Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomedicine & Pharmacotherapy 57 (2003) 296–300
BIOMEDICINE & PHARMACOTHERAPY
www.elsevier.com/locate/biopha

Dossier: Immunity and medical pathologies

Immunomodulatory effects of agents of plant origin

Lilly Ganju *, Dev Karan, Sudipta Chanda, K.K. Srivastava, R.C. Sawhney, W. Selvamurthy

Immunomodulation Laboratory, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi 110054, India

Received 13 June 2003

Abstract

The immunomodulatory properties of amla (*Emblica officinalis*) and shankhpushpi (*Evolvulus alsinoides*) were evaluated in adjuvant induced arthritic (AIA) rat model. Injecting Complete Freund's Adjuvant (CFA) in right hind paw of the animals induced inflammation. The crude extracts of both the herbs were administered intraperitonially following a repeated treatment profile. The anti-inflammatory response of both the extracts was determined by lymphocyte proliferation activity and histopathological severity of synovial hyperplasia. Both the extracts showed a marked reduction in inflammation and edema. At cellular level immunosuppression occurred during the early phase of the disease. There was mild synovial hyperplasia and infiltration of few mononuclear cells in amla or shankhpushpi treated animals. The induction of nitric oxide synthase (NOS) was significantly decreased in treated animals as compared to controls. These observations suggest that both the herbal extracts caused immunosuppression in AIA rats, indicating that they may provide an alternative approach to the treatment of arthritis.

Keywords: Amla; Shankhpushpi; Immunomodulation; Arthritis

1. Introduction

Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. Fruits of *Emblica officinalis* (family Euphorbiaceae) commonly known as "amla" or the Indian gooseberry, member of a small genus *Emblica*, are claimed to have anti-fungal, anti-bacterial, anti-diabetic, anti-clastogenic and hepatoprotective properties [7,10] besides having significant anti-oxidant [4], adaptogenic [15] and anti-tumor activities [11]. Amla fruit has also been demonstrated to possess cytoprotective properties in acute cadmium toxicity [12]. Recent in vitro studies have also demonstrated that fruit extract of amla is able to relieve the immunosuppressive effects of chromium in rat lymphocytes [16]. Similarly, the whole plant of shankhpushpi, *Evolvulus alsinoides* (family Convolvulaceae) has been extensively used in ayurvedic practices as an alternative, antiphlogistic, febrifuge and as a brain tonic [14] to treat nervous debility and scrofula [3]. The juice of shankhpushpi has also been found to promote the healing of ulcers [2], and the leaf extract has been used to treat whitlow in the fingers and toes [3]. These effects of amla and shankhpushpi may be due to the presence of immunomodulatory activity in these plants. Despite many therapeutic effects of amla and shankhpushpi, there is a paucity of information on the immunomodulatory effects of these plants as anti-inflammatory agents. Therefore, the present study has been undertaken to determine the immunomodulatory effects of amla and shankhpushpi extracts in adjuvant induced arthritic (AIA) rat model. AIA is an erosive autoimmune polyarthritis involving both humoral and cell mediated immune responses that resemble human rheumatoid arthritis (RA).

2. Materials and methods

2.1. Plant extracts

Fruits of amla and whole plant of shankhpushpi were collected from authentic sources, dried under shade and powdered in the laboratory. Crude powder of both the herbs were soaked in water over night, centrifuged at 3000 rpm and the supernatant was used for immunomodulatory studies.

* Corresponding author.
E-mail address: lganju@rediffmail.com (L. Ganju).

doi:10.1016/S0753-3322(03)00095-7

2.2. Animals

The study was approved by Institute's animal ethical committee and confirmed to national guidelines on the care and use of laboratory animals. Both male and female albino rats (Sprauge Dawley Strain), 10–12 weeks old, weighing 125–150 g were used for the study. The animals were maintained at 25 ± 2 °C in the institute's animal house with food and water ad libitum.

2.3. Development of arthritis

Animals were divided into five groups of 10 each: Gr. I received PBS only; Gr. II—CFA; Gr. III—Amla + CFA; Gr. IV—Shankhpushpi + CFA and Gr. V—Dexamethasone + CFA. Rats were injected with 300 μl of Complete Freund's Adjuvant (CFA) (Sigma, USA) in the right hind footpad and left overnight for development of inflammation.

2.4. Evaluation of arthritis

Measuring the thickness of inflamed ossicular tissue using a dial gauge caliper assessed degree of arthritis. Severity of inflammation was classified using a six-point scale based on enlargement, erythema and edema of the tissue:

Degree	Severity
++++	Severe
+++	Moderate
++	Mild
+	Positive
±	Doubtful
–	Nil

2.5. Treatment

To determine the optimum dose, animals were initially treated with 100, 50, 25, 12.5, and 6.25 mg/kg body weight of the amla and shankhpushpi herbal extracts (HEs) separately. Intraperitoneal administration of 25 mg/kg body weight of HE was found to be the optimum dose for immunomodulatory property, hence used for further studies. All the animals received HEs for 25 days.

2.6. Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed using mitogen Con-A (Sigma, USA). Both the treated and untreated animals were sacrificed to remove the spleen in RPMI-1640 medium (Sigma, USA). Spleen was crushed and cell suspension was washed with plain medium. Cells were lysed with 0.9% ammonium chloride and resuspended in complete medium with 10% fetal calf serum (Sigma, USA). Cells were cultured at a final concentration of 3×10^5 cells/100 μl/well in triplicate in flat bottom microtiter tissue culture plates (Tarson, India). The optimal concentration of 0.05 μg/ml (in vitro concentration titration done earlier) of both the extracts was added to the wells separately and in combination with Con-A (5.0 μg/ml). After 3 days of incubation at 37 °C under humidified air supplemented with 5% CO_2, 1 μci ^{3}H-thymidine (BARC, Mumbai, India) was added to each well. Six to eight hours later cells were harvested and aspirated on to glass-fiber filter papers using NUNC, Automatic Cell Harvester (The Netherlands) and the radiolabel incorporated into DNA was counted using LKB autobeta counter.

2.7. Histology of joint tissues

Rats were sacrificed to remove the knee joints. Specimens were fixed for 24 h in 2% glutaraldehyde in phosphate buffer saline, bisected, decalcified and returned to 2% glutaraldehyde and submitted for routine paraffin embedding. Tissue sections were stained using hematoxylin and eosin stain. The histological findings were graded on the basis of synovial hyperplasia and mononuclear cell infiltration.

2.8. Measurement of induction of NO in activated macrophages

Macrophage activation assay was performed by injecting rats with thioglycolate intraperitoneally 72 h prior to sacrifice. On the day of sacrifice, cold RPMI-1640 incomplete medium was administered intraperitoneally to flush out the activated macrophages. The process was repeated 8–10 times to obtain a good yield of macrophages. Once the activated macrophages were recovered, cells were centrifuged and washed thrice with plain medium, counted and plated at a final concentration of 5×10^5 cells/well. Plates were incubated for 4 h at 37 °C in CO_2 incubator (Heraeus, Germany). Floating cells were removed and the wells were washed twice with warm medium to avoid the leaching of the adhered macrophages. Cells were then treated with 0.05 μg/ml of HEs. Culture soup was collected for estimation of NO production by Griess Reagent method [17]. Briefly, 100 μl of cell supernatant mixed with 1% sulfanilamide/0.1% naphthylethylenediamine/2.5% H_3PO_4 was incubated at room temperature for 10 min to form a chromophore. The absorbance was read at 550 nm and NO was measured using $NaNO_2$ as standard. All values are mean ± S.E.M. of n experiments. They were analyzed by ANOVA and student 't'-test and $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Clinical analysis

All the animals injected with CFA developed severe inflammation within 24 h. In HE treated animals edema began to subside gradually and showed a significant reduction ($P < 0.01$) in swelling of joints and progression of inflammation as compared to untreated animals (Fig. 1). The reduction in swelling in HE treated groups was at par with dexamethasone treated animals. After 17 days of treatment, the swelling

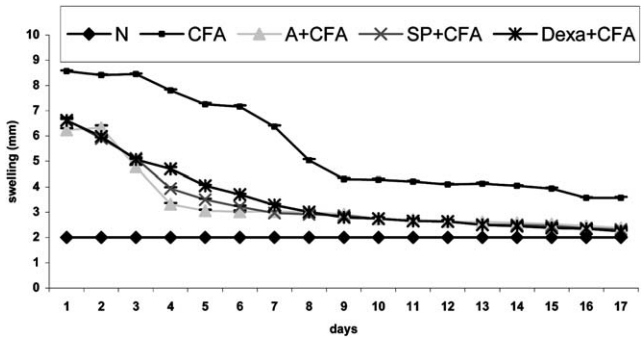

Fig. 1. Daily measurement of swelling in hind paw of treated and untreated AIA rats. Results are mean ± S.E.M. (N, normal; CFA, Complete Freund's Adjuvant; A, amla; SP, shankhpushpi; Dexa, dexamethasone).

in treated animals was reduced almost equal to normal controls, whereas it persisted longer in untreated animals.

3.2. Cell mediated immunity

In untreated CFA injected animals the lymphocyte proliferation on day five and 10 was significantly increased ($P < 0.01$) as compared to control animals whereas on day 15 and 20 the proliferation was not significantly different ($P > 0.05$) than the controls (Fig. 2). Treatment with HE caused a significant suppression ($P < 0.01$) in lymphocyte proliferation when observed after 5, 10 and 15 days of treatment. However, proliferation rate on day 20 was not significantly different ($P > 0.05$) than that of the CFA injected or normal controls.

3.3. Histopathological analysis

Results of histopathological examination are shown in Fig. 3. All rats injected with CFA developed pronounced

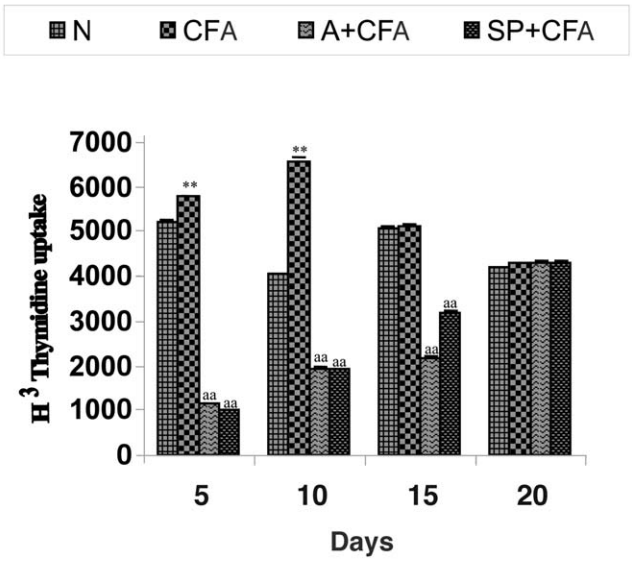

Fig. 2. Effects of amla and shankhpushpi on the proliferative response of lymphocytes derived from spleens of treated and untreated AIA animals. Results are mean ± S.E.M. * vs. normal $P < 0.01$, *a* vs. CFA $P < 0.01$. (N, normal; CFA, Complete Freund's Adjuvant; A, amla; SP, shankhpushpi).

edema and exhibited marked synovial hyperplasia with significant infiltration of mononuclear cells, whereas in animals treated with HEs there was less cartilage erosion and bone destruction and mild synovial hyperplasia with very few mononuclear cell infiltration.

3.4. Nitric oxide production

The NO levels varied between 0 and 0.05 μM in peritoneal macrophages of normal animals. After 10,15 and 20 days of CFA administration in untreated animals, the NO production was significantly higher ($P < 0.01$) as compared to mean values of normal animals (Fig. 4). In amla treated animals the NO production was significantly lower ($P < 0.01$) as compared to CFA injected animals on all the days, whereas in shankhpushpi treated animals a significant decrease ($P < 0.01$) was observed on day 10 only. The decline in NO production was significantly higher ($P < 0.01$) in amla treated animals as compared to animals, which received shankhpushpi.

4. Discussion

Results from the present study demonstrate that amla and shankhpushpi have a significant anti-inflammatory activity in AIA animals. There was a significant reduction in swelling and redness of inflamed areas in treated animals than in untreated controls. The ability of both the HEs to suppress the lymphocyte proliferation in response to AIA is consistent with the findings of earlier studies on the effect of commercially available drugs or cytokines or herbal preparations [5,9,13,19]. Both are as potent as dexamethasone, a traditionally used immunosuppressant for arthritis. The dosing regimen used in these experiments covered the clinically relevant stage of the immune response. There was no toxicity, anorexia or weight loss observed in any of the treated animals.

Selective modulation of T-cell response in autoimmunity has been achieved convincingly in experimental model of autoimmunity and in that regard, AIA has proven to be a suitable model to elaborate new strategies of immunotherapy. In our study it was observed that both the HEs used for treatment of AIA in rats resulted in immunosuppression during the acute phase of the disease. The individual effects of both, amla and shankhpushpi have anti-arthritic properties in AIA as they selectively inhibited T-cell activation, which was clearly indicated by decreased lymphocyte proliferation. The anti-inflammatory effect of both the HEs were also associated with disappearance of follicular aggregation of lymphocytes from the inflamed synovium. Particularly remarkable was the significant reduction of synovitis in joints, which was evident from the histology of the inflamed tissues. These observations support the T-cell directed immunosuppression by amla and shankhpushpi and confirm the observations of earlier investigations using immunomodulators of plant origin [6,18,20].

Production of NO in appropriate magnitude serves as a key-signaling model in various physiological processes. But

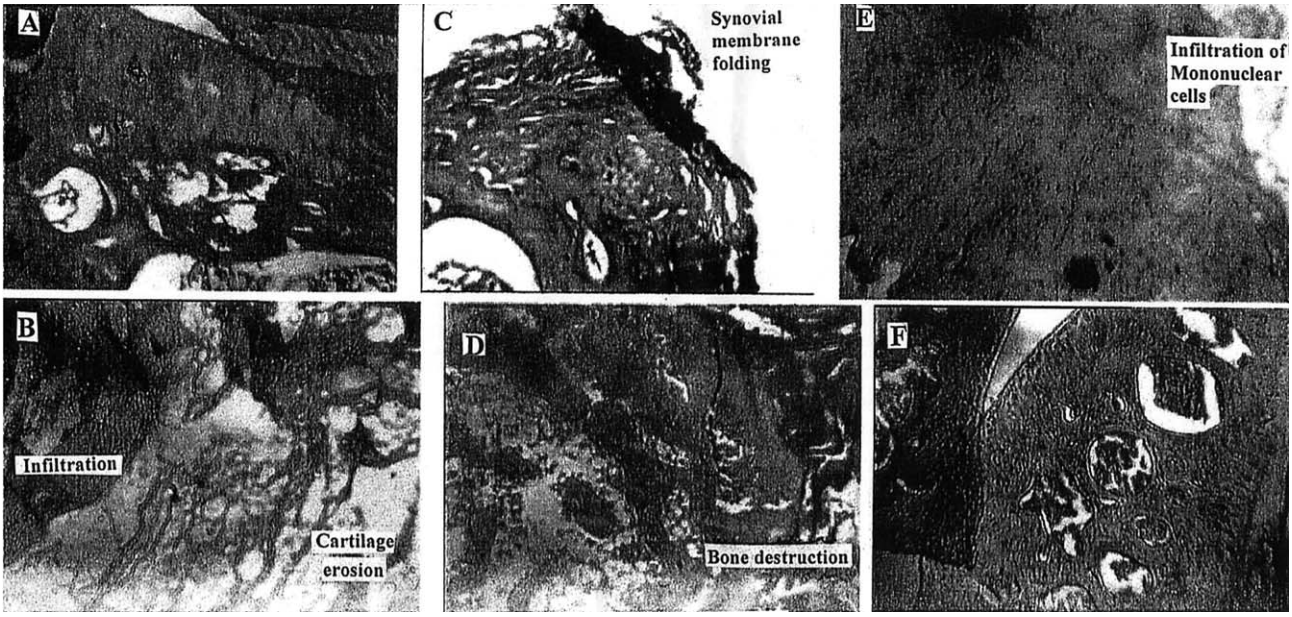

Fig. 3. Histopathological analysis: knee joints sections of treated and untreated AIA animals stained with hematoxylin–eosin. (A) Normal joint; (B, C) CFA injected untreated rats with cartilage erosion, severe synovial hyperplasia and synovial folding; (D–F) HE treated rats showing mild synovial hyperplasia, infiltration of fewer mononuclear cells and less bone destruction.

at the same time NOS has been found to cause pathological conditions such as RA [8]. Our observation that NO production in amla and shankhpushpi treated animals was significantly lower as compared to controls, suggests that one of the possible mechanisms for curtailing the progression of AIA in animals might have been a decreased cellular production of NO by inhibiting NOS activity. Therefore, the inhibitory activity of amla and shankhpushpi might have been due to their anti-inflammatory properties and ability to counteract NO induced oxidative damage, which eventually helps in remodeling of cells.

Treatment of RA include drugs to control inflammation e.g. nonsteroidal anti-inflammatory drugs (NSAIDs) and slow acting anti-inflammatory drugs (SAARDs) which probably induce regression or arrest progression of the disease and also have tissue protective effects. The limitations of SAARDs therapy, however, are their well-known toxicity and the variation in clinical efficacy [1]. Hence, there has been an interest in using an alternative to conventional chemotherapy, which could provide the protection and prevention for such type of diseases where host immune response is impaired or a selective immunosuppression is required.

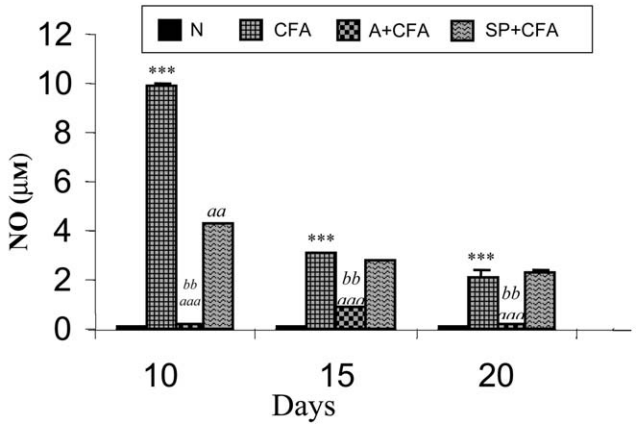

Fig. 4. Induction of NO synthase activity in peritoneal macrophages by HEs in treated and untreated AIA animals. Results are mean ± S.E.M. * vs. normal $P < 0.01$, *a* vs. CFA $P < 0.01$, *b* vs. shankhpushpi $P < 0.01$. (N, normal; CFA, Complete Freund's Adjuvant; A, amla, SP, shankhpushpi).

With respect to the possible future clinical potential of this study is the observation that therapeutic dosing with amla and shankhpushpi inhibited established arthritis in rats. Both the herbal preparations proved to be as useful as dexamethasone or any other drug in the treatment of AIA, and can be used as an adjunct to chemotherapy. The plant-derived immunomodulators thus have tremendous future potential for developing new pharmaceutical products. Phytochemical analysis of these HEs is in progress to identify bioactive molecules responsible for immunomodulatory properties in these plants.

References

[1] Anseth J, Hangen H, Foore O. Rheumatoid arthritis and metal compounds perspectives on the role of O_2 radical detoxification. Analyst 1998;123:3–6.
[2] Asolkar LV, Kakkar KK, Chakre OJ. Second supplement to glossary of Indian medicinal plant with active principles. Part I 1965–85. New Delhi: NISCAIR; 1992.
[3] Bhatt DC, Mehta SR, Mitaliya KD. Ethanomedicinal plants of Shetrunjaya hill of Palitans Gujrat. Ethanobotany 1999;11:22–5.
[4] Bhattacharya A, Chatterjee A, Ghosal S, Bhattacharya SK. Antioxidant activity of active tannoid principles of *Emblica officinalis* (Amla). Ind J Exp Biol 1999;37:676–80.
[5] Chattopadhyay RR. Possible biochemical mode of anti-inflammatory action of Azadirachta indica A. Juss in rats Ind J Exp Biol 1998;36: 418–20.

[6] Chaturvedi AK, Parmar SS, Bhatnagar SC, Misra G, Nigam SK. Anticonvulsant and anti-inflammatory activity of natural plant coumarins and Triterpenoids. Res Commun Chem Path Pharm 1974;9: 11–22.

[7] Dhir H, Roy AK, Sharma A, Talukdar G. Modification of clastogenicity of lead and aluminium in mouse bone marrow cells by *Phyllanthus emblica* fruit extract. Mutation Res 1991;24:305–12.

[8] Gross SS, Wolin MS. Nitric oxide: pathophysiological mechanism. Ann Rev Physiol 1995;57:737–69.

[9] Jackson CG, Williams HJ. Disease modifying antirheumatic drugs. Drugs 1998;56:337–44.

[10] Jeena KJ, Joy KL, Kuttan R. Effect of *Emblica officinalis, Phyllanthus amarus* and *Picrorhiza kurroa* on *N*-nitrosodiethylamine induced heptocarcinogenesis. Cancer Letters 1999;136:11–6.

[11] Jose JK, Kuttan G, Kutan R. Antitumor activity of *Emblica officinalis*. J Ethanopharmacol 2001;75:65–9.

[12] Khandelwal S, Shukla LJ, Shanker R. Modulation of acute Cadmium toxicity by *Emblica officinalis* fruit in rat. Ind J Exp Biol 2002;40: 564–70.

[13] Kubo M, Matsuda H, Tokuoka K, Ma S, Shimoto H. Anti-inflammatory activities of methanolic extract and alkaloidal component from corydalis tuber. Biol Pharm Bull 1994;17:262–5.

[14] Nadkarni KM. In: Nadkarani AK, editor. Indian material medica, vol. 1. Bombay: Popular Prakashan; 1982. p. 531.

[15] Rege NN, Thatte UM, Dahanukar SA. Adaptogenic properties of six rasayana herbs used in ayurvedic medicine. Phytotherapy Res 1999; 13:275–91.

[16] SaiRam M, Neetu D, Yogesh B, Anju B, Dipti P, Pauline T, et al. Cyto-protective and immunomodulatory properties of Amla (*Emblica officinalis*) on lymphocytes: an in-vitro study. J Ethanopharmacol 2002;81:5–10.

[17] Stucher DJ, Nathan CF. Nitric oxide, a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 1989;169:1543–55.

[18] Vazquez B, Avile G, Segura D, Escalanta B. Anti-inflammatory activity of extracts from Aloe vera gel. J Ethanopharmacol 1996;55:69–75.

[19] Yuhe M, Sherry T, Laura ED, Gregory PB, Edward HG, Jeffrey ML, et al. Inhibition of collagen induced arthritis in mice by viral-IL-10 gene transfer. J Immunol 1998;161:1516–24.

[20] Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B. Studies on the immunomodulatory effects of Ashwagandha. J Ethanopharmacol 1996;50:69–76.