Stimulation of Immune Function Activity of the Extract of Heliotropium Indicum Leaves

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Citation

Abstract
The objective of present study is to assess the immunostimulatory activity of H. indicum, using different in vitro and in vivo experimental models. The immunostimulatory potential of the test compound was investigated by in vitro phagocytic index and lymphocyte viability tests using interferon α-2b, a standard immunostimulant drug. Other tests such as carbon clearance, antibody titer and delayed type hypersensitivity were studied in mice using levimasole as the standard drug. The dried leaves extract (200mg/ml) significantly increased the in vitro phagocytic index and lymphocyte viability in all assays. They also showed a significant increase in antibody titer, carbon clearance and delayed type hypersensitivity in mice. To conclude H. indicum exhibited a dose-dependent immunostimulant effect, which could be attributed to the alkaloid content or due to the combination with other component(s).

INTRODUCTION
Heliotropium indicum (Boraginaceae) - commonly called as Indian Turnsole, is a herb with slightly woody at base. It is distributed in the tropical and temperate regions of the world and found throughout India [1]. The whole plant is claimed to possess medicinal properties. In ayurveda the juice of leaves applied on boils, pimples, ulcers, sores and wounds to cure. In Belize, the plant used for diarrhea, malaise or vomiting in infants. The leaves are used for the treatment of ophthalmic disorders, erysipelas, pharyngodynia, and anti-inflammatory, anti-tumor. The roots are used as astringent, expectorant and febrifuge. The extract of leaves was proved to be active against Schwartz’s leukemia, wound healing and anti-inflammatory activities [2].

Immunostimulators have been known to support T-cell function, activate macrophages and granulocytes, and complement natural killer cells apart from affecting the production of various effector molecules generated by activated cells (Paraimmunity) [3]. It is expected that these non-specific effects offer protection against different pathogens, including bacteria, fungi, viruses and so on, and constitute an alternative to conventional chemotherapy [4]. Immunostimulatory therapy is now being recognized as an alternative to conventional chemotherapy for a variety of disease conditions, involving the impaired immune response of the host [5]. In view of the above, the present investigation was undertaken to evaluate the immunostimulatory potential of H. indicum leaves, using in vitro and in vivo models.

MATERIALS AND METHODS
PLANT EXTRACT
The fresh leaves of H. indicum were collected from the Udupi district, Karnataka between September-October. The leaves were authenticated by Prof Gopal Krishna Bhat, Department of Botany, Poorna Prajna College Udupi, India. A voucher specimen was preserved in our laboratory for future reference. The fresh leaves were shade-dried and reduced to a fine powder with a mechanical grinder. The powdered leaves are stored in an airtight, hard polyethylene container with silica pouch up to 10-12 days. About 700 g of dry powder was extracted with chloroform using soxhlet apparatus. Freshly prepared H. indicum extract was subjected to phytochemical screening tests for the detection of various constituents using conventional protocol [6]. The extract was concentrated to dryness using a rotary evaporator attached to a vacuum pump and stored at a temperature of -4°C until use. The yield was 7 g, in a dark brownish green residue form.

ANIMALS
Swiss albino mice (17-25 g) of either sex are procured from Indian Institute of Sciences. They are maintained under...
standard conditions (temperature 22 ± 2°C, relative humidity 60±5% and 12 h light/dark cycle). The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding. They had free access to standard pellet diet and water ad libitum. The Institutional Animal Ethics Committee approved the experimental protocol. All the animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the “National Academy of Sciences” and published by the “National Institute of Health”.

**DRUGS AND CHEMICALS**

EDTA, RPMI-1640, Hank's balanced salt solution (HBSS), dextran, phosphate buffered saline, fetal calf serum, streptomycin, penicillin, amphotericin, and Trypan blue were purchased from Himedia. Phytohaemagglutinin, ficoll hypaque and L-glutamine were purchased from Sigma Diagnostic, USA. Interferon α-2b and levimasole were obtained as gift samples from Fulford (I) Ltd. and Khandelwal Laboratories Ltd., Mumbai, respectively.

**ANTIGEN**

Fresh blood was collected from a healthy sheep from the local slaughter house. Sheep red blood cells (SRBCs) were washed thrice with normal saline and adjusted to a concentration of 0.1 ml containing 1X10^8 cells for immunization and challenge.

**IN VIVO CARBON CLEARANCE TEST**

The mice were divided into six groups, each consisting of 10 animals. Group I (Control) was given 2% gum acacia in water (0.3 ml/mouse, p.o) for 7 days, Group II-VI were given different concentrations of chloroform extract (50-200mg/kg, p.o.) and standard drug (Levimasole 50 mg/kg, p.o.) respectively for 7 days. At the end of 7 days, the mice were injected carbon ink suspension (10 µl/g body weight) through tail vein. Blood samples were drawn (in EDTA solution 5 µl), from the retro orbital vein, at intervals of 0 and 15 min, a 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and the absorbance measured at 660 nm. The carbon clearance was calculated using the following equation: \( \frac{\log OD_1 - \log OD_2}{15} \), where OD_1 and OD_2 are the optical densities at 0 and 15 min, respectively [6].

**IN VIVO HUMORAL ANTIBODY (HA) TITER AND DELAYED TYPE HYPERSENSITIVITY (DTH)**

**RESPONSE HUMORAL ANTIBODY (HA)**

The mice were divided into six groups, each consisting of 6 mice. Group I (Control) was given 2% gum acacia (0.3 ml/mouse) for 7 days, Group II-VI were given drug treatment which was exactly the same as with the carbon clearance test. The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing 1x10^8 cells, intraperitoneally on day zero. Blood samples were collected in micro centrifuge tubes from individual animals of all the groups by retro orbital vein puncture on day 8. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the haemagglutination technique [7]. Briefly, equal volumes of 50µl individual serum samples of each group were pooled. Serial two-fold dilutions of pooled serum samples were made in 50µl volumes of RPMI-1640 in micro titration plates. To this 50 µl of 1% suspension of SRBC in RPMI-1640 was added. After mixing, the plates were incubated at 37ºC for 1 h and examined for haemagglutination under the microscope (button formation). The reciprocal of highest dilution, just before the button formation, was observed and titre values were calculated.

**DELAYED TYPE HYPERSENSITIVITY TEST (DTH)**

On Day 8, the thickness of the right hind footpad was measured using Vernier calipers. The mice were then challenged by injection of 1x10^8 sub SRBCs in the right hind footpad. The footpad thickness was measured again after 24 h of challenge. The difference between the pre- and post challenge footpad thickness, expressed in mm, was taken as a measure of the DTH response [8].

**IN VITRO PHAGOCYTIC INDEX**

**PREPARATION OF MICROORGANISM**

Escherichia coli 832 (E. coli) was grown and kept on a slope of solid agar medium. Before use, the microorganism was cultured in 100 ml of 2.5% nutrient broth (oxoid) for about 18 h at 37°C. The culture was then washed twice with phosphate buffer saline and re-suspended in gelatin-HBSS to a concentration of 1x10^7 sub cells/ml. During each experiment, the number of viable microorganisms was determined microbiologically by counting colony forming units (cfu), using nutrient agar plates after incubation, at 37°C for 18 h [9].

**PREPARATION OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES**
Human blood was collected from a local blood bank and the RBCs removed by sedimentation in 5% (w/v) solution of dextran in buffered saline (m.w. 200,000; 3 ml of solution to 10 ml of blood) for 30 min at 37°C. The PMNC-rich supernatant layer was washed twice with heparin-saline, concentrated by centrifugation (10 min at 110 g), counted with a hemocytometer, and suspended in gelatin-HBSS to make up a concentration of 1x10⁷ cells/ml.

**MICROBIOLOGICAL ASSAY FOR THE PHAGOCYTOSIS**

To assess phagocytosis, different concentrations of chloroform extract (50-200µg/ml) and the standard drug, Interferon α-2b (0.5 million IU), in the final volume of 0.1ml, were incubated respectively with 2 ml of the PMNCs suspension (1x10⁷ sub cells/ml), 2 ml of the suspended microorganisms (1x10⁷ cells/ml) and 0.4 ml of fetal calf serum at 37°C for 1h in 5% CO₂ atmosphere in a slanting position. At 30 min intervals up to 120 min, 0.5 ml aliquot of the suspension was removed and added to 1.5 ml of the ice-cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 110 g for 4 min. Under this condition, the non-ingested microorganisms remained in the supernatant fluid. The viable count of the microorganisms was undertaken using the colony counter [9].

Phagocytosis was expressed as the percentage decrease in the initial number of viable extra cellular bacteria according to the formula: 

\[
P(t) = \left(1 - \frac{N_t}{N_0}\right) \times 100,
\]

where P(t) is the phagocytic index at time t, N₀ and Nₜ are the number of viable extra cellular bacteria at time t = 0 and t = 30, 60, 90 and 120 min, respectively [10].

**IN VITRO CELL PROLIFERATION ASSAY**

This test was performed with peripheral mononuclear blood cells, following their separation from the blood by using ficoll-hypaque gradient centrifugation, according to manufacturer's instructions (Sigma Diagnostic, USA). The rate of proliferation of mononuclear cells, under the influence of mitogens, was measured [11]. Under sterile conditions, the cells were diluted to 1x10⁷ sub cells/ml with RPMI-1640 (supplemented with 20% fetal calf serum). The cell suspension (2 ml) was transferred into a sterile culture tube and to each sample. Different concentrations of the plant extract (50-200µg/ml, filtered through 0.22 µ pore size filter) and standard drug Interferon α-2b (0.5 million IU), in the final volume of 0.1 ml, were added, respectively. The proliferation of cells was induced by 50 µl phythaemagglutinin (PHA, 0.1 mg/ml). The prepared samples were incubated for 72 h at 37°C in a CO₂ atmosphere, supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin and 0.25 µg/ml amphotericin. The control incubated with cells minus the plant extract. The viability of the cells was assessed after incubation with test compounds, using the Trypan blue dye exclusion method [12]. Briefly, 20 µl of the incubation mixture was mixed with 20 µl of Trypan blue dye. The total number of mononuclear cells and mononuclear stained blue (dead cells) were counted under an inverted microscope (Olympus, Japan), using the hemocytometer. The percentage of cell viability was taken as a measure of cell proliferation and calculated as per the following formula.

![Figure 1](image1.png)

Similarly, the percentage of cell stimulation was calculated as per the following formula [13].

![Figure 2](image2.png)

**STATISTICAL ANALYSIS**

Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test. The significance in difference was accepted at P <0.05.

**RESULTS**

The chloroform extract, 50-150mg/kg, p.o. exhibited a significant increase in carbon clearance from the blood in a dose-dependent manner (Table1). The higher dose of test drug (200mg/kg) shown maximum carbon clearance was seen.

The results (Table 1) also indicate that animals treated with 50, 100,150 and 200mg/kg of chloroform leaves extract and standard drug levamisole (50mg/kg) produced a significant increase in HA titer (humoral immunity) as evident from haemagglutination after incubation of serum with SRBCs.

In the DTH response (cell mediated immunity) test, the chloroform extract at higher doses (150 and 200mg/kg) and the standard drug levimisole (50mg/kg) showed a statistically significant increase in mean paw edema in mice.
compared to control animals.

**Figure 3**
Table 1: Effect of Heliotropium indicum leaves extract on immunostimulatory activity in mice

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Carbon clearance</th>
<th>HA titer</th>
<th>DTH response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (2% ascia)</td>
<td>0.068±0.012</td>
<td>0.48±0.21</td>
<td>0.38±0.10</td>
</tr>
<tr>
<td>Extract (50)</td>
<td>0.12±0.016*</td>
<td>185±3.31*</td>
<td>0.86±0.13</td>
</tr>
<tr>
<td>Extract (100)</td>
<td>0.132±0.014*</td>
<td>172.7±1.6*</td>
<td>0.28±0.13</td>
</tr>
<tr>
<td>Extract (150)</td>
<td>0.164±0.016*</td>
<td>282.5±1.6*</td>
<td>0.45±0.10*</td>
</tr>
<tr>
<td>Extract (200)</td>
<td>0.156±0.018*</td>
<td>320±1.6*</td>
<td>0.48±0.21*</td>
</tr>
<tr>
<td>Levamisole (50)</td>
<td>0.14±0.016</td>
<td>430.6±8.3*</td>
<td>0.57±0.21*</td>
</tr>
<tr>
<td>One-way F</td>
<td>230</td>
<td>521</td>
<td>6.32</td>
</tr>
<tr>
<td>ANOVA P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=6 in each group; df=7, 40; *P<0.05 in comparison with control. (+) indicates increase and (-) indicates decrease in cell stimulation.

**Figure 4**
Table 2: Effect of Heliotropium indicum leaves extract on phagocytic index of polymorphonuclear leukocyte

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Phagocytic Index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (2% ascia)</td>
<td>61.14±1.56</td>
</tr>
<tr>
<td>Extract (50)</td>
<td>63.53±1.52*</td>
</tr>
<tr>
<td>Extract (100)</td>
<td>64.12±1.18*</td>
</tr>
<tr>
<td>Extract (150)</td>
<td>67.38±1.24*</td>
</tr>
<tr>
<td>Extract (200)</td>
<td>69.54±1.32*</td>
</tr>
<tr>
<td>Interferon α-2b</td>
<td>70.27±1.64*</td>
</tr>
<tr>
<td>One-way F</td>
<td>1.123</td>
</tr>
<tr>
<td>ANOVA P</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=6 in each group; df=7, 40; *P<0.05 in comparison with control.

**DISCUSSION**

The present study established the immunostimulatory activity of the chloroform extract of *H. indicum*. Prophylactic treatment of *H. indicum* enhanced the rate of carbon clearance from the blood (more than a two-fold increase) when compared with the control group. The result is owing to a mechanism related to phagocytosis by macrophages. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b, leading to a more rapid clearance of foreign particulate matter from the blood [10]. *H. indicum* was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance.

The chloroform extract, at a dose of 200mg/kg, body weight showed almost a four-fold increase in HA titer, compared to untreated controls. This could be due to the presence of alkaloids which augment the humoral response, by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis [8]. The DTH response, which directly correlates with cell-mediated immunity (CMI), was found to be the highest at the maximum dose tested in the leaves extract (150mg/kg). The mechanism behind this elevated DTH during the CMI responses could be due to sensitized T-lymphocytes. When challenged by the antigen, they are converted to lymphoblasts and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction [13]. The infiltrating cells are probably immobilized to promote defensive (inflammatory) reaction [14]. An increase in DTH response indicates that the leaves extract of *H. indicum* and
Stimulation of Immune Function Activity of the Extract of Heliotropium Indicum Leaves

its isolated compound have a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction \([13]\).

The in vitro immunostimulatory activity of the chloroform extract was tested on human polymorphonuclear and mononuclear cells. The phagocytosis and intracellular killing of microorganisms by polymorphonuclear phagocytes was determined by the direct measurement of the microbicidal activity \([15]\). Phagocytosis was expressed as the phagocytic index, in which the percentage decrease in the initial number of viable extra cellular bacteria was determined microbiologically after incubation with polymorphonuclear leukocytes. In our study, the phagocytic index of H. indicum leaves extract was found to be increased in a time and dose dependent manner. The leaves extract (200µg/ml) showed significant phagocytic index as compared to control.

Further, the immunostimulatory effect of the extract was tested in mitogen-activated cultured mononuclear cells. PHA was used to activate the mononuclear cells in the culture. The mitogenic PHA are polyclonal activators, in that they activate mononuclear cells including memory type cells, irrespective of their antigenic specificity \([16]\). The leaves extract (higher concentrations) caused a significant stimulation of the mononuclear cells. This is attributed to the fact that the chloroform extract may stimulate the PHA-activated mononuclear cells and induce the release of cell proliferating factors such as interleukin and TNFα \([11]\).

Recent reports indicate that several types of alkaloids stimulate human peripheral blood leukocyte proliferation. They significantly increase the activity of helper T cells, cytokines, interleukin 2, g-interferon and macrophages and are thereby useful in the treatment of several diseases caused by immune dysfunction \([13]\). It is thus apparent that the immunostimulatory effect produced by the chloroform extract of H.indicum, containing Indicine N-Oxide, may be due to cell mediated and humoral antibody mediated immune responses.

The present finding provides scientific evidence to the ethno medicinal use of this plant by tribal. The plant H.indicum has the potential for new therapeutic applications in the future.

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References

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