Assessment of Immunomodulatory Activity of *Euphorbia hirta* L.

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Ramesh and Padmavathi: Immunomodulatory Activity of *Euphorbia hirta* L.

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
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 $2 \times 10^8$ 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 ($\times 100$) 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.,

$$\text{Stimulation (\%)} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI (control)}} \times 100$$

The change in macrophage phagocytic 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.

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
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\textsuperscript{[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>
<thead>
<tr>
<th>CONCENTRATION (mg/ml)</th>
<th>PHAGOCYTIC INDEX</th>
<th>% IMMUNOSTIMULATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>43</td>
<td>23.30</td>
</tr>
<tr>
<td>250</td>
<td>66</td>
<td>57.10</td>
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<td>125</td>
<td>78</td>
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<td>62.5</td>
<td>83</td>
<td>97.60</td>
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<td>31.25</td>
<td>80</td>
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<tr>
<td>15.60</td>
<td>65</td>
<td>54.80</td>
</tr>
<tr>
<td>7.80</td>
<td>61</td>
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</tr>
<tr>
<td>1.95</td>
<td>47</td>
<td>11.90</td>
</tr>
<tr>
<td>0.98</td>
<td>44</td>
<td>4.80</td>
</tr>
<tr>
<td>0.48</td>
<td>33</td>
<td>-21.40</td>
</tr>
<tr>
<td>0.24</td>
<td>31</td>
<td>-26.10</td>
</tr>
<tr>
<td>0.12</td>
<td>25</td>
<td>-40.50</td>
</tr>
<tr>
<td>0.06</td>
<td>21</td>
<td>-50.00</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>TIME (MIN)</th>
<th>CONTROL 40 MG/KG</th>
<th>40 MG/KG</th>
<th>80 MG/KG</th>
<th>160 MG/KG</th>
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<tbody>
<tr>
<td>3</td>
<td>0.31±0.005</td>
<td>0.55±0.024</td>
<td>0.33±0.017</td>
<td>0.41±0.017</td>
</tr>
<tr>
<td>6</td>
<td>0.87±0.016</td>
<td>0.72±0.027</td>
<td>0.77±0.014</td>
<td>0.43±0.015</td>
</tr>
<tr>
<td>9</td>
<td>0.64±0.022</td>
<td>0.87±0.004</td>
<td>0.84±0.031</td>
<td>0.81±0.019</td>
</tr>
<tr>
<td>12</td>
<td>0.48±0.024</td>
<td>0.64±0.018</td>
<td>0.62±0.014</td>
<td>0.61±0.039</td>
</tr>
<tr>
<td>15</td>
<td>0.47±0.014</td>
<td>0.57±0.012</td>
<td>0.57±0.008</td>
<td>0.48±0.014</td>
</tr>
<tr>
<td>18</td>
<td>0.46±0.018</td>
<td>0.43±0.005</td>
<td>0.51±0.015</td>
<td>0.47±0.040</td>
</tr>
</tbody>
</table>

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

Table 1: IN VITRO PHAGOCYTOSIS EVALUATION

Table 2: IN VIVO EFFECT OF ETHANOLIC EXTRACT OF EUPHORBIA HIRTA ON MACROPHAGE PHAGOCYTIC ACTIVITY
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 immunomodulatory 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 myeloperoxidase. 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

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Determination of Site of Absorption of Propranolol in Rat Gut Using In Situ Single-Pass Intestinal Perfusion

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Nagare, *et al*.: Determination of Site of Propranolol Absorption in Rat Gut

Previously, permeability and site of intestinal absorption of propranolol have been reported using the Ussing chamber. In the present study, the utility of Single-Pass Intestinal Perfusion to study permeability and site of intestinal absorption of propranolol was evaluated in rats. Drug permeability in different regions of rat intestine viz. duodenum, jejunum, ileum and colon was measured. Propranolol (30 μg/ml) solution was perfused *in situ* in each intestinal segment of rats. Effective permeability (Peff) of propranolol in each segment was calculated and site of absorption was determined. The Peff of propranolol in rat duodenum, jejunum, ileum and colon was calculated to be 0.3316×10^{-4} cm/s, 0.4035×10^{-4} cm/s, 0.5092×10^{-4} cm/s and 0.7167×10^{-4} cm/s, respectively. The above results suggest that permeability of propranolol was highest through colon compared to other intestinal sites, which is in close agreement to that reported previously. In conclusion, *in situ* single pass intestinal perfusion can be used effectively to study intestinal permeability as well as site of intestinal absorption of compounds in rats.

Key words: Intestine, permeability, propranolol, single-pass intestinal perfusion, site of absorption

*Accepted 15 September 2010*

*Revised 01 April 2010*

*Received 29 August 2009*

*Indian J. Pharm. Sci., 2010, 72 (5): 621-625*