008), Vancouver, Canada, 6–11 July 2008.
13. Barstow, S. B. and Kollstad, T., Field trials of the directional waverider. In Proceedings of the First International Offshore and Polar Engineering Conference, Edinburgh, 1991, pp. 55–63.
14. Kuik, A. J., Ph van Vledder, G. and Holthuijsen, L. H., A method for the routine analysis of pitch-and-roll buoy wave data. *J. Phys. Oceanogr.*, 1988, **18**, 1020–1034.
15. Mørk, G., Barstow, S., Kabuth, A. and Pontes, M. T., Assessing the global wave energy potential. In Proceedings of OMAE2010, 29th International Conference on Ocean, Offshore Mechanics and Arctic Engineering Shanghai, China, 6–11 June 2010.
16. Boyle, G., *Renewable Energy: Power for a Sustainable Future*. Oxford University Press, UK, 2004, 2nd edn, p. 452.
17. Philip, S. C., Kumar, V. S., Johnson, G., Dora, G. U. and Vinayaraj, P., Interannual and seasonal variations in nearshore wave characteristics off Honnavar, west coast of India. *Curr. Sci.*, 2012, **103**, 286–292.
18. Johnson, G. *et al.*, Variations in swells along eastern Arabian Sea during the summer monsoon. *Open J. Mar. Sci.*, 2012, **2**, 43–50.
19. Joseph, P. S. and Baba, M., Linking of coastal wave energy utilization with coastal protection. *ARPN J. Sci. Technol.*, 2012, **2**, 169–174.
20. Barstow, S., Gunnar, M., Mollison, D. and Cruz, J., The wave energy resource. In *Ocean wave energy* (ed. Cruz, J.), Springer, Berlin, 2008, pp. 93–132.

ACKNOWLEDGEMENTS. We thank the Earth System Sciences Organization, Ministry of Earth Sciences, Government of India for financial support to conduct this research. We also thank the Director, CSIR-National Institute of Oceanography, Goa and the Director, Indian National Centre for Ocean Information System, Hyderabad for encouragement. This is CSIR-NIO contribution number 5359.

Received 26 January 2013; revised accepted 25 March 2013

Immunomodulatory constituents from *Annona squamosa* twigs provoke differential immune response in BALB/c mice

Vishal Kumar Soni[1], Manisha Pathak[1], Dinesh Kumar Yadav[2], Rakesh Maurya[2], Mahendra Sahai[3], Swatantra Kumar Jain[4] and Shailja Misra-Bhattacharya[1,*]

[1]Division of Parasitology, and
[2]Medicinal and Process Chemistry Division, Central Drug Research Institute, 10/1, Sector 10, Janakipuram Extension, Lucknow 226 001, India
[3]Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India
[4]Department of Biotechnology, Faculty of Science, Jamia Hamdard University, Hamdard Nagar, New Delhi 110 062, India

***Annona squamosa* (AS) has traditionally been used as ethnomedicine and various parts of the plant have been used to combat several disorders including dysentery, cancer and hyperthyroidism. Since the twig of this plant is reported to contain a large number of alkaloids, we chose to study its medicinal properties on the immune response of BALB/c mice. The present study, thus, aims at evaluation of immunomodulatory activity in the crude ethanolic extract and its four fractions, viz. hexane (F1), chloroform (F2), *n*-butanol (F3) and aqueous (F4) prepared from the twigs of AS to locate the active constituents in the fractions. The extract and fractions were fed orally at 3, 10 and 30 mg/kg for 14 consecutive days and mice were euthanized to assess various immune parameters. The ethanolic extract and its three fractions F2, F3 and F4 were found active since they increased splenic T and B cellular proliferation with a significant accentuation in peritoneal macrophage function, differentially increased the CD4+, CD8+ T lymphocytes and CD19+ B lymphocytes. The extract and its active fractions also demonstrated significant Th1 or Th2 mixed cytokine response at almost all doses tried in a dose-dependent manner. Its hexane fraction, however, could only induce reactive oxygen species production in peritoneal macrophages and could not induce lymphocytes; thus, it remained inactive. Thus, the activity could be localized distributed in its three fractions (chloroform, *n*-butanol and aqueous). Further purification and evaluation of the active molecule/s is underway in our laboratory.**

Keywords: *Annona squamosa*, flow cytometry, immunomodulatory activity, lymphocytes, reactive oxygen species.

INDIA is a rich biodiversity hotspot. It bears several plant species claimed to have traditional medicinal impor-

*For correspondence. (e-mail: shailja_cdri@rediffmail.com)

tance[1–3]. *Annona squamosa* (AS; Annonaceae) is one such plant commonly known as custard apple, sugar apple or sweetsop[4,5]. It is a native of Tropical America and the West Indies and is assumed to have been introduced before 1590 in southern India[6–8]. It bears a well-known edible fruit, the seeds of which have been reported to exhibit insecticidal and abortifacient properties[9]. The plant has traditional use against epilepsy, cardiac problems, constipation and ulcers[10]. The crushed leaves are sniffed to overcome hysteria, fainting spells and also applied on ulcers and wounds. The seed decoction is used as an enema, bark as a tonic and root has strong purgative action[9,11,12]. The plant contains a variety of compounds, e.g. monoterpenes[13], alkaloids[14,15], flavanoids[16], etc. and the extracts of leaves and stem are reported to have cancer[17] and hyperthyroidism ameliorating properties[18]. The plant also possesses hepato-protective activities[10,19]. In spite of the diverse biological properties in the leaves and fruits of AS we have chosen to study the twigs of this plant as they are reported to contain a large number of compounds[20], however, there is no report on their immunomodulatory activities. The twig was chosen to study its medicinal properties on the immune response of BALB/c mice. The present preliminary study has been attempted to assess the immunomodulatory activities of the ethanolic extract and its four fractions, namely hexane (F1), chloroform (F2), *n*-butanol (F3) and aqueous (F4) derived from the AS twigs by evaluating the immunological response of BALB/c mice after their oral administration. The immune response studies were *in vitro* T and B lymphocyte proliferation, cell-surface markers on T- and B-cells, measurement of Th1 (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-10), apart from macrophage activation in BALB/c mice.

The twigs of AS were collected from Lucknow, India. A voucher specimen number (4738) is kept in the investigators' laboratory.

Plant material (6.0 kg) was shade-dried, finely powdered and placed in a percolator with ethanol (18 l) to stand at room temperature for 48 h. Then the percolate was collected. This process of extraction was repeated four times, till the plant material was exhaustively extracted. The total percolate was collected, filtered and concentrated under vacuum using a rotavapor at 40–45°C, which yielded 520 g (8.66%) extract.

The ethanolic extract (500 g) was triturated with *n*-hexane (250 ml × 15); the hexane-soluble fraction (F1) was then concentrated (100 g) under reduced pressure at 40°C. The residue obtained after triturating with hexane was triturated with chloroform (250 ml × 15) and chloroform soluble fraction (F2) was concentrated (95 g) under reduced pressure. The residue obtained after triturating with chloroform was then suspended in distilled water (2 l) and fractionated with *n*-butanol saturated with water (500 ml × 10). *n*-Butanol-soluble fraction (F3) was concentrated (136 g) under vacuum at 50°C. Water-soluble fraction (F4) was also concentrated (168 g) under vacuum at 45–50°C.

Medium RPMI-1640 (Sigma, USA) containing phenol red was used for culturing splenocytes. The medium was fortified with 1% antibiotic–antimycotic cocktail (Sigma, USA), 10% foetal bovine serum (GIBCO, USA) and HEPES (Sigma, USA). 2′,7′-Dichlorofluorescin diacetate (DCF-DA) to quantitate reactive oxygen species (ROS) levels was also procured from Sigma, USA, whereas the remaining chemicals were of analytical grade available locally.

Inbred BALB/c mice (18–20 g, either sex) were randomly distributed in four separate groups (four groups each for extract and fractions) according to the experimental protocol (five mice in each group). The animals were housed under standard conditions of temperature (23° ± 1°C), relative humidity (55 ± 10%) and 12/12 h light/dark cycles at the National Laboratory Animal Centre (NLAC), Central Drug Research Institute (CDRI), Lucknow and fed with standard pellet diet and water *ad libitum*. All the animal handling and experimental protocols, including the number of animals employed in the present study were duly approved by CDRI Institutional Animal Ethics Committee (IAEC) vide its approval no. 38/08/PARA/IAEC dated 08.02.2008.

The ethanolic extract was prepared as a suspension in distilled water and administered orally at three doses of 3, 10 and 30 mg/kg to BALB/c mice for 14 consecutive days using canula. Control mice received water only as vehicle under identical conditions. At the end of treatment, the animals were euthanized humanely to collect splenocytes and peritoneal macrophages to determine the macrophage function (ROS production), T- and B-cell proliferation, T- and B-cell surface markers (CD4, CD8 and CD19) and measurement of Th1/Th2 cytokines. Once the extract was found active, it was further fractionated into F1, F2, F3 and F4, which were also administered and assessed using the same doses and method as described above.

ROS in peritoneal macrophage cells were determined through a fluorometric assay using DCF-DA on flow cytometer (FACS Calibur, Becton Dickinson or BD, USA), according to the protocol of Zurgil *et al.*[21] after minor modifications[22]. Briefly, freshly harvested macrophage cells of both treated and untreated groups were adjusted to a concentration of 1×10^6 cells/ml in phosphate buffer saline (PBS), washed with PBS × 3 and transferred to FACS tubes (1×10^6 cells/tube). For probe-loading, the cells were incubated with the DCF-DA at a final concentration of 1 μM, for 15 min at 37°C, and then washed twice in PBS. ROS levels in individual living cells were determined by measuring their fluorescence intensity on FACS Calibur (BD Biosciences, USA). Data were analysed using CellQuest software and mean ROS values were evaluated for cell populations.

Splenic T and B lymphocyte proliferative responses of treated and untreated mice were determined in triplicate

wells of 96-well flat bottom culture plates (NUNC) after stimulation with an optimal concentration (2.5 μg/ml) of concavaline-A (Con-A) (Sigma, USA) and lipopolysaccharide (LPS, Sigma, USA) at 37°C for 48 h in 5% CO_2 in air. Cells were harvested after 16 h of pulsing with [3H]-thymidine (0.5 μCi/well) and radioactive incorporation was measured on β-counter (Beckman coulter) to evaluate stimulation indices[23].

Immunophenotyping was carried out to assess lymphocyte subset population by flow cytometry on FACS Calibur using fluorochrome conjugated monoclonal antibodies (BD, USA) directed against different cell-surface CD antigens, CD4 (fluorescein isothiocyanate or FITC), CD8 (phycoerythrin or PE) and CD19 (FITC) following the manufacturer's protocol[24]. Splenocyte suspensions were prepared from spleens of treated and untreated (control) mice on day-15 after commencement of treatment. Initially 1×10^6 cells were blocked with mouse seroblock FcR at room temperature (RT) for 10 min, washed and labelled with rat anti-mouse CD4+ (FITC) antibodies for 10 min at RT for further incubation with rat anti-mouse CD8+ (PE) antibodies for another 10 min. The third tube served as control with no labelling. Cell pellet was suspended in sheath fluid and analysed on FACS using CellQuest analysis software (Becton-Dickinson, USA) after gating the forward- and side-scatter settings to exclude debris. For each determination, 10,000 cells were analysed and the results expressed as percentage of each cell population.

The measurement of intracellular cytokines in the splenocytes was done according to the manufacturer's instructions using antibodies (BD, USA) and reagents (leukoperm; Serotec, UK). Briefly, splenocytes (4×10^6/ml) were incubated with brefeldin A (10 μg/ml) in dark for 6 h in CO_2 incubator at 37°C. Cells were reincubated with mouse Seroblock FcR for another 10 min and washed in PBS. Now FITC-rat anti-mouse CD4+ antibody was added to the cells. Leucoperm A and leucoperm B (Serotech, UK) were added at RT for 15 min each and cells were dispensed in four tubes each containing 1×10^6 cells/100 μl PBS. PE-rat anti-mouse monoclonal antibodies to cytokines IL-2, IL-4, IL-10 and IFN-γ were added to separate tubes; cells were washed and finally suspended in 250 μl of PBS containing 0.5% para formaldehyde for FACS readings.

Statistical analyses were carried out by employing the Student's *t*-test and analysis of variance by one-way ANOVA (Dunnett's multiple comparison test). Data have been expressed as the mean ± standard error (SE) and a conventional $P < 0.05$ (*) was taken as evidence of significant differences and $P < 0.01$ (**) was considered as highly significant, and $P > 0.05$ was considered as not significant.

Significant induction in ROS generation was observed in the peritoneal macrophages isolated from mice after treatment with crude ethanolic extract with high significance ($P < 0.01$) at all the three doses in a dose-dependent manner (Figure 1). The three fractions (F2, F3 and F4) also showed a dose-dependent increase, but the increase was highly significant at higher doses. In contrast, hexane fraction (F1) downregulated the oxidative burst (Figure 1).

Treatment of mice with AS ethanolic extract led to a dose-dependent increase in B ($P < 0.01$; Figure 2 *a*) and

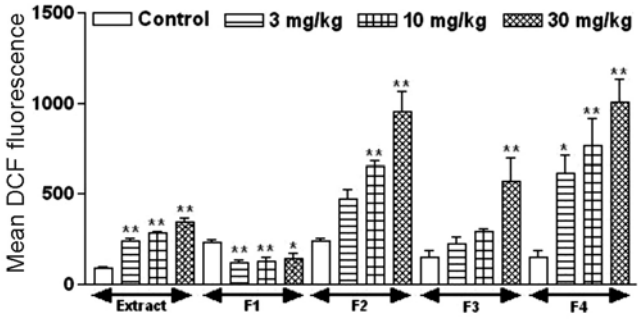

Figure 1. Macrophages were collected from the peritoneal cavity of BALB/c mice fed orally with ethanolic extract, hexane (F1), chloroform (F2), *n*-butanol (F3) and aqueous (F4) fractions (each at 3, 10 and 30 mg/kg) of *Annona squamosa* twigs, and untreated control mice. Cells were washed with phosphate buffer saline and adjusted to a concentration of 1×10^6 cells/ml. Reactive oxygen species levels were determined using fluorescent probe 2′,7′-dichloroflourscein diacetate and fluorescence intensity measured on FACS Calibur.

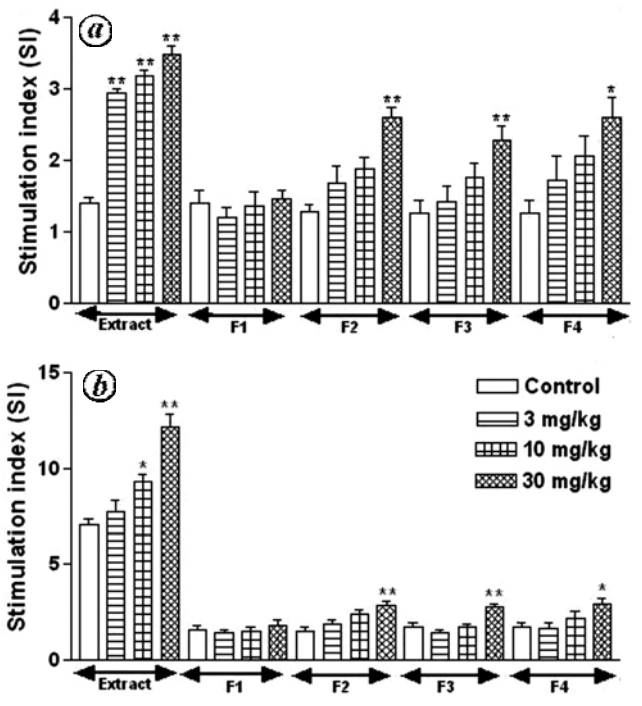

Figure 2. Effect of *A. squamosa* ethanolic extract and its hexane (F1), chloroform (F2), *n*-butanol (F3) and aqueous (F4) fractions (each at 3, 10 and 30 mg/kg) on *in vitro* splenic B (***a***) and T (***b***) lymphocyte proliferation. B-/T-cell mitogens lipopolysaccharide (***a***) and concanavaline-A (***b***) were added at an optimal concentration of 2.5 μg/ml and the results are expressed as stimulation indices (SI). Untreated control mice received vehicle under identical conditions.

T ($P < 0.05$ to $P < 0.01$; Figure 2 *b*) lymphocyte proliferation *in vitro* in the presence of nonspecific B-cell (LPS) and T-cell (Con A) mitogens ($P < 0.01$). F2, F3 and F4 also showed a dose-dependent increase in cellular proliferation (Figure 2). However, the data were significant ($P < 0.01$) only at 30 mg/kg. F1 did not cause cellular proliferation in splenic lymphocytes (Figure 2).

There was a dose-dependent upregulation in CD4+ T-cell population in crude extract and F2 (chloroform) fraction-treated mice, with the values being significant at the highest dose of 30 mg/kg F1 (hexane) and F3 (butanol) did not significantly influence CD4+ cells, whereas F4 (aqueous) showed marginal downregulation though statistically insignificant (Figure 3 *a*). Barring F1 (hexane), all other fractions stimulated a significant ($P < 0.01$) dose-dependent increase in the CD8+ cell population, which was comparatively much better than the crude ethanol extract (Figure 3 *b*). On the other hand, CD19+ cells (B-cells) demonstrated a significant expansion in crude ethanol and F2 (chloroform) fraction-treated mice. However, a decreasing trend was noticed as the dose increased, though still being statistically higher than the untreated control values at 3 and 10 mg/kg doses (Figure 3 *c*). The other fractions did not have any noticeable effect on the B-cells.

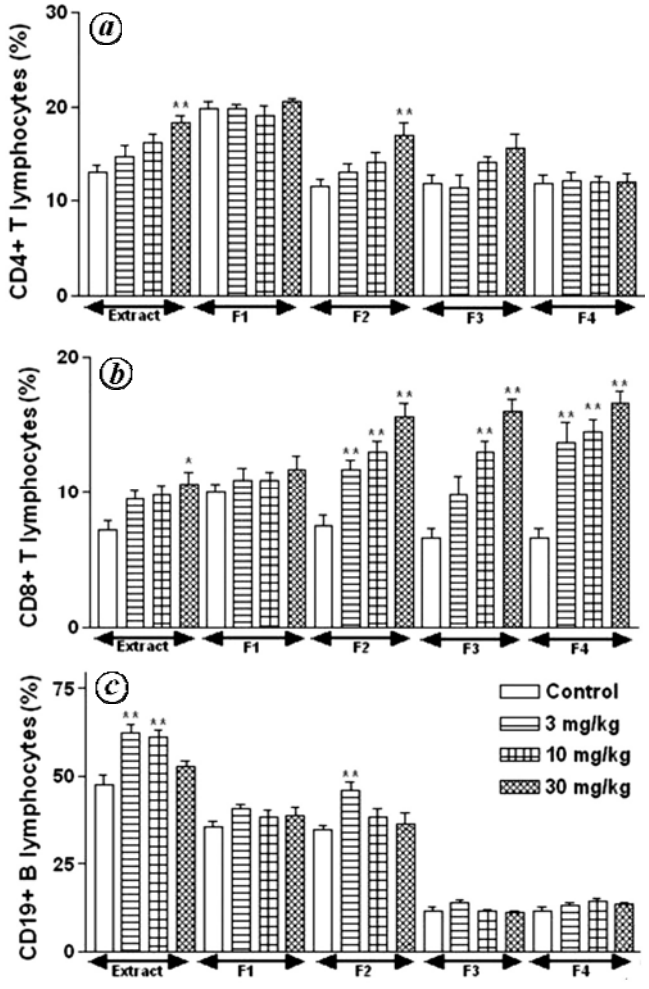

Figure 3. Flow cytometric measurements of splenic CD4+ (***a***) and CD8+ (***b***) T-cell sub-populations and CD19+ (***c***) B-cell populations in mice fed with various doses (3, 10 and 30 mg/kg) of *A. squamosa* ethanolic extract and fractions (hexane (F1), chloroform (F2), *n*-butanol (F3) and aqueous (F4)). Untreated control mice received vehicle under identical conditions.

The proinflammatory Th1 cytokine contents (IL-2 and IFN-γ) were found to increase intracellularly in the splenic cell population after treatment of mice with the crude extract and F2 (chloroform) fraction in contrast to their downregulation in F3 (butanol) and F4 (aqueous) fractions (Figure 4 *a* and *b*). The Th2 (anti-inflammatory) cytokines (IL-4 and IL-10) contents, however, were observed to increase in the crude extract, F2 (chloroform), F3 (butanol) and F4 (aqueous) fractions. Nevertheless, these followed a dose-dependent decreasing pattern in spite of being higher than that of untreated control (Figure 4 *c* and *d*). The F1 (hexane) fraction influenced neither Th1 nor Th2 cytokine responses.

Table 1 depicts the fold increase in each immune parameter to clearly display the action of crude ethanolic extract and its four chromatographic fractions on different immunological responses in BALB/c mice. Table 1 also shows that the crude extract along with the three fractions (F2, F3, F4) stimulates both T- and B-cell proliferation. All the fractions, except F1 (hexane) stimulate CD8+ T-cell. Both the crude extract as well as chloroform fraction stimulate the production of both Th1 and Th2 cytokines, while butanol (F3) and aqueous (F4) fractions principally induce the production of Th2 cytokines.

Several plant extracts in the past have been shown to modulate the host immune system[3,22,25,26]. AS is one such plant which has various traditional uses against epilepsy, cardiac problems, constipation, ulcers[10], hysteria, fainting spells, cancer[17] and hyperthyroidism[18]. The fruit of this plant contains high sugar content and the twig is reported to contain a large number of alkaloids[20]. The extraction and bioactivity-guided fractionation of the twigs yielded four fractions: the hexane, chloroform, *n*-butanol and aqueous fractions which were subsequently exploited for immunomodulatory activity *in vivo* in BALB/c mice. The crude ethanolic extract of AS twig and its three fractions (chloroform, butanol and aqueous) were found to be highly efficient in augmenting both cellular and humoral immune responses by stimulating the proliferation of T and B lymphocytes as also the simultaneous activation of antigen-presenting cells. One of the four fractions, hexane, did not show any immune involvement and all the immune parameters studied remained unaffected by this fraction. The crude ethanolic extract demonstrated immunostimulating characteristics closer to the chloroform fraction. The immunostimulation appeared to be mostly dose-dependent. The highest oxidative burst was caused by aqueous fraction which was dose-dependent, followed by chloroform and crude ethanol extract. All the three

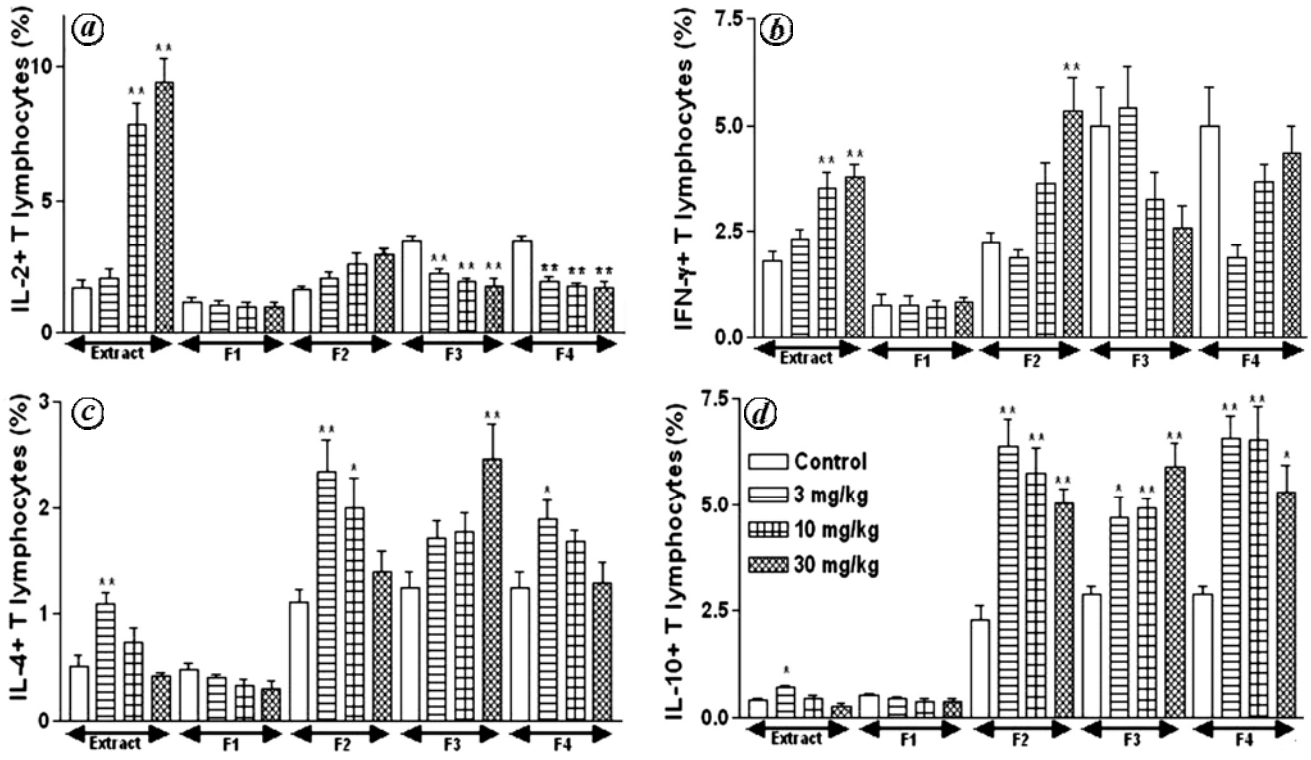

Figure 4. Flow cytometric detection of intracellular type-1 cytokines IL-2 (***a***), IFN-γ (***b***) and type-2 cytokines IL-4 (***c***) and IL-10 (***d***) in splenocytes of mice fed with ethanolic extract and fractions (hexane (F1), chloroform (F2), *n*-butanol (F3) and aqueous (F4)) of *A. squamosa* twigs at various doses (3, 10 and 30 mg/kg). Untreated control mice received vehicle under identical conditions.

Table 1. Fold increase in immune parameters of *Annona squamosa* ethanolic extract and its four fractions (hexane, chloroform, *n*-butanol and aqueous) treated BALB/c mice compared to their respective untreated groups after a 14 days oral dose schedule

	Annona squamosa EtOH extract dose (mg/kg)			Hexane fraction (F1) dose (mg/kg)			Chloroform fraction (F2) dose (mg/kg)			*n*-Butanol fraction (F3) dose (mg/kg)			Aqueous fraction (F4) dose (mg/kg)		
Parameters	3	10	30	3	10	30	3	10	30	3	10	30	3	10	30
Reactive oxygen species synthesis by activated macrophages	**2.623**	**3.153**	**3.797**	0.511	0.554	0.619	1.969	**2.707**	**3.952**	1.484	1.891	**3.699**	**4.038**	**4.978**	**6.553**
B-cell	**2.114**	**2.282**	**2.504**	0.854	0.971	1.043	1.305	1.461	**2.015**	1.133	1.399	1.816	1.375	1.647	**2.070**
T-cell	1.103	1.322	1.727	0.910	0.971	1.181	1.257	1.597	1.867	0.823	0.975	1.604	0.957	1.247	1.701
CD4	1.13	1.241	1.405	1.003	0.961	1.037	1.135	1.220	1.466	0.969	1.197	1.319	1.024	1.008	1.020
CD8	1.327	1.373	1.478	1.085	1.087	1.165	1.562	1.735	**2.082**	1.761	**2.331**	**2.871**	**2.083**	**2.202**	**2.525**
CD19	1.309	1.287	1.106	1.148	1.083	1.093	1.329	1.107	1.054	1.206	0.987	0.962	1.129	1.217	1.148
IL-2	1.194	**4.610**	**5.549**	0.883	0.830	0.874	1.252	1.608	1.836	0.640	0.552	0.495	0.552	0.504	0.486
IL-4	**2.124**	1.428	0.822	0.848	0.679	0.630	**2.121**	1.801	1.266	1.371	1.418	1.976	1.514	1.349	1.038
IL-10	1.794	1.103	0.684	0.885	0.683	0.686	**2.778**	**2.499**	**2.193**	1.630	1.712	**2.049**	**2.278**	**2.269**	1.836
IFN-γ	1.283	1.954	**2.101**	1.013	0.923	1.074	0.846	1.634	**2.388**	1.080	0.657	0.522	0.375	0.737	0.876

The increases at least two times or more are indicated in bold font.

fractions along with the crude extract possessed lymphocyte proliferative capability in the presence of non-specific mitogens. Once the crude ethanolic extract showed strong immune stimulating potential, it was further fractionated and the fractions were also bioevaluated to localize the activity. The results are summarized in Table 1 to demonstrate fold increase or decrease in the various immune parameters over controls. The cellular immune response plays an important role in the host defence against intracellular pathogens by limiting replication and accelerating clearance of infected cells, and in the generation of both humoral and cell-mediated responses. Macrophages play an important role in the non-specific defence mechanism against host infection and the

killing of tumour cells. The enhanced phagocytic activity of activated macrophages by various biological response modifiers is accompanied by the production of ROS which are involved in killing and digesting microbial pathogens[27,28]. The AS extract and its three fractions increased the oxidative burst in peritoneal macrophages, thereby indicating AS to be a potent stimulant of naïve immune response. A marginal increase in the CD4+ T-cells by the crude extract and chloroform fraction shows the augmentation of T helper cell activity. CD8+ or T cytotoxic (Tc)-cells were stimulated by all the three active fractions. Tc-cells are responsible for killing a tumour cell or a virus-infected cell. In the present study, significant upregulation of the CD4, CD8 and CD19-positive cell populations was observed after AS and F2 treatments. CD19 is a cell surface marker of B-cells which are responsible for humoral immune response against the foreign antigens. Humoral immune response is mediated by antibodies secreted by plasma cells (effector B-cells), which function by neutralizing extracellular microbial toxins. The increased population of CD19+ cells after treatment with the crude ethanol extract and its chloroform fraction thus may be correlated with the increased humoral activity in the host. F1, F3 and F4 could not stimulate the CD4+ and CD19+ cells significantly. However, F2, F3 and F4 significantly upregulated the CD8+ T-cells indicating their support for humoral immune response. After activation, CD4+ T-cells can be subdivided into either Th1-type cells that secrete IL-2 or IFN-γ in mice, or Th2-type cells that secrete IL-4 or IL-10 (ref. 29). In the above study AS and F2 significantly stimulated the production of pro-inflammatory cytokines IL-2 and IFN-γ at increasing doses and anti-inflammatory cytokines IL-4 and IL-10 at decreasing doses, demonstrating induction of both Th1 and Th2 T_H-cell subpopulations and thus may be useful for treating various Th1 or Th2-dominant pathological disorders. It was interesting to observe that maximum stimulation of Th2 cytokines was observed at the lowest dose tried, i.e. 3 mg/kg and the doses higher than this led to continuous and sustained decrease in the Th2 cytokine levels, be it IL-4 or IL-10. This phenomenon correlated well with the CD19+ population, where similar trend was noticed with regard to dose. It appears that doses lower than 3 mg/kg, if used, may have resulted in higher Th2 stimulation. These results demonstrate that the selection of dose of immunostimulant is crucial in mounting desired Th1- or Th2 arm of helper immune response. Thus the findings indicate that AS contains molecules which act via both Th1 and/or Th2 biased pathway and dose selection would be the determining factor for polarization of the type of cytokine secretion. The F3 and F4 fractions upregulated the Th2 cytokine response with concomitant downregulation of the Th1 cytokines, supporting the presence of both Th1 and Th2 stimulating as well as downregulating active principles in AS twigs.

The hexane fraction did not possess any immune regulatory properties; this could be due to absence of immunomodulatory constituents in this fraction. Based on the comparative fold increase data, F2 appears to be the most effective fraction as it stimulated almost all the immunological responses included in the present study. The active molecules need to be isolated and explored further to localize the Th1 or Th2 or mixed T-helper cell response, which would help in further exploitation of the active molecules either against intracellular or extracellular pathogens as chemotherapy adjunct or as immunoprophylactant, or to boost specific immune machinery of the immunocompromized host after careful escalation of the proper dose.

Thus, the findings of the present study indicate that ethanolic extract of AS twigs and its three fractions (F2–F4) stimulate proliferation of splenic T and B lymphocytes in the form of increased T- and B-cell populations, including increased CD4, CD8 and CD19-positive cells and also stimulate peritoneal macrophages to produce greater amount of ROS. They also regulate the Th1 and Th2 cytokines based upon the dose. The present study supports the traditional claim of ethnomedicinal use of AS and suggests that ethanolic extract and its three fractions (F2–F4) derived from the twigs have therapeutic potential and could serve as an effective immunomodulatory candidate and may stimulate both cellular and humoral immune responses and therefore, molecules responsible for the above activity may be further studied. Further studies on the localization of bioactivity in the pure molecules, the molecular mechanism of immunostimulation, are warranted to establish the therapeutic potential of AS for the prevention of immune disorders and infectious/parasitic diseases, where infections are often associated with immunosuppression.

1. Atal, C. K., Sharma, M. L., Kaul, A. and Khajuria, A., Immunomodulating agents of plant origin. I: Preliminary screening. *J. Ethnopharmacol*., 1986, **18**, 133–141.
2. Puri, A., Sahai, R., Singh, K. L., Saxena, R. P., Tandon, J. S. and Saxena, K. C., Immunostimulant activity of dry fruits and plant materials used in Indian traditional medical system for mothers after child birth and invalids. *J. Ethnopharmacol*., 2000, **71**, 89–92.
3. Pathak, M., Bano, N., Dixit, P., Soni, V. K., Kumar, P., Maurya, R. and Misra-Bhattacharya, S., Immunosuppressive activity of hexane and ethanolic extracts of *Pterospermum acerifolium* seeds in BALB/c mice. *Med. Chem. Res*., 2011, **20**, 1667–1673.
4. Yu-Liang, Y., Kuo-Feng, H., Pei-Hsuan, C., Shih-Hsiung, W, Kuen-Yuh, W., Fang-Rong, C. and Yang-Chang, W., New cyclic peptides from the seeds of *Annona squamosa* L. and their anti-inflammatory activities. *J. Agric. Food Chem*., 2008, **56**, 386–392.
5. Ranjan, R. and Sahai, M., Coumarinolignans from the seeds of *Annona squamosa* Linn. *E-J. Chem.*, 2009, **6**, 518–522.
6. Kirtikar, K. R. and Basu, B. R., *Indian Medicinal Plants I*, L M Basu Publishers, Allahabad, 1984, vols I–V, p. 66.
7. Morton, J. F., Sugar apple. In *Fruits of Warm Climates*, Miami, USA, 1987, pp. 69–72.

8. *The Wealth of India. Raw Materials, A Dictionary of Indian Raw Materials and Industrial Products*, Council of Scientific and Industrial Research, New Delhi, 1984, vol. 1, p. 80.
9. Chopra, R. N., Nayar, S. L. and Chopra, I. C. (eds), *Glossary of Indian Medicinal Plants*, CSIR, New Delhi, 1956, p. 20.
10. Shirwaikar, A., Rajendran, K. and Kumar, C. D., *In vitro* antioxidant studies of *Annona squamosa* Linn. Leaves I. *J. Exp. Biol*., 2004, **42**, 803–807.
11. Quisumbing, E., *Medicinal Plants of Philippines*, Bureau of Printing, Manila, 1951, pp. 310–311.
12. Chopra, R. N., Chopra, I. C. and Varma, B. S., *Glossary of Indian Medicinal Plants*, CSIR, New Delhi, 1969, p. 6.
13. Fransworth, N. R., Henry, L. K., Svoboda, G. H., Blomster, R. N., Yates, M. J. and Euler, K. L., Biological and phytochemical evaluation of plants. I: Biological test procedures and results from 200 accessions. *Lloydia*, 1966, **29**, 101.
14. O'Farrell, P. H., High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem*., 1975, **25**, 4007–4021.
15. Mukhlesur, R. M., Parvin, S., Ekramul, H. M., Ekramul, I. M. and Mosaddik, M. A., Antimicrobial and cytotoxic constituents from the seeds of *Annona squamosa*. *Fitoterapia*, 2005, **76**, 484–489.
16. Kotkar, H. M., Mendki, P. S., Sadan, S. V. G. S., Jha, S. R., Upasani, S. M. and Maheshwari, V. L., Antimicrobial and pesticidal activity of partially purified flavonoids of *Annona squamosa*. *Pest. Manage. Sci*., 2002, **58**, 33–37.
17. Bhakuni, D. S., Dhar, M. L., Dhar, M. M., Dhawan, B. N. and Mehrotra, B. B., Screening of Indian plants for biological activity: Part-II. *Indian J. Exp. Biol.*, 1969, **7**, 250–262.
18. Sunanda, P. and Anand, K., Possible amelioration of hyperthyroidism by the leaf extract of *Annona squamosa*. *Curr. Sci*., 2003, **84**, 1402–1404.
19. Mohamed, S. T. S., Christina, A. J. M., Chidambaranathan, N., Ravi, V. and Gauthaman, K., Hepatoprotective activity of *Annona squamosa* Linn. on experimental animal model. *Int. J. Appl. Res. Nat. Prod.*, 2008, **1**, 1–7.
20. Yadav, D. K., Singh, N., Dev, K., Sharma, R., Sahai, M., Palit, G. and Maurya, R., Anti-ulcer constituents of *Annona squamosa* twigs. *Fitoterapia*, 2011, **82**, 666–675.
21. Zurgil, N., Shafran, Y., Afrimzon, E., Fixler, D., Shainberg, A. and Deutsch, M., Concomitant real-time monitoring of intracellular reactive oxygen species and mitochondrial membrane potential in individual living promonocytic cells. *J. Immunol. Methods*, 2006, **20**, 27–41.
22. Singh, M., Shakya, S., Soni, V. K., Dangi, A., Kumar, N. and Bhattacharya, S. M., The *n*-hexane and chloroform fractions of *Piper betle* L. trigger different arms of immune responses in BALB/c mice. *Int. Immunopharmacol*., 2009, **9**, 716–728.
23. Misra, S., Mukherjee, M., Dikshit, M. and Chatterjee, R. K., Cellular immune response of gerbils and mastomys in experimental filariasis. *Trop. Med. Int. Health*, 1998, **3**, 124–129.
24. Vedi, S., Dangi, A., Hajela, K. and Misra-Bhattacharya, S., Vaccination with 73 kDa recombinant heavy chain myosin generates high level of protection against *Brugia malayi* challenge in jird and mastomys models. *Vaccine*, 2008, **26**, 5997–6005.
25. Shukla, S., Mehta, A., John, J., Mehta, P., Vyas, S. P. and Shukla, S., Immunomodulatory activities of the ethanolic extract of *Caesalpinia bonducella* seeds. *J. Ethnopharmacol*., 2009, **125**, 252–256.
26. Yu, L., Zhao, M., Yang, B. and Bai, W., Immunomodulatory and anticancer activities of phenolics from *Garcinia mangostana* fruit pericarp. *Food Chem*., 2009, **116**, 969–973.
27. Rainard, P., A colorimetric microassay for opsonins by reduction of NBT in phagocytosing bovine polymorphs. *J. Immunol. Methods*, 1986, **90**, 197–201.
28. Kang, N. S., Park, S. Y., Lee, K. R., Lee, S. M., Lee, B. G., Shin, D. H. and Pyo, S., Modulation of macrophage function activity by ethanolic extract of larvae of *Holotrichia diomphalia*. *J. Ethnopharmacol*., 2002, **79**, 89–94.
29. Kidd, P., Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern. Med. Rev.*, 2003, **8**, 223–246.

ACKNOWLEDGEMENTS. Financial assistance in the form of research fellowships to D.K.Y. by the Council of Scientific and Industrial Research, New Delhi, and V.K.S. and M.P. by the Indian Council of Medical Research, New Delhi, is acknowledged. We thank A. L. Vishwakarma and Madhu Chaturvedi (CDRI, Lucknow) for help in acquiring and analysing FACS data. This manuscript bears CDRI communication no. 8432.

Received 16 June 2011; revised accepted 18 March 2013