Immunomodulatory Properties of the Aerial parts of Local Populace of *Hypericum perforatum* (Ethanolic extract) in Balb/c mice

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ABSTRACT

Aerial parts of the *Hypericum perforatum*, commonly known as St.Johns wort belonging to Family Guttiferae was evaluated for its immunomodulatory properties. *Hypericum perforatum* obtained from higher reaches of Kangdoori, Gulmarg was administered orally at doses of 50, 100 and 200 mg/kg body weight day to healthy Balb/c mice divided into six groups consisting of six animals each. The assessment of immunomodulatory activity was carried out by testing the humoral (antibody titre) and cellular (Delayed type hypersenstivity reaction) immune responses to the antigenic challenge by Sheep Red Blood Corpuscles (SRBCs).

On oral administration the successive ethanol extract was found to exhibit a dose related increase in the hypersensitivity reaction, to the SRBC antigen, at concentrations of 100 and 200 mg/kg body weight. It also resulted in a significant increase in the antibody titer value, to SRBC, at doses of 100 and 200 mg/kg in animal studies. The successive ethanol extract was found to stimulate cell mediated and antibody mediated immune responses in mice. There was increase in immunostimulation compared to control group and this difference was statistically significant.

Key words: *Hypericum perforatum*, haemagglutinating antibody titre, delayed type hypersensitivity, cell mediated immunity, sheep RBC

INTRODUCTION

Immunomodulation using plant-material can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the condition of impaired immune response (Srikumar *et al.*, 2006).There are many plants, which are having immunostimulatory where as other have immunosuppressant activity (Oladunmoye, 2007). The plant products have long been used as

immunomodulators by the traditional healers. Scientific literature is continuously reporting plant drugs having immunomodulatory activity (GangXle *et al*., 2008).The modulation of immune response with the aids of various medicinal plants in order to alleviate certain diseases is hence an active area of interest.

Hypericum perforatum belongs to family Guttiferae. The genus *Hypericum* comprises approximately 400 species of which several species have been used in folk medicine. There is a growing interest in constituents of this genus because a number of species have been found to harbour biological properties. *Hypericum perforatum* itself is an herbaceous, perennial plant native to Europe and Asia (Bilia *et al*., 2002). It is also called as St. Johns Wort and is a long lived, wild growing herb that has been used for centuries to treat a variety of ailments (Di Carlo *et al*., 2001). Tradionally, Hypericum extracts were used both externally for the treatment of inflammation, wounds, skin disease and internally for the treatment of anxiety, headache, mild to moderate depression, bedwetting, neuralgia and inflammation,

At the global level it is also used to treat anxiety, seasonal affective disorders and sleep disorders (Linde *et al*., 1996; Gaster and Holroyd, 2000). It has been reported that hypericum contains unique natural products, *hypericicn, pseudohypericin* and *hyperforin* (Bilia *et al*., 2002) Biochemically hyperforin is *prenyalted acylpholoroglucinol* (polyphenol) It is reported to be unique among all antideprssants for being a potent uptake inhibitor of three nuerotransmiiters, serotonin, noradrenaline and dopamine (Nathan, 2001).Certain constituents of *Hypericum perforatum* have been shown to produce significant anti-inflammatory effects based on carragenean induced edema models. Further *Hypericum perfoartum* extracts have been shown to exhibit significant inhibitory effects on 5-LOX and Cycloxygenase enzyme (Albert *et al*., 2007). Literature survey revealed that no scientific investigation has been made in regard to the immunomodulatory activity of *H. perforatum* . Therefore the aim of the present study was to evaluate the *H. perforatum* for immunomodulatory activity in said experimental models.

MATERIAL AND METHODS

Plant Material

Collection

Hypericum perforatum was collected from higher reaches of Kangdoori, Gulmarg (2310-2650 mts in height) in the month of May-June. The plant was identified at Department of Botany, Universityof Kashmir, Srinagar.

Extraction

The authentically identified plant material was shade dried and then powdered. Powdered plant material (1 kg) was subjected to Soxhlet extraction with absolute ethanol for 48 hours. The ethanolic extract was then evaporated under reduced pressure using a rotary flash evaporator. The percentage yield of ethanol extract was 17 gms. The crude extract was stored at 4°C for experimental use. The test materials for experimentation were prepared as fresh suspensions each time using 1% sterile gum acacia. The material was ensued to be free from pathogens, aflatoxins, pesticidal residues and heavy metals according to WHO guidelines of purity and safety (WHO, 1998).

Animals and Treatment

The study was conducted on male Balb/c mice obtained from healthy animal colony at the Department of pharmacology, IIIM. Balb/c mice (male, 3-4 weeks old) were randomly distributed in groups as per experimental protocols (n=6), weighing 18-22gms. The ethical committee of the regional research laboratory (CSIR) instituted for animal handling approved all protocols. The animals were bred and maintained under standard laboratory conditions; temperature (25°C) and photoperiod of 12 hours. Commercial pellet diet and water were given ad libitum.

Antigenic Stimulus

Preparation of B and T cell dependant SRBC antigen

Fresh sheep blood was extracted aseptically from the jugular vein and stored in Alsevers solution. SRBCs collected in Alsevers solution were washed three times in large volumes of pyrogen free 0.9% normal saline at 2000 rpm for 10 minutes.

Treatment

Animals were divided into six groups of 5 animals each: Group I: Normal control: Received *normal saline*. Group II: Vehicle control: Received *1% gum acacia*. Group II: Positive control: Received *Levamisole (immunostimulant)*. Group III: Negative control: *Received Cyclophosphomide (immunosuppressant)*. Group IV: Received ethanolic extract *H. perforatum, 50mg/kg body weight*. Group V: Received ethanolic extract *H. perforatum, 100mg/kg body weight*. Group VI: Received ethanolic *extract H. perforatum, 200mg/kg body weight*.

Normal and vehicle control mice received normal saline and 1% gum acacia administered orally while the negative and positive control received

cyclophosphomide and levamisole administered orally. The ethanolic extracts of *H. perforatum* were dissolved in 1% gum acaia and was administered orally for 14 days. The dose volume was 0.2 ml (200µl).

Haemaggultnation titre (In vivo effect of SRBC on specific humoral immune response)

The animals were immunized by injecting 0.2ml of 10% of fresh SRBC suspension intraperitoneally on DAY 0. Blood samples were collected in micro centrifuge tubes from individual animals by retro-orbital plexus on DAY 7 for primary antibody titre and for secondary antibody titre on DAY 15. Serum was separated and antibody levels were determined by Standard Hamgglutination Test (Nelson and Midenhall, 1967). After mixing the plates were incubated at room temperature for 1 hour and examined for haemaggltination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre. The mean titre values of the drug treated groups were compared of the control.

Delayed type hypersensitivty reaction (In vivo effect of SRBC specific on cell mediated immune response)

Doherty's (1981) method was employed to assess SRBC induced Delayed type hypersensitivity (DTH) response in mice.On DAY 7th SRBC primed mice were challenged and immunized with giving 50µl of SRBC antigen (5×10^8 cells) in right hind footpad and 50µl of Normal Saline was given in left hind footpad. The plant extract (drug) was administered 2 hr after SRBC injection and once daily on consecutive days. The foot pad thickness was measured using speromicrometer (0.01mm pitch) after 24hrs, 48hrs and 72hrs. The thickness of the left hind paw was taken as control. A graph was plotted between DTH thickness and time period at 0hrs, 24hrs, 48hrs and 72hrs.

Statistical Analysis

The experimental results were expressed as mean ±standard error of mean (SEM) of six experiments .Where applicable, the data were subjected to one way analysis of variance (ANOVA). P values <0.05 were regarded as significant and P values <0.001 as very significant.

RESULTS

Hypericum perforatum was evaluated for immunomodulatory effect. The immunomodulatory influence of the ethanolic extract of *H. perforatum* was

explored in vivo in Balb/c mice through modulation of both B-cell and T-cell activation in relation to serum antibody titres and delayed type hypersensitivity response against SRBC antigen.The treatment with *Hyp* induced marked enhancement of humoral (Table 1) and DTH response in the animals (Fig.1). From the study it may be inferred that *Hyp* promotes immunomodulation and thus rationalizing its traditional claim.

Table 1. ***In vivo effect of H. perforatum on Humoral response***

Treatment	Doses (mg/kg)	Primary antibody titre(IgM) Mean±SEDAY 7	Stimulation index (% change)	Secondary anti-body titre (IgG) Mean±SEDAY 15	Stimulation index (%) change)
Control SRBC	-	7.6	-	6.8	
Levamisole (Positive control)	2.5	10.6 (↑)***	42.10 (↑)	9.2(↑)***	35.29 (↑)
Cylophosphomide (Negative control)	100	5.2 (↓)***	15.38 (↓)	4.4(↓)**	35.29(↓)
HYP -50	50	8 ***±0.09	7(↑)	7.9 ***±0.11	7.9(↑)
HYP-100	100	8.9 **±0.13	14(↑)	8.3 ***±0.06	10(↑)
HYP-200	200	9.3 ***±0.07	23(↑)	9 ** ±0.17	19(↑)

Each value represents mean ±SEM of six experiments and statistically significant P values :
**** P<0.001 with respect to their control; **P<0.01 with respect to their control; * P<0.05 with respect to their control*

The modulation of the humoral response was evaluated after immunization with SRBC antigen by determination of Primary and Secondary antibody titres at Day 7 and Day 14 .The cell mediated response was assessed using DTH reaction post immunization with SRBC after a period of 24 hours ,48 hours and 72 hours.

Assessment of the Humoral Response (Primary and Secondary Antibody Synthesis)

The Ethanolic extracts were tested for any possible role of B-cell activation by determination of Haemagglutination titer (Doherty, 1981). This is a universally accepted model to screen the modulation of the humoral immune response by any agent. The extract was tested in vivo conditions at doses of 50 mg/kg, 100 mg/kg and 200 mg/kg b.w and was assessed by determination of primary antibody levels, IgM and secondary antibody levels IgG at Day 7 and Day 14. Results as summarized in Table 1 and Fig. 1 demonstrate that all the concentrations of the extract exhibited some level of immunostimulation and a dose dependant increase was observed for both the primary and secondary antibody titres. Levamisloe and

cyclophoshomide were used as immunostimulatory and immunosuppressive controls respectively

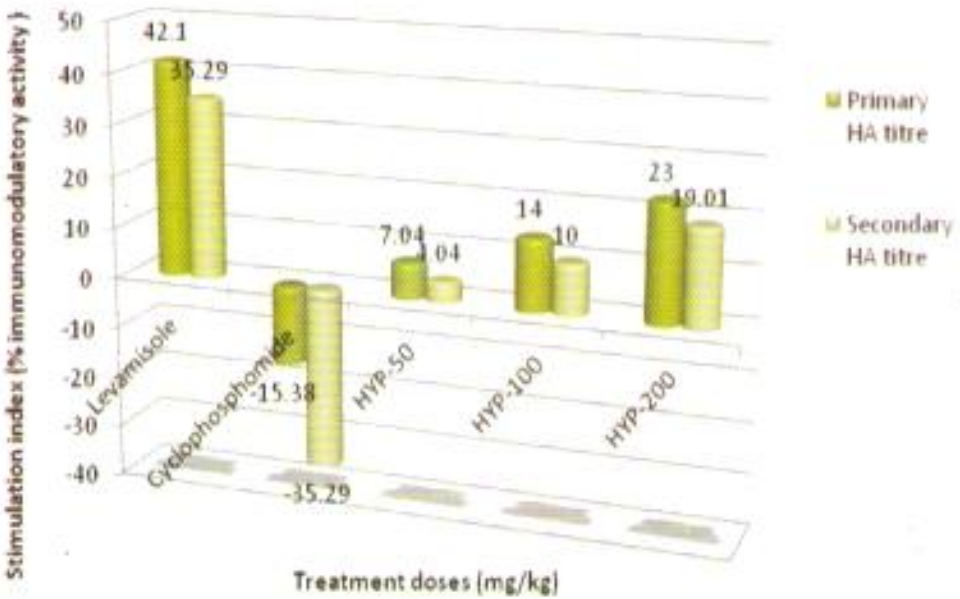

Fig. 1. Comparative stimulation index of H. perforatum for HA titre
(Each value represents mean ±SEM of six experiments)

Asssesment of the Cell mediated response (Delayed Type Hypersensitivty)

The ethanolic extract was tested in vivo conditions at doses of 50 mg/kg, 100 mg/kg and 200 mg/kg b.w in Balb/c mice and the DTH response was recorded following immunization on day 14 after a time period of 24 hours, 48 hours and 72 hours. Fig. 2 and 3 depicts the DTH response in comparison to levamisole and cylophosphomide. A significant dose dependant increase in footpad thickness was found at 24 hours, 48 hours and 72 hours. The food pad thickness after 24 hrs increased from 0.94 to 1.11 at a dose of 50 mg/kg but significantly decreased from 1.06 to 0.89 in a dose range of 100-200 mg.kg b.w (Fig. 2). Maximum effect was found to be at 24 hours and minimum after 72 hours. At a dose of 50 mg/kg of *H. perforatum* maximal immunostimulatory effect was observed (Fig. 3).

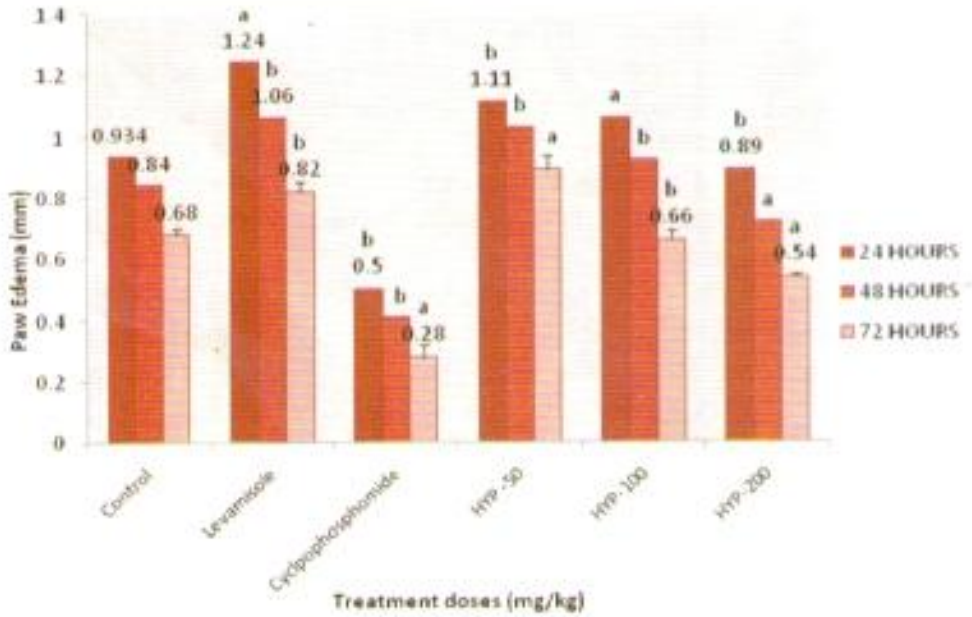

Fig. 2. *In vivo* Effect of *H. perforatum* on Delayed Type Hypersensitivity (Cell Mediated Response)

Each value represents mean ±SEM of six experiments and statistically significant P values : [a]P<0.001 with respect to their control; [b]P<0.01 with respect to their control; [c]P<0.05 with respect to their control)

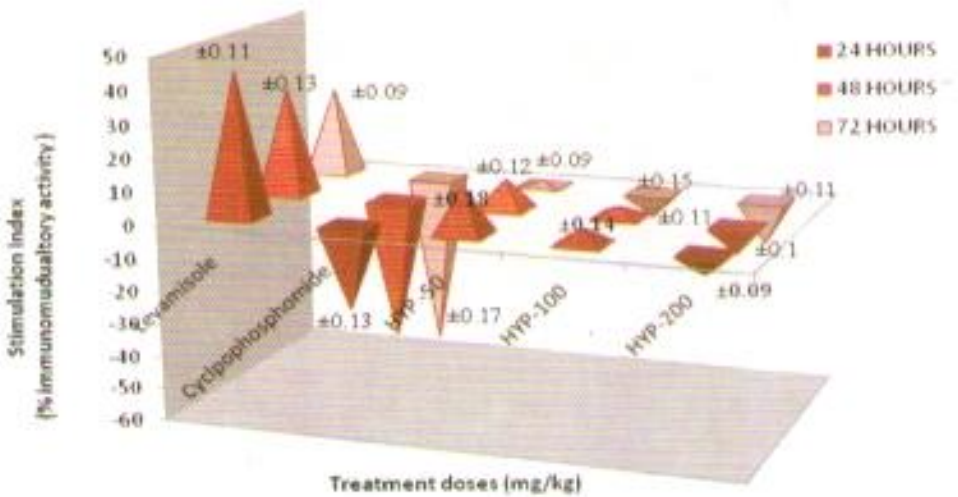

Fig. 3. Stimulation Index of *H. perforatum* on Delayed Type Hypersensitivity (Cell Mediated Response)

(Each value represents mean ±SEM of six experiments)

DISCUSSION

Immunomodulatory agents of plant enhance the immune responsiveness of an organism against a pathogen by activating the immune system. However these agents should be subjected to systematic studies to substantiate the therapeutic claims made with regard to their clinical utility. In the present study *Hyp* when orally administered, significantly produced immunostimulant effects on both humoral and cell mediated responses.

The augmentation of the humoral response was evidenced by an enhancement of antibody responsiveness to SRBC in mice as consequence of both pre and post immunization protein treatment indicates the enhanced responsiveness of macrophages and B-lymphocytes subsets involved in antibody synthesis (Mungantiwar, *et al.*, 1999). The DTH response,which is a direct correlate of Cell Mediated Immunity (CMI), was found to be increased by the administration of *Hyp*. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive. In out studies, foot volume was enhanced after *H. perforatum* treatment suggests cell mediated enhancement (Sen *et al.*, 1992). Increase in both, HA titre and DTH response indicated the *H. perforatum* potentiates humoral as well as the cellular immunity. One of the explanations forwarded to justify the beneficial effects of indigenous plant extracts in disease states is the non specific enhancement of immune states of the organism (Patil *et al.*, 1998). In conclusion, the results obtained in the present study have shown the immunomodulatory activity of *Hyp in vivo*, further studies are warranted for understanding the exact mechanisms responsible for immunomodulation.

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