

**IN VIVO AND IN VITRO IMMUNOMODULATORY EFFECTS OF INDIAN
AYURVEDIC HERBAL FORMULATION TRIPHALA ON EXPERIMENTAL
INDUCED INFLAMMATION**

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Summary

In the present study, an attempt has been made to evaluate the immunomodulatory effects of the Indian ayurvedic herbal formulation Triphala on experimental induced inflammation. The effect of Triphala was investigated on complement activity, humoral immune response, and cell mediated immune response in mice, and in mitogen (phytohemagglutinin)-induced T-lymphocyte proliferation *in vitro*. Triphala administration significantly inhibited the complement activity, humoral and cell mediated immune response (delayed type hypersensitivity reaction (DTH)), and mitogen (phytohaemagglutinin)-induced T-lymphocyte proliferation in a dose dependent manner. These observations suggest that Triphala caused immunosuppression in experimental-induced inflammation, indicating that they may provide an alternative approach to the treatment of inflammatory and autoimmune diseases.

Keywords: *Triphala; complement; humoral and cell mediated immune response*

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Introduction

Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions (1). With the objective to expand the therapeutic resources within nature, we need to understand the logic that governs the clinical or biological activity of various agents. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defence mechanism has to be activated under the conditions of impaired immune responsiveness. Ayurveda, the Indian traditional health care system (ayus-life, veda-knowledge, meaning the science of life) is the oldest medical system in the world, which exploits the potential of various herbs generally in polyherbal formulations as drugs (2).

Triphala is the most commonly used Indian ayurveda herbal formulation, comprising the fruits of three trees, Indian goose berry (*Emblica officinalis* Gaertn, family-Euphobiaceae), Belleric myrobalan (*Terminalia bellerica* Linn, family-Combretaceae), and Chebulic myrobalan (*Terminalia chebula* Retzr, family-Combretaceae). Triphala has been reported to be a rich source of Vitamin C, ellagic acid, gallic acid, chebulinic acid, bellericanin, β -sitosterol and flavanoids (3). Its components *Emblica officinalis*, *Terminalia bellerica*, *Terminalia chebula* are reported to possess anti-inflammatory, antimutagenic, antioxidant, cytoprotective, gastroprotective activity, myocardial necrosis, hepatoprotective, antibacterial, hypolipidemic, and anticancer activity (4), (5), (6). Our preliminary studies confirm its anti-inflammatory and lysosomal membrane stabilizing effect on adjuvant-induced arthritis in mice (7). In the present study, we primarily explored the underlying mechanism of Triphala's anti-inflammatory activity by evaluating the immunomodulatory effects of Triphala on complement activity, lymphocyte proliferation, cellular immunity, and humoral immunity in mice.

Materials and Methods

Animals

The study was performed with Swiss albino mice, 25-30g, of either sex. The mice were brought from the Tamil Nadu Veterinary College, Chennai, India. The mice were acclimatized for a week in a light and temperature-controlled room with a 12 hr dark-light cycle. The mice were fed commercial pelleted feed from Hindustan Lever Ltd. (Mumbai, India) and water was made freely available. The animals used in this study were treated and cared for in accordance to the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Culture, Chennai. Experimental protocol was approved by the ethical committee of our department.

Drug

The commercially available Triphala powder (mixture of dried and powdered fruits of three plants, *T. chebula*, *E. officinalis* and *T. bellerica* in equal proportions (1:1:1)) was obtained from Indian Medical Practitioners Co-operative Stores and Society (IMCOPS), Adyar, Chennai, India and its aqueous suspension in 2% gum acacia was used.

The Triphala powder used in this study was found to contain approximately 50% polyphenols as investigated by High Performance Thin Layer Chromatography (HPTLC) densitometer analysis (7). All other reagents used were standard laboratory reagents of analytical grade and were purchased locally.

Anti-complement activity

Induction of arthritis

Two groups each containing six mice, were inoculated with 0.1 ml of Complete Freund's Adjuvant (CFA) in the right hind paw (7). The adjuvant (Tuberculosis Research Center, Chennai) contained dry heat killed *Mycobacterium tuberculosis* in sterile paraffin oil (10 mg/ml). Triphala was orally administered (500/1000mg/kg/b.wt) by gavage needle, 1 h before induction of adjuvant, then daily for 5 days to the one group of mice whereas the other group of six mice served as control group.

Complement test

The mice were bled immediately before and after the administration of Triphala and adjuvant, and thereafter, bled daily for five days. Complement activity and immunohaemolysing effect of test samples via the classical pathway were determined spectrophotometrically (8).

Veronal buffer (25 mM, pH 7.3, containing 0.15 mM Ca²⁺ and 0.5 mM Mg²⁺) was used as diluent in the complement assay and mouse serum was used as a source of complement. Sensitized sheep erythrocytes were incubated with the serum (complement) of treated and control mice, respectively. Degree of haemolysis was determined spectrophotometrically at 413 nm.

Lymphocyte proliferation assay

Heparinized human peripheral blood (20 ml) was obtained from a healthy donor. The blood was centrifuged at 2500 rpm, 4 °C for 10 min to remove plasma. Blood cells were then diluted with PBS buffer and centrifuged in a Ficoll-Hypaque discontinuous gradient at 1500 rpm in room temperature for 30 min. Human mononuclear cells (HMNC) layer was collected and washed with cold distilled H₂O and 10× Hank's buffer saline solution to remove red blood cells (9). Under sterile conditions, the cells were diluted to 2×10⁶ cells per ml RPMI 1640 (supplemented with 10% FCS) and dispensed in 96-wells flat bottom microtitre tissue culture plates (50 µl per well) (Tarson, India). The cells were then co-cultured in triplicate with or without Triphala (50/100µg/ml) and phytohemagglutinin (PHA), a T lymphocyte activator (5µg/mL). The viability of cells was measured by trypan blue dye exclusion method. After 4-day incubation at 37°C, T-cell proliferation was determined using a modified colorimetric MTT assay (10). Twenty micro liters of MTT solution (5 mg/mL in RPM 1640 medium) was added and incubated for another 4 h under the same conditions for the dye to be metabolized. The plates were centrifuged (500 × g, 10 min); the supernatant was removed and 100 µL of DMSO was added under agitation. The plates were read on an ELISA reader at 450 nm wavelength after keeping at room temperature for 15 min.

Humoral antibody response

The mice were intraperitoneally immunized with 0.2 ml of SRBC (5×10^9 cells/ml) on day zero according to the technique described by Subramoniam et al (11). Triphala (500/1000 mg/kg b.wt) was administered orally by gavage needle on days - 3, - 2, - 1, 0, + 1, + 2 and + 3. Blood samples were collected from individual animals from the orbital plexus on day 7. Two fold dilutions of sera were performed in 0.15 phosphate buffered saline (pH 7.2) and 50 μ l of each dilution was aliquoted into 96-well microtitre plates. A 25 μ l of fresh 1% SRBC suspension in the above buffer was dispersed into each well and mixed. The plates were incubated at 37 °C for 2 h and examined visually for agglutination. The value of the highest serum dilution causing haemagglutination was taken as the antibody titre. Antibody titres were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1, and the mean ranks of different groups were compared for statistical significance. Each experimental group contains 6 animals.

Delayed-type hypersensitivity response (DTH)

The effect of the Triphala on the antigen specific cellular immune response in experimental animals was measured by determining the degree of DTH response using the foot paw swelling test (12). The mice were injected intraperitoneally with a suspension containing 1×10^6 SRBC in 0.2 ml of phosphate buffered saline (PBS) on day zero sensitization. Seven days later (day + 7), the same animals were injected subcutaneously with 1×10^6 SRBC suspended in 50 μ l of PBS into the right hind foot pad for elicitation of the DTH reaction. The left hind foot pad was injected with 50 μ l of PBS as control. Foot pad swelling was measured on day + 8 with a caliper. The difference between the means of right and left hind footpad thickness gave a degree of foot pad swelling which was used for group comparisons. To establish the effect of on this immune response, a daily dose of Triphala (500/1000 mg/kg b.wt) was administered by gavage needle at the induction phase (+ 4 to + 7 days). Simultaneously, another group of animals (controls) was inoculated in the same condition with 0.1 ml of PBS. Each experimental group contains 6 animals.

Data were expressed as the percentage of inflammation relative to control

$$\% = \frac{\text{mean of foot pad swelling of treated animals}}{\text{mean of foot pad swelling of control animals}} \times 100$$

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean \pm S.D. Student's t-test was used to analyze statistical significance of the differences between the control and the treated values. *p* value at 0.05 was taken as significant.

Results

Complement activity

In arthritic control mice, the complement activity was increased by 83% at 72 h when compared with 0 h and there was a decrease at 96 h (Table 1). However in Triphala treated arthritic mice, the complement activity was increased significantly by 16% and 10% in 96 h at both the dose levels (500/1000mg/kg/b.wt) respectively, when compared to the arthritic control mice. The results obtained clearly showed that triphala at the both dosages was found to have inhibitory effect towards complement activity.

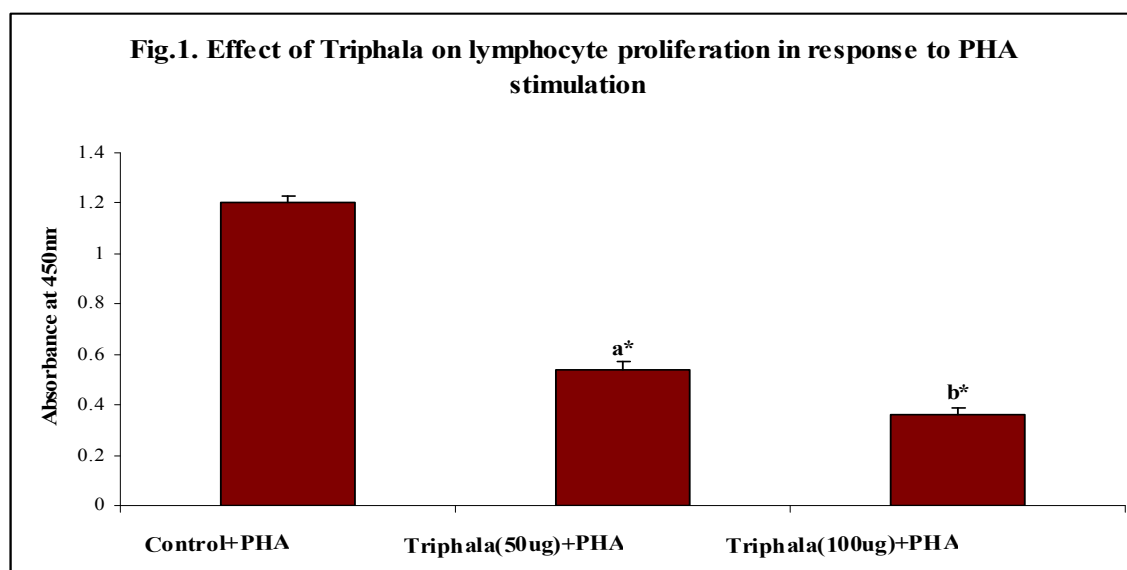
Table 1. Effect of Triphala on complement activity in adjuvant-induced arthritis

| Time (hr) | Arthritis | | Arthritis+Triphala (500mg/kg/b.wt) | | Arthritis+Triphala (1000mg/kg/b.wt) | |
|-----------|----------------------------------|-----------|------------------------------------|-----------|-------------------------------------|-----------|
| | Complement activity (absorbance) | % Changes | Complement activity (absorbance) | % Changes | Complement activity (absorbance) | % Changes |
| 0 | 0.30 ±0.03 | | 0.30± 0.02 | | 0.30± 0.02 | |
| 24 hr | 0.45± 0.03* | 50 % | 0.42± 0.02* | 40 % | 0.40± 0.03* | 33 % |
| 48 hr | 0.48± 0.05* | 60 % | 0.40± 0.04* | 33 % | 0.38± 0.02* | 26 % |
| 72 hr | 0.55± 0.04* | 83 % | 0.37± 0.03* | 23 % | 0.35± 0.03* | 16 % |
| 96 hr | 0.52± 0.03* | 73 % | 0.35± 0.03* | 16 % | 0.33 ±0.02* | 10 % |

Comparisons are made with arthritic control group vs Triphala (500/1000mg/kg/b.wt) treated arthritic groups. Symbols represent the statistical significance at * $p < 0.05$.

T-lymphocyte proliferation assay

A significant inhibition in phytohemagglutinin stimulated lymphocyte proliferation was observed at both concentration of Triphala tested ($p < 0.05$) in these experiments (Fig. 1), when compared with the control cells treated only with phytohaemagglutinin. In order to ascertain that Triphala itself was not cytotoxic to the cells, trypan blue dye exclusion test was carried out and it was shown that Triphala by itself does not alter cell viability.



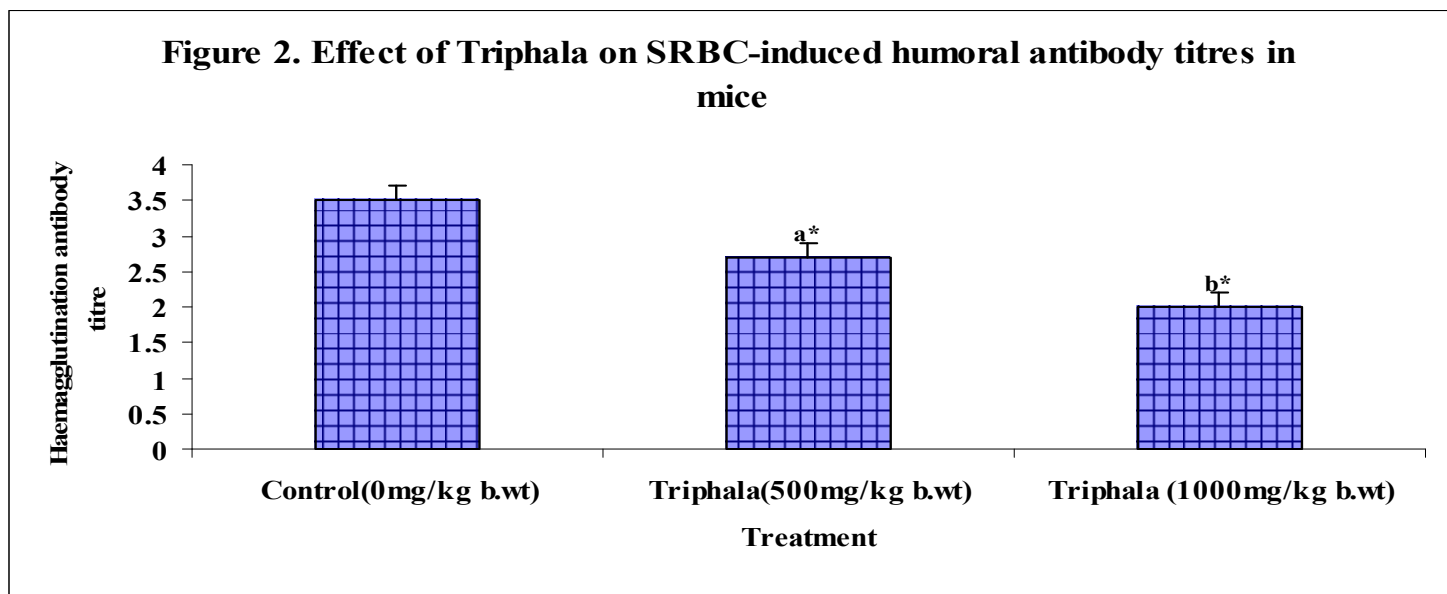
The comparisons are made as follows; a- Triphala (50ug) +PHA vs Control+PHA

b- Triphala (100ug) +PHA vs Control+PHA

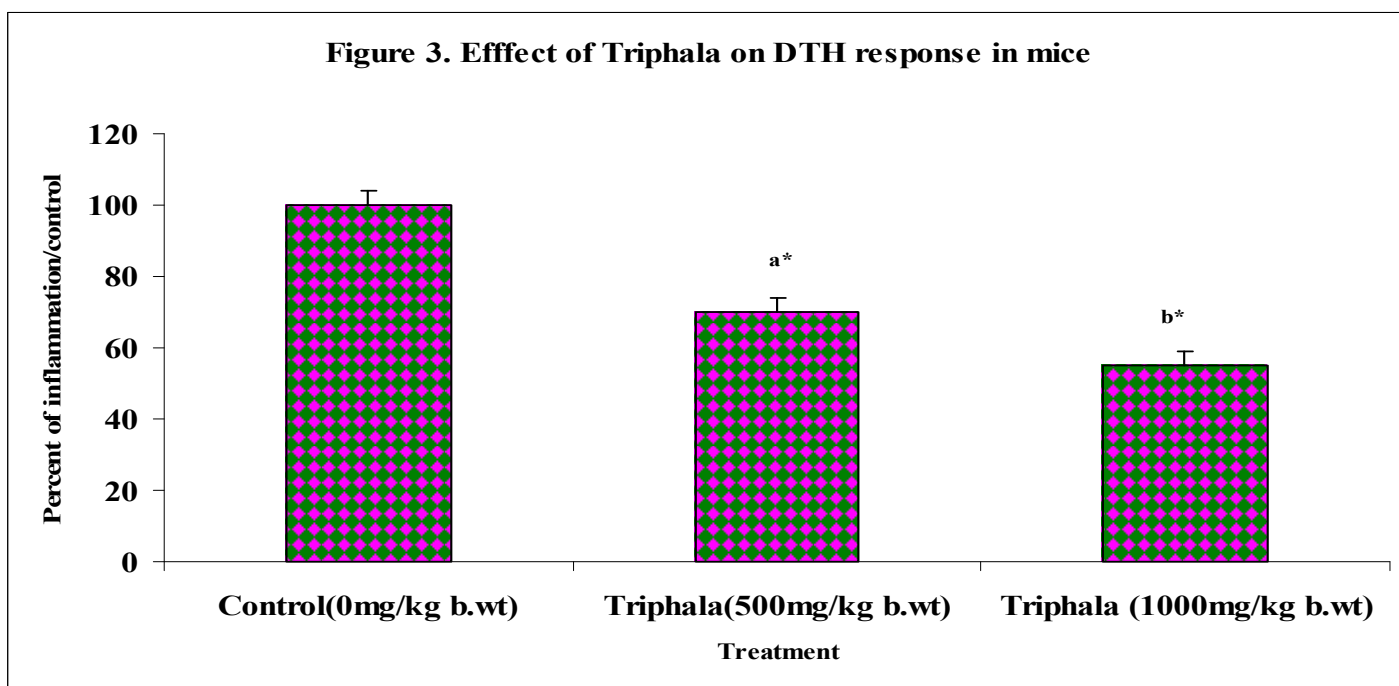
The symbols represent statistical significance at: $p < 0.05$.

Humoral antibody and delayed-type hypersensitivity response

Triphala at the both doses (500/1000 mg/kg b.wt) showed significant inhibition on both humoral (Fig. 2) and DTH response (Fig. 3), when compared to control mice.



The comparisons are made with control group vs Triphala(500/1000mg/kg/b.wt) treated mice. The symbols represent statistical significance at: $p < 0.05$.



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Discussion

Rheumatoid arthritis (RA) is a kind of chronic immunological disease that primarily presents as a chronic symmetric polyarthritis associated with inflammation and cartilage destruction (13). Investigations on the pathogenesis have revealed the primary involvement of cellular immune response (CIR), such as an increase in interleukin-1 production by macrophage, as well as a decrease in functions of suppresser T cells (14). Keeping this in view, the present study was carried out to investigate the effect of Triphala on some immunological parameters like complement activity, T-lymphocyte proliferation, humoral immune response and cell mediated immune response (DTH), which are closely related to inflammatory processes.

During complement activation, the complement components induce the release of mediators such as proinflammatory cytokines from the mast cells, lymphocytes and macrophages (15). A strong correlation between complement inhibitory and anti-inflammatory activity in experimental models has been observed (16). Our present study is in agreement with previous reports on inhibition of complement dependent inflammation by using *Withania somnifera* root powder (17) oleanolic acid (18) rosmarinic acid (19) and boswellic acid (8). From the above results, it is confirmed that complement plays a vital role in inflammation. It would be predicted that inhibition of complement activity might be a possible mechanism for inhibiting inflammatory disorders. The inhibition of the classical complement pathway can be due to several effects. Active compounds like gallic acid in the Triphala can bind or alter particular components of the complement system, or inactivate converting enzymes, eliminating their involvement in the cascade. Thus Triphala inhibits the production of complement triggered by inflammation. This inhibitory action may be due to its anti-inflammatory effect (7) and inhibition of inflammatory mediator TNF-alpha (20). Further research has to reveal the nature of the effects observed and the identity of the active constituents in complement inhibition.

Selective modulation of T-cell response in autoimmunity has been achieved convincingly in experimental model of autoimmunity and in that regard; adjuvant-induced arthritis has proven to be a suitable model to elaborate new strategies of immunotherapy. It is well known that T-cells respond to stimulation by non-specific polyclonal activators like mitogen such as phytohaemagglutinin (PHA), concanavalin A, pokeweed mitogen etc and enter into cell division. Then mitogen acts first by binding to cell surface receptor which in turn, initiates a cascade of biochemical reactions leading to cell proliferation (21). This situation mimics the unrestricted proliferation of T cells *in vivo*, in disease conditions such as arthritis. Our present report shows that Triphala exerts inhibitory effects on lymphocyte proliferation stimulated by phytohaemagglutinin. Therefore, the result observed suggests that Triphala may exert their antiarthritic activity through the inhibitory effect on cell proliferation.

The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. To evaluate the effect of Triphala on humoral response, its influence was tested on sheep erythrocyte-specific haemagglutination antibody titre in mice. Triphala at both the doses (500/1000 mg/kg, p.o) showed significant inhibition in antibody titre response.

On the other hand, Triphala was able to diminish the inflammation, occurring during delayed type hypersensitivity reaction (DTH) in mice. DTH reaction requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release mediators such as histamine, products of arachidonic acid metabolism and cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation (22). Therefore the inhibition of DTH response observed in our study could be due to an influence of Triphala on the biological mediators.

Many studies have revealed that the constituents of Triphala, *Terminalia chebula*, *Embllica officinalis* and *Terminalia bellerica* contain bellericanin, ellagic acid, gallic acid, ethyl gallate and β -sitosterol (23). In fact, the immunosuppressive effect of Triphala observed in our study might be due to the immunomodulatory effect of *Embllica officinalis* which has been described (24). However, further investigations at a molecular level to establish the mechanism of the action of the drug are under way.

References

1. Sainis KB, Sumariwalla PF, Goel A, Chintalwar GJ, and Sipahimalani AT, Banerji A. Immunomodulatory properties of stem extracts of *Tinospora cordifolia*: cell targets and active principles. In: S.N. Upadhyay, Editor, *Immunomodulation*, Narosa Publishing House, New Delhi, India 1997.
2. Dash B, and Kashyap P. *Materia Medica of Ayurveda*. Concept Publishing Company, New Delhi 1980.
3. Swayamjot Kaur, Husheem Michael, Saroj Arora, Pirkko, Härkönen, and Subodh Kumar. The in vitro cytotoxic and apoptotic activity of Triphala-an Indian herbal drug, *J. Ethanopharmacol.* 2005; 10: 15-20.
4. Pulok Mukherjee, Sujay Rai, Sauvik Bhattacharyya, Pratip Kumar Debnath, Tuhin Kanti Biswas, Utpalendu Jana, Srikanta Pandit, Bishnu Pada Saha, and Pradip K, Paul. Clinical study of Triphala- A well known phytomedicine from India, *Iranian Journal of Pharmacology&Therapeutics.* (2006) 5: 51-54.
5. Sandhyaa T, Lathikaa KM, Pandeyb BN, and Mishraa KP. Potential of traditional ayurvedic formulation, Triphala, as a novel anticancer drug. *Cancer Letters* 2006 ;231 (2): 206- 214.
6. Saravanan S, Srikumar R, Manikandan S, Jeya Parthasarathy N, and Sheela Devi R. Hypolipidemic effect of triphala in experimentally induced hypercholesteremic rats. *Yakugaku Zasshi.* 2007;127(2): 385-8.
7. Rasool M, and Sabina EP. Anti-inflammatory effect of Triphala on adjuvant-induced arthritis in mice. *Phyt. Res.* 2007; 21(9): 889-94.
8. Kapil A, and Moza N. Anticomplementary activities of boswellic-acids—an inhibition of C3-convertase of the classical complement pathway. *Int. Immunopharmacol.* 1992;14: 1139–1143.
9. Boyan A. Isolation of mononuclear cells and granulocytes from human blood. *Scand.J. Clin.Lab.Invest.* 1968; 21; 77.

10. Hansen MB, Nielsen SE, and Berg KJ. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Meth.* 1989; 119: 203–210.
11. Subramoniam DA, Evans S, Rajasekharan S, and Pushpangadan P. Effect of *Trichopus zeylanicus* Gaertn (active fraction) on phagocytosis by peritoneal macrophage and humoral immune responses in mice. *J. Ethanopharmacol.* 2000; 32: 221–225.
12. Fabian Benencia, Maria Cecilia Courreges, and Felix Carlos Coulombie. In vivo and in vitro immunomodulatory activities of *Trichilia glabra* aqueous leaf extracts. *J. Ethanopharmacol.* 2000; 69: 199–205.
13. Costello JC, and Halverson PB. A new era in rheumatoid arthritis treatment, *Wisc. Med. J.* 2003;102 (7):29–33.
14. Firestein GS, Tsai V, and Zvaifler NJ. Cellular immunity in the joints of patients with rheumatoid arthritis and other forms of chronic synovitis. *Clin. Rheum. Dis.* 1987; 13: 191–213.
15. Bacle F, Cavaillon Haeffner N, and Laude M. Induction of IL-1 release through stimulation of the C3b/C4b complement receptor type one (CR1, CD35) on human monocytes. *J. Immunol.* (1990) 144: 147-152.
16. Noordhock J, and Bonta IL. The mechanism of anti-inflammatory effect of carragenan pouch exudates. In: future trends in inflammation, Velo, G.P., Willoughby, D.A., Giroud, J.P. (ed.), Peuda, London. 1974; 249-259.
17. Rasool M, and Varalakshmi P. Immunomodulatory role of *Withania somnifera* root powder on Experimental induced Inflammation: an *in vivo* and *in vitro* study. *Vasc. Pharmacol.* (2006) 44: 406-410.
18. Kapil A, and Sharma S. Effect of oleanolic acid on complement in adjuvant and carragenan-induced inflammation in rats. *J. Pharm. Pharmacol.* 1995; 47: 85-587.
19. Englberger W, Hadding U, Eschenberg E, Graf E, Leyek S, Winkelmann, J, and Parnham MJ. Rosmarinic acid: A new inhibitor of complement C3-convertase with anti-inflammatory activity. *Int. J. Immunopharmacol.* 1988; 10: 729-737.
20. Sabina EP, and Rasool M. An in vivo and in vitro potential of Indian ayurvedic herbal formulation Triphala on experimental gouty arthritis in mice. *Vasc. Pharmacol.* 2008; 48: 14-20.
21. Talwar A. *Handbook of Practical Immunology*, Vikas Publishing House Pvt. Ltd. 1983; 318–327.
22. Gokhale AB, Damre AS, and Saraf MN. Investigations into the immunomodulatory activity of *Argyrea speciosa*. *J. Ethanopharmacol.* 2002; 84: 109-114.
23. Jagetia GC, Baliga ML, Malagi KJ, and Sethukumar Kamath M. The evaluation of the radioprotective effect of Triphala (an ayurvedic rejuvenating drug) in the mice exposed to γ -radiation. *Phytomedicine* 2002; 9: 99-108.
24. Sai Ram M, Neetu D, Yogesh B, Anju B, Dipti P, Pauline T, Sharma SK, Sarada SK, Ilavazhagan G, Kumar D, and Selvamurthy W. Cyto-protective and immunomodulating properties of Amla (*Emblica officinalis*) on lymphocytes: an in-vitro study. *J. Ethanopharmacol.* 2002; 81(1):5-10.