Assessment of Immunomodulatory Activity of *Euphorbia hirta* L.

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Immune system is the major target for development of treatment strategies to improve the management of infections. Many species of Indian medicinal plants have been reported to possess active principles with immunomodulating properties. *Euphorbia hirta*, a pantropic herb has been reported to be pharmacologically active. This study reports one another not widely reported property of the plant, immunomodulatory activity, which has been proved using simple techniques like the macrophage activity testing, carbon clearance test and mast cell de-granulation assay.

Key words: *Candida albicans*, *Euphobia hirta*, mast cells, phagocytosis

Plant products have been used in the treatment of human diseases since time immemorial. Indian subcontinent is endowed with a rich flora and more than 1500 plant species have been known to possess therapeutic properties. The modulation of immune response by various herbal formulations in order to alleviate diseases has been of interest over many decades. Many plants have been evaluated for immunostimulant and immunosuppressive properties using simple techniques. The ayurvedic concepts of preventive health care and the therapeutic potential of immunomodulatory agents from plants have been reviewed exhaustively[1]. *Azadirachta indica* leaf extracts is found to induce cell mediated immune response as seen from the enhancement of macrophage migration inhibition[2]. In human volunteers, it stimulated humoral immunity by increasing antibody levels and cell mediated immunity by increasing total lymphocyte T cell count in 21 days.

Euphorbia hirta, a small herb/common garden weed has been reported to have antimicrobial activity specific to enteropathogens[3]. There has been comprehensive reports on the plant (whole plant) to have a 45% immunomodulation activity by way of inhibition of nitric oxide production[4]. In this paper, the *in vitro* and *in vivo* immunomodulatory properties of *Euphorbia hirta* are reported.

Euphorbia hirta Linn. locally called 'garden spurge' was used for the study. The plant was locally collected and was authenticated at the Department of Botany, Presidency College, Chennai, India. Voucher specimen was deposited in the Herbarium (#8413). Swiss albino mice, 25-30 g in weight, were used for the *in vivo* experiments. The animals were maintained in cages at a temperature of 24° and 10 h light; 14 h dark cycle throughout the experimental period. All the animals were fed a standard diet and water *ad libitum.*

Aerial portion of *E. hirta* was washed well, inflorescence collected and shade dried. Ethanol was used for extraction since it being a polar solvent could bring into solution all the metabolites present. The shade dried inflorescence of *E. hirta* (25 g) was immersed in 250 ml of ethanol and was left a room temperature for 24 h. The extract was filtered through Whatman No. 1 filter paper. Solvent was removed completely by evaporation under vacuum.

Capillary blood (0.2 ml) was obtained by finger prick method and was placed on a clean grease free glass slide and spread to 1.5×1.5 cm. Blood was allowed to clot at 37° for 25 min. The clot was removed using sterile normal saline. The polymorphonuclear

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leukocytes (PMN's) were found adhered to the glass surface while the rest of the blood components are washed away. Slides in duplicates were prepared and used for each dilution of the plant extract.

Candida albicans was confirmed using germ tube test and was inoculated in Saboraud Dextrose Broth. Overnight culture was centrifuged at 2000 rpm for 15 min. The cell pellet was washed four times with sterile Hank's balanced salt solution (HBSS). The final cell button was suspended in sterile HBSS and human serum in a proportion of 4:1 and the cell density was adjusted to 2×10^8 cfu/ml with the help of MacFarlands standard.

Different dilutions of the ethanol extracts (0.1 ml) were flooded over the PMN layer on the slides, after which the slides were incubated at 37° for 15 min followed by the addition of 100 µl of *C. albicans* cell suspension. The slides were further incubated at 37° for 60 min. After incubation, the film was washed twice with sterile normal saline. The film was fixed with methanol for 5 min. Diluted Giemsa stain was flooded over the film and was left undisturbed for 25 min. The excess stain was removed using HBSS and air dried. The slides were observed under the oil immersion (×100) objective. The mean number of *Candida* cells phagocytosed by PMNs on the slide were determined microscopically for 100 granulocytes using morphological criteria[5]. This number was taken as the phagocyte index (PI) and was compared with the PI of the control. Immuno stimulation (%) was calculated by using the following Eqn., Stimulation (%) = $PI_{(test)} - PI_{(control)}\times100/PI_{(control)}$. The change in macrophage phagocyte activity following the administration of crude ethanol extract to Swiss albino mice was determined by using carbon clearance assay. Swiss albino mice of either sex weighing (25-28 g) were used in duplicates for the study.

The working solution of the extract was prepared by dissolving in dimethylsulfoxide (DMSO) and then in normal saline to yield a dose of 40, 80 and 160 mg/kg. Mice used for the control studies received 0.2 ml of normal saline, while the experimental mice received 0.2 ml of the extracts intraperitonealy for 5 d prior to injection of carbon particles. Phagocytic activity of the reticuloendothelial system was assessed[6]. On the 6th day, mice (both experimental and control were injected with 0.1 ml of 1% carbon suspension intravenously through the tail vein. Blood sample (20 µl) was drawn from retro-orbital plexus immediately before and at 3, 6, 9, 12, 15 and 18 min after injection of carbon suspension. The individual aliquots were lysed with 2 ml of 1% acetic acid. Absorbance was measured spectrophotometrically at a wavelength of 660 nm until the absorbance equivalent to standard (original pre-injection blood sample) was obtained[7]. The rate of carbon clearance (phagocytic index K) was calculated form the slope of each time-concentration curve drawn by plotting absorbance as ordinate against time as abscissa.

Plant extract (0.2 ml of 25 mg/ml) was injected in to mice intraperitoneally for six days prior to the isolation of peritoneal mast cells. Tyrode solution (10 ml) containing 5 units /ml heparin was injected in mice intraperitoneally. After a gentle massage for about 30-45 s, the mast cell rich peritoneal fluid was collected over ice and centrifuged at 2000 rpm for 5 min. The cells were washed twice with chilled Tyrode solution and resuspended in 1 ml tyrode solution. These isolated peritoneal mast cells were used to study polysorbate 80 induced mast cell degranulation *in vitro.*

To study the effect of plant extract on mast cells[8], 0.1 ml of ethanol extract of *Euphorbia hirta* (25 mg/ml) was added to 0.1 ml peritoneal mast cells suspension, and incubated at 37° for 15 min. Then 0.1 ml of the degranulating agent (polysorbate 80) was added and further incubated for 10 min. The cells were stained with neutral red and % protection of degranulation of mast cells in control and treated groups were calculated by counting the mast cells under high power of light microscope.

The *in vitro* cytotoxicity studies[9] were performed on the continuous cell line of African green monkey (Vero) procured from the Department of Animal Biotechnology, Madras Veterinary College, Chennai, India. The Vero cells were split at a ratio of 1:4 and grown in 6 well plates. The plates were incubated at 37° overnight in 5% CO_2 atmosphere. Once the monolayers were formed, the growth medium was removed from all the cell monolayers.

Filter sterilized extract of *E. hirta* (160 mg/ml) were diluted (1:2, 1:4, 1:8) and 4 sets of 2 Vero cell monolayers were used for the study. Fifty microlitres of the different dilutions were added to each well in duplicates. Sterile PBS was added to 2 wells which

served as controls. Sterile maintenance medium was added to all the wells and the plates were incubated at 37° for 48 h at 5% CO_2 atmosphere.

Morphological changes in the Vero cells were observed at 24 h and at 48 h were graded as 4+, 3+, 2+, 1+ using an inverted phase contrast microscope (ie) 100%, 75%, 50%, 25% CPE. CPE of 2+ (50%) and above were marked positive.

For the *in vivo* cytotoxicity studies in Swiss albino mice model[10], 4 groups each of 4 Swiss albino mice were maintained at standard laboratory conditions. They were fed daily with mouse chow (PRS Chennai) and water *ad libitem*. Saline was used as the control. Animals were observed every h for 8 h and next day, mortality and change in other physiological and behavioural aspects like the nature of urine, co-ordination of movements, presence of convulsion if any and presence of salivation or tremor was noted.

While performing the *in vitro* evaluation of phagocytosis, ethanol extract of *Euphorbia hirta* was found to be cytotoxic at a concentration of 1000 µg/ml. Maximum phagocytic activity was evident at 62.5 µg/ml. With gradual increase in the dilutions of the plant extract there was a corresponding decrease in phagocytic activity. A concentration dependent switch from immunostimulation to immunosuppression was observed at concentration 0.48 µg/ml (Table 1).

From the *in vivo* evaluation, the ethanol extract of *E. hirta* was found to be increasing the phagocytic index at a concentration of 80 mg/ml and 160 mg/ml. *Euphorbia hirta* is found to possess immunostimulatory activity at the above said concentrations but at a concentration of 40 mg/ml, it is found to possess immunosuppressive activity as assessed by carbon clearance assay (Table 2).

From the *in vitro* cytotoxicity tests, it was seen that the ethanol extract of *E. hirta* at neat dilution, resulted in 100% CPE as observed in the Vero cells after 24 h i.e. rounding of cells, multinucleated giant cell formation and detachment of cells from the plate surface. The percentage of CPE decreased proportionately with the increase in dilution of the plant extract.

Fifty percent CPE was observed after 48 h for the plant extract at 1:2 dilution and at 1:4 dilution, the plant extract was non cytotoxic at 24 h and 48 h. In the *in vivo* cytotoxicity tests, no mortality was observed in all the three doses i.e. 160 mg/kg, 80 mg/kg, 40 mg/kg. All the mice exhibited normal behavioral and physiological conditions. The mice were active and returned to normal position immediately when placed on their back. There was no salivation or tremor. The urine was normal in appearance and the mice fed well. Mortality was not observed in any group of extract fed animals for a period of 7 d after the day of experiment. *Euphorbia hirta,* at a concentration of 25 mg/ml was found to inhibit polysorbate 80 induced mast cell degranulation as assessed by the *in vitro* method. (Table 3).

The present study revealed the immunomodulating potential of an Indian medicinal plant *Euphorbia hirta*. This plant's immunomodulatory potential has not been reported thus far to our knowledge. However, antibacterial activity of this plant has been

TABLE 1: *IN VITRO* PHAGOCYTOSIS EVALUATION

Concentration of ethanol extract of *E. hirta* (mg/ml)	Phagocytic index	% Immunostimulation
500	43	23.30
250	66	57.10
125	78	85.70
62.5	83	97.60
31.25	80	90.50
15.60	65	54.80
7.80	61	45.20
3.90	53	26.20
1.95	47	11.90
0.98	44	4.80
0.48	33	-21.40
0.24	31	-26.10
0.12	25	-40.50
0.06	21	-50.00

Table showing the % immunostimulation with reference to the concentration of extract

TABLE 2: *IN VIVO* EFFECT OF ETHANOLIC EXTRACT OF *EUPHORBIA HIRTA* ON MACROPHAGE PHAGOCYTIC ACTIVITY

Time (min)	Control	40 mg/kg	80 mg/kg	160 mg/kg
3	0.313±0.005	0.556±0.024	0.331±0.017	0.413±0.017
6	0.872±0.016	0.725±0.027	0.772±0.014	0.434±0.015
9	0.648±0.022	0.872±0.004	0.843±0.031	0.812±0.019
12	0.486±0.024	0.645±0.018	0.622±0.014	0.615±0.039
15	0.472±0.014	0.575±0.012	0.578±0.008	0.482±0.041
18	0.469±0.018	0.431±0.005	0.511±0.015	0.478±0.040
Mean phagocytic index	-0.0055	-0.0124	0.001	0.003

Table showing the statistically analysed values of phagocytosis in *in vivo* condition

TABLE 3: EFFECT OF PLANT EXTRACT ON POLYSORBATE 80- INDUCED DEGRANULATION OF ISOLATED PERITONEAL MAST CELLS

Mice	*In vitro* treatment	Number of mast cells	% Inhibition
Control	DMSO	8±2	-
	E. hirta	38±4	32.60
Test	DMSO	15±2	7.61
	E. hirta	39±5	33.60

Table showing the percentage inhibition values of mast cell de-granulation by the plant extract.

reported[3]. The antibacterial activity is attributed in part to the flavanoids present in the plant. Flavanoids have also been reported to responsible for producing antiinflammatory and humoral antibody responses[11]. In the present study, inflorescence part of the plant was used since a previous study from this laboratory has revealed the presence of flavanoids as a major fraction in the inflorescence.

The precise mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substances being isolated. The plant parts were shade dried since there is every possibility of losing some unstable plant constituents in bright sunlight. One of the most important and fundamental considerations in designing a phytochemical screening procedure is the selection of a proper solvent. Solvent extracts of plants has already been proven to exhibit a well marked biological activity than when compared to the aqueous extract[12].

Several methods for preparing an initial extract of the plant material have been reported and 80% ethanol and methanol appear to be the most useful solvents[13]. Similar observation was found in our study where we noted good extractability as well as biological activity only in the ethanol extracts. The ethanol extract of *E. hirta* was found to possess immunostimulatory activity both *in vitro* and *in vivo*. On screening for cytotoxic activity, the ethanol extract of the plant was found to be non toxic to the cultured Vero cells at a concentration of 2 mg/ml after 48 h, whereas 50% CPE was observed at a concentration of 4 mg/ml after 48 h. Our previous report[3] also corroborates this finding where no cytotoxic effect was observed till 3.125 mg/ml. Maximum immunostimulatory activity was observed at 0.063 mg/ml. Thus this plant may be a promising initiative towards drug development in the cue of immunomodulatory agents in future.

Ethanol extract of *E.hirta* showed a dose-dependent shift on neutrophils in *in vitro* phagocytosis. Also intraperitoneal injection of *E. hirta* was found to stimulate macrophages as evident from the increase in phagocytic index when compared with control using carbon clearance assay. *Euphorbia hirta* was found to be immunostimulatory at concentrations ranging from 0.98 to 500 µg/ml, but at concentrations below 0.98 µg/ml the plant exerted an immunosuppressive activity. Hence the concentration of the plant extract plays a major role in determining the immnomodulatory activity.

Correlation between antimicrobial activity and peroxidase content of leucocytes have been already studied[5]. The fungicidal activity of PMN's from healthy humans was found to be relatively much higher when compared to PMN's of pigs and chickens. The plant extract was found to elevate the phagocytic index, and once *Candida albicans* enters inside the secondary lysosomes of the intact PMN's, they are killed probably by the activity of myelopeorixdase. The viability of intra cellular *Candida albicans* could be determined by staining. The dead phagocytosed *C. albicans* appeared blue while the viable cells remained unstained.

Ethanol extract of *E. hirta* was found to inhibit polysorbate 80 induced degranulation of isolated peritoneal mast cells *in vitro*. This is indicative of its mast cell stabilizing activity. Thus the anti-inflammatory activity of *E. hirta* could be attributed to its ability to stabilize mast cell membrane, thereby inhibit the release of inflammatory mediators.

The overall efficacy (97%) stimulation of the phagocytosis could be attributed to the synergistic effect of the key constituents present in the ethanol concentrate or could be due to a single active component. Further studies are required to single out an active principle from the plant, which can be put to use.

REFERENCES

1. Upadhyay SM. Therapuetuic potential of immunomodulatory agents from plant products. In: SN Upadhyay Ed. Immunomodulation. New Delhi: Narosa Pub House; 1997. p. 149-54.
2. Ansari KH, Kasturi M, Tewarson SL, Singh S, Pandey RC. An experimental and clinical evaluation of immunomodulating potential of *Azadirachta indica* (Neem) extract. Indian J Clin Pharmacol Ther 1997;18:82-5.
3. Vijaya K, Ananthan S, Nalini R. Antibacterial effect of theaflavin,

polyphenon 60 (*Camellia sinensis*) and *E. hirta* on *Shigella* spp. – a cell culture study. J Ethanopharmacol 1995;49:115-8.
4. Jae-Ha R, Hanna A, Hwa Jin L, Wen He Q, Yong Nam H, Byung Hoon H. Inhibitory activity of Chinese Medicinal plants on Nitric oxide synthesis in Lipopolysaccharide activated macrophages. J Appl Pharmacol 2001;9:183-7.
5. Brune K, Schmid L, Glatt M, Minder B. Correlation between antimicrobial activity and peroxidase content of leukocytes. Nature 1973;245:209-10.
6. Hudson L, Hay FC. Practical Immunology, 2nd ed. Oxford: Blackwell Scientific Pub; 1980. p. 73-82.
7. Ponkshe CA, Indap MM. *In vivo* and *in vitro* evaluation for immunomodulatory activity of three marine animal extracts with reference to phagocytosis. Indian J Exp Biol 2002;40:1399-402.
8. Kim YC, Lee EH, Lee YM, Kim HK, Song BK, Lee EJ, *et al*. Effect of the aqueous extract of *Aquilaria agallocha* stems on the immediate hypersensitivity reactions. J Ethanopharmacol 1997;58:31-8.
9. Toda M, Okubo S, Ikigai H, Suzuki T, Suzuki Y, Shimamura T. The protective activity of tea against infection by *Vibrio cholerae* O1. J Appl Bacteriol 1990;70:109-12.
10. Lanhers MC, Fleurentin J, Cabalion P, Rolland A, Dorfman P, Misslin R, *et al*. Behavioural effects of *E. hirta* L. Sedative and anxiolytic properties. J Ethanopharmacol 1990;29:189-8.
11. Rezaeipoor R, Saeidnia S, Kamalinejad M. Immuno suppressive activity of *Achillea talagonia* on humoral immune responses in experimental animals. J Ethanolpharmacol 1999;65;273-6.
12. Vijaya K, Ananthan S. Effect of three Indian medicinal plants on enteropathogens. Biomedicine 1994;14:21-3.
13. Farnsworth NR. Biological and phytochemical screening of plants. J Pharm Sci 1966;55:225-75.

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