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Immunomodulation by *Hibiscus rosa-sinensis*: Effect on the Humoral and Cellular Immune Response of Mus Musculus

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Abstract: In West India, the *Hibiscus rosa-sinensis* L. (Malvaceae) is traditionally used as tea as a natural diuretic. Extract of this plant contains Vitamin C and is used traditionally as a mild medicine. In spite of a long history of traditional medicinal value of *H. rosa-sinensis* still no data are available for immunomodulatory activity. In present investigation, aqueous extract of *H. rosa-sinensis* (AEHrs) (500 mg kg^{-1} BW) was intraperitoneally (IP) injected to the male Swiss albino mice (Mus musculus) to evaluate the immunomodulatory property of extract. In addition for evaluation of phytochemical constituents of flowers of *H. rosa-sinensis* HPTLC was performed. The crude extract of *H. rosa-sinensis* has immunomodulatory activity. After the 15 days treatment, the number of plaque forming cells increased by 0.6%, antibody titre was increased 38.15% and significant increase of 52% was observed in DTH response. At the same concentration of dose the level of serum IL-1α enhanced significantly (14.27%) whereas a considerable decrease (32.70%) in the concentration of IL-2 was observed among AEHrs treated mice in comparison to the control mice. HPTLC chromatogram revealed that *H. rosa-sinensis* posses alkaloid (Rf 0.93) and flavonoids (Rf 0.02, 0.06, 0.14) on the basis of Rf values. Results of investigation supports for the immunomodulatory activity of *H. rosa-sinensis* aqueous extract.

Key words: Delayed type hypersensitivity, high performance thin layer chromatography, *Hibiscus rosa-sinensis*, interleukin-1α, interleukin-2, immunomodulatory, plaque forming cells

INTRODUCTION

The term immunity infers protection from diseases. Immune system is persistently vigilant in regard to regulate itself to ensure that its cellular components behave and interact symbiotically to generate protective immune responses that ensure good health (Coico *et al*., 2003).

Immunity may be of innate or acquired type. Innate immunity conferred by all those elements which is present in body from the time of birth of an individual and protects at very short notice while adaptive immunity is more specified than innate and it supplement the protection provided by the innate immunity. Adaptive immunity is compressed of humoral and cellular immunity. Immune system can be modulated by immunostimulant or immunosuppressant. Intonation of immune response to alleviate the diseases has been of interest from many years and the concept of Rasayana in Ayurveda was developed from related principles (Shah and Juvekar, 2010). The concept of Rasayana in Ayurveda, lays an emphasis on improving the health and strengthen the host defence against different diseases (Thatte and Dahanukar, 1986).

Immunomodulators are being sold in market but they are expensive and having several side effects. Immunomodulation using medicinal plants is a major breakthrough in the field of immunity. These can provide a conventional chemotherapy to the number of diseases, including when immune response of body impaired. It can be activated by immunostimulant while, suppressed in case of autoimmune disorders. A large number of plants and their phytoconstituents have been reported for their potentiality towards immunomodulation (Attard and Cuschieri, 2009; Ghaisas *et al*., 2009; Patil *et al*., 2009; Sharififar *et al*., 2009; Agrawal *et al*., 2010; Lee *et al*., 2010).

Plants are rich source of phytoconstituents which are reported to incite para-immunity and also affects the non-specific immunomodulation especially of granulocytes macrophages, natural killer and complement system (Sainis *et al*., 1997). It has been suggested that plants labelled as Rasayana have been gifted with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the psychoneuroimmune axis (Katiyar *et al*., 1997).

Hibiscus rosa-sinensis Linn. (Family-Malvaceae) known in Sanskrit as Japa or Rudhrapushpa is a native of

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south East Asia, but now it is commonly available throughout the world (Nadkarni, 1976). Besides, its flowers are reported to be refrigerant, emollient, demulcent and aphrodisiac and emmenagogue, it was also reported to be useful in menorrhagia, strangury, cystitis and other conditions of the genitor-urinary tract (Kirtikar and Basu, 2005). The present study has been undertaken to find out the possible interaction of *H. rosa-sinensis* flowers on immune response.

MATERIALS AND METHODS

Blooms of *H. rosa* sinensis was collected from University campus in the month of August-September 2011.

Extraction of the plant material and experimental schedule: Flowers of plant oven dried and homogenised to get coarse powder. The fine powdered material was extracted using Soxhlet method. Extract was further dried, and used for the study. Male Swiss albino mice were divided randomly into two groups having nine mice each and were treated intraperitoneally for the period of 15 days. Group I, Control (Normal saline, 0.9%); Group II, AEHrs (500 mg kg^{-1} b.wt.).

Animals: Adult male and female Swiss albino mice procured from CCS Haryana Agricultural University, Hisar were mated and resulting progeny was maintained in a well ventilated animal house with 12:12 light/dark cycle. Tap water was made available ad libitum. As far as possible, necessary sterile conditions were provided and cleanliness was maintained in the animal cages as well as in the room. Prior for experiments approval was taken from Institutional Animal Ethics Committee as per CPCSEA (Govt. of India) norms.

Immunization schedule: Sheep Red Blood Cells (SRBC) were used as a source of T-dependent antigen. For this purpose, the blood was withdrawn from a healthy sheep in Alsever's solution (Chakraborthy, 2009). SRBC used for immunization were prepared in pyrogen-free normal saline.

Isolation of peritoneal macrophage: Peritoneal macrophages were obtained from mice that had been intraperitoneally injected with normal saline. Peritoneal exudates obtained were harvested in plastic tubes using syringe. Cell suspension was centrifuged at 1000 rpm for 10 min at 4°C. The supernatant was discarded and pellet was washed with 5 mL of ice cold saline, mixed and recentrifuged for 5 min. Pelleted cells were resuspended in 0.5 mL of ice cold saline. The cell number was determined by a hematocytometer and cell viability was tested by trypan blue dye exclusion technique (Boyum, 1968).

Determination of the Phagocytic index: The aqueous extract of *H. rosa-sinensis* Phagocytic index was determined by following Boyum method (Boyum, 1968).

Determination of the circulating antibody titre: Antibody titre was estimated by indirect ELISA method. Microtitre wells were incubated with 200 μL of diluted antigen (1 mg mL^{-1}, diluted to 5 μg mL^{-1}) for 24 h at 4°C. The wells were rinsed with distilled water and filled with 200 μL of blocking buffer and left for 1 h at room temperature. After rinsing the wells with water, 200 μL of diluted test serum samples (1 μL of serum with 2 mL of sample diluents) were poured into wells and placed for incubation for 30 mins. Discarded the well contents and filled the wells with PBST for 30 mins. Next, discarded the contents and added 200 μL of secondary antibody-HRP conjugate and incubated them for 30 min. Rinsed the wells again with PBST and added 200 μL of TMB/H_2O_2 (Substrate). As soon as the colour developed, 100 μL stop solution was added. Absorbance was recorded at 450 nm on ELISA reader.

Determination of the plaque forming cell (PFC) assay: PFC assay was done by Jerne and Nordin method (Jerne and Nordin, 1963). Mice were immunized with 2.5×10^8 SRBC intraperitoneally. The mice of 1st group were given normal saline (Control) and 2nd group were treated with AEHrs, daily for five days prior to the immunization. Then animals were sacrificed on day 5, 10, 15. Then, the spleen was processed and the number of plaque forming cells counted.

Determination of delayed type hypersensitivity (DTH) response: DTH was performed as described by (Langrange *et al*., 1974).

Determination of cytokines: For cytokine estimation blood sample was collected from retro sinus after 5, 10, 15 days of treatment. Serum was separated and cytokine levels of IL-1α and IL-2 were determined by ELISA method using kits (Bender MedSystems).

High performance thin layer chromatography of the plant material: For HPTLC, *H. rosa-sinensis* flowers samples were prepared in methanol. For characterization of phytoconstituents aluminium silica gel $60F_{254}$ (Merck No. 5564) was used with mobile phase chloroform and methanol in the ratio of 9:1. 15 μL leaves sample was applied to the plate and plate was developed in a Camag

twin trough chamber to a distance of 8 cm. Chamber was previously equilibrated with the mobile phase for 45 min. The developed HPTLC plate was dried using dryer for 2 min. It was scanned at 366 nm (λ_{max}) for quantification using Camag scanner having deuterium lamp.

Statistical analysis: Results are expressed as mean±standard deviation (SD). Statistical significance between the different groups was determined by one way Analysis of Variance (ANOVA) using the SPSS (Ver. 16) ($p<0.05$).

RESULTS

Humoral immune response of AEHrs was checked by ELISA antibody titre (Fig. 1). It was noticed that in AEHrs treated mice, antibody titre increased up to 2.39 ng mL^{-1} on day 15th while it was 1.73 ng mL^{-1} in control mice. The increase in titre value was siginificant at $p<0.05$. maximum numbers of PFCs were observed on day 15th of treatment (782±10.60) when compared to controls Administration of AEHrs significantly enhanced the number of plaque forming cells in spleen (777±11.42) (Fig. 2). The paw oedema measured at 24 h after injection of SRBC showed a significant reduction in AEHrs dose of 500 mg kg^{-1} treated compared to control group ($p<0.05$) (Fig. 3). AEHrs administration increased the level of cytokine IL-1α (Table 1) while the level of IL-2 was decreased after treatment (Table 2). IL-1α is a cytokine which posses wide spectrum of haematopoietic activity and plays major role in the immune responses whereas IL-2 is a leukocytotrophic hormone and it is responsible for cell mediated immune response. Macrophage yield and viability of macrophages was increased after AEHrs dose (Table 3) while AEHrs possess macrophage stimulatory activity as evidenced by increased phagocytic index in Boyum test. The phagocytic index for control was 72.85±1.07 whereas it was significantly increased by 76.76±1.40 for AEHrs treated mice after 15th day treatment (Table 3). These results indicate that AEHrs has significant effect on the cytokines involved in the humoral immune response. Figure 4 depict the HPTLC analysis of *H. rosa-sinensis* profile which shows the presence of 4 peaks of *H. rosa-sinensis* extract at RF 0.02, 0.06, 0.14 and 0.93.

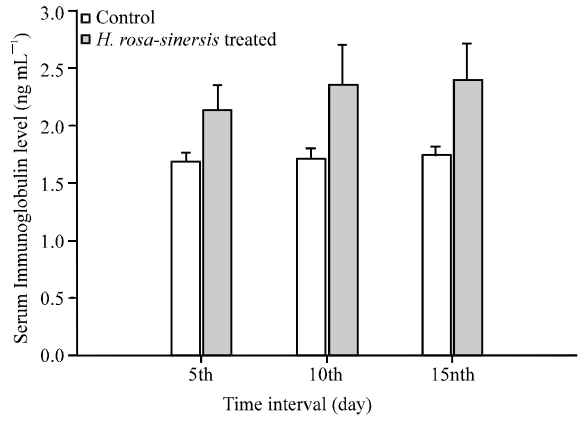

Fig. 1: Effect of AEHrs on serum immunoglobulin level (ng mL^{-1})

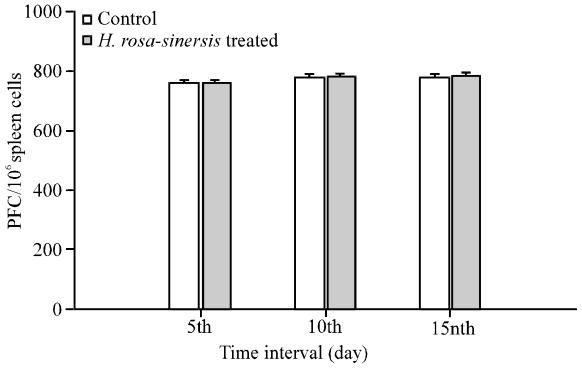

Fig. 2: Effect of AEHrs on plaque forming cells (FPC)

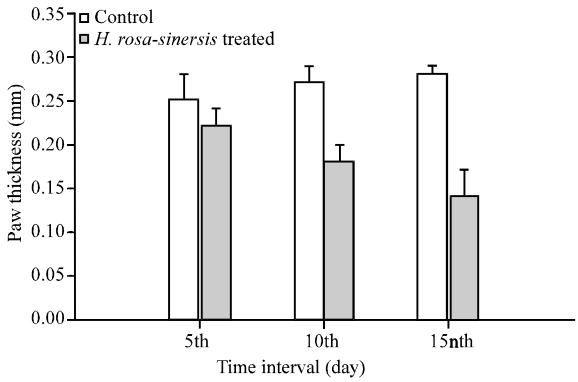

Fig. 3: Effect of AEHrs on paw thickness

Table 1: Effect of aqueous extract of *H. rosa-sinensis* (AEHrs) on IL-1α

Treatment	5th day	10th day	15nth day
Control	21.43±0.59	22.12±0.26	22.56±0.64
H. rosa-sinensis	24.58±0.60*	25.25±0.33*	25.78±0.40*

Values are mean±SD from 9 mice in each group, *Data are significant at $p<0.05$ as compared to control (pg mL^{-1})

Table 2: Effect of aqueous extract of *H. rosa-sinensis* (AEHrs) on IL-2

Treatment	5th day	10th day	15nth day
Control	66.58±0.73	67.45±1.32	68.06±0.19
H. rosa-sinensis	36.40±0.40*	44.99±0.61*	45.08±2.64*

Values are mean±SD from 9 mice in each group, *Data are significant at $p<0.05$ as compared to control (pg mL^{-1})

Table 3: Effect of aqueous extracts of *H. rosa-sinensis*/*B. spectabilis* on macrophage yield, viability of macrophage and phagocytic index

	Day after treatment		
Treatment	5th	10th	15nth
Macrophage yield ($\times 10^6$ cells mL^{-1})			
Control	8.23±0.20	8.19±0.23	8.21±0.27
H. rosa-sinensis	8.51±0.14	8.61±0.21	8.77±0.22
B. spectabilis	9.08±0.15	9.25±0.11	9.33±0.14*
Viability of macrophages (%)			
Control	68.47±0.33	68.62±0.45	68.15±0.28
H. rosa-sinensis	68.75±0.45	68.73±0.48	69.38±0.64
B. spectabilis	68.94±0.60	69.50±1.36	70.51±1.81
Phagocytic index			
Control	73.21±0.65	72.36±0.46	72.85±1.07
H. rosa-sinensis	74.58±0.52	74.81±0.68	76.76±1.40*
B. spectabilis	75.81±0.62	77.83±0.99*	78.19±0.77*

*Data are significant at $p<0.05$ as compared to control (pg mL^{-1})

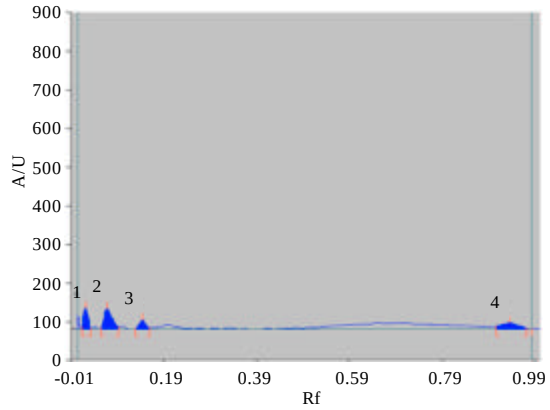

Fig. 4: HPTLC chromatogram showing the developed bands of aqueous extract of *H. rosa-sinensis* and their respective Rf value

DISCUSSION

H. rosa-sinensis is primarily described as a popular herb in traditional medicine system. Ethnomedicinal information shows that this plant possesses powerful antioxidant, anti-inflammatory, antidiabetic, cardioprotective hepatoprotective and anticancer activity on various animal models (Masaki *et al*., 1995; Anonymous, 1956; Sachdewa and Khemani, 2003; Gauthaman *et al*., 2006; Obi and Uneh, 2003). Hence, in the present study the interaction of AEHrs has been investigated with respect to not only on phagocytic index but DTH activity and plaque forming cells was counted and its effect on the titre of antibodies and cytokines (IL-2 and IL-1α).

It is well established, that neutrophils and monocytes play a major significant role in the process of phagocytosis. Phagocytosis primarily help in the removal of microorganism, foreign bodies and also helpful in the removal of dead and injured cells. It is considered to be the main non-specific defense mechanism and it was pointed out that potentiation of non-specific defense mechanism occur during microbial invasion leading to more efficient clearance and destruction of pathogens or other harmful substances. Macrophages play an pivotal role in coordinating the processing and presentation of antigen to B-cells. AEHrs was evaluated for its effect on macrophage phagocytic activity. Macrophage yield, Viability of macrophages and phagocytic index was significantly increased after the treatment with AEHrs on 10 and 15th days (Fig. 1). Determination of the phagocytic index, gives an idea about the activity and efficiency of phagocytizing cells. The rate of the clearance of carbon from blood by macrophages is governed by an exponential equation. Results of present investigation indicate that AEHrs (500 mg kg^{-1}, oral administration) produced a significant rate of elimination of carbon particles in *H. rosa-sinensis* with respect to controls (Table 1). This can be taken as general way in which inert particulate matter is cleared from the blood. In this study, AEHrs showed a significant effect on phagocytosis. This indicates, that this plant have the ability to boost phagocytic index as has been earlier reported in *Bauhinia variegata* (Ghaisas *et al*., 2009), *Urena lobata* Linn (Rinku *et al*., 2009), *Nothapodytes foetida* (Pur *et al*., 2007), *Tinospora* and ashwagandha rasayana (Mathew and Kuttan, 1999).

In order to further understand the effect of *H. rosa-sinensis* extract on humoral response, the titre of immunoglobulins was examined. Antibody gathered to act as an effector of the humoral response by binding to the antigen and normalize it or via facilitating its elimination bye cross-linking to form clusters that are more readily ingested by phagocytic cells. The effect of the extract of *H. rosa-sinensis* administration on antibody titre in mice is summarized in Fig. 2. It was noticed that in *H. rosa-sinensis* treated mice, antibody titre increased up to 2.39 ng mL^{-1} on day 15th this incline was significant at $p<0.05$. Increased titre of antibody could be due to the presence of flavonoids which augment the humoral response, by stimulating the macrophage and B-lymphocytes subsets involved in antibody synthesis (Shah *et al*., 2008). Earlier studies on different plants, have also pointed out an increase in antibody titre after administration of plant extract (Sharififar *et al*., 2009; Makare *et al*., 2001; Madan *et al*., 2008; Amirghofran *et al*., 2007).

It is to be mentioned here that IL-2 was considered as the representative cytokines of TH response and IL-1α as that of monocytes, macrophages and B cells. Although,

there are many interactions of IL-1α with other cytokines, it stimulates fibroblast proliferation, induces synthesis of protease, release of all types of amino acids, stimulates active phase protein synthesis, increases blood neutrophils, activates lymphocytes proliferation and induces fever. To be more precise IL-1α induces proliferation of CD4 cells. It also co-stimulates CD8 cells and IL-1 receptors on antigen presenting cells along with proliferation of mature B cells and immunoglobulin secretion. In the present work effect of AEHrs was studied against IL-1α and IL-2. The results are summarized in Table 1, 2. The data reveal that AEHrs used in the study, showed a significant increase in the level of IL-1α but there was significant decrease in IL-2 was observed.

Besides, in recent years, a few reports have appeared indicating an increase in IL-1 alpha and IL-2 in serum after the administration of plants extracts (Magbhraby *et al*., 2010; Kumar *et al*., 1999; Egger *et al*., 1996). It is thereby, suggested that the plants used in the present study i.e. *H. rosa-sinensis* have the potentiality to stimulate humoral immune response from day 5 onwards of treatment. It can be said that enhanced activity of macrophages and I1-α involved in antibody synthesis (Leung and Young, 1987).

Moreover, a gradual significant increase in the number of plaque forming cells from day 5 onwards of the treatment with AEHrs and an increase in the titre of antibodies further support the results of present study. It is assumed that these cells are also antibody secreting cells forming plaques in the layer of RBC with the help of complement system. Administration of AEHrs significantly enhanced the number of plaque forming cells in spleen (Fig. 3). The maximum number of PFCs was observed on day 15th of treatment when compared to controls. Earlier, the effect of aloe vera (Madan *et al*., 2008) and *Clerodendrum phlomidis* (Gokani *et al*., 2007) extracts has been studied on Plaque forming cells. Delayed type Hypersensitivity is directly related to Cell mediated immunity and is a part of graft rejection, tumour immunity and most important it exhibits a significant role in many intracellular infectious microorganisms, those causing chronic diseases (Delves and Roitt, 1998) and it was found that AEHrs enhance the DTH reactivity which leads to subsidise the SRBC effect. AEHrs produce a significant inhibition of edema as compared to control (Fig. 4). An increased response in DTH shows stimulatory effect of plant which intiated on lymphocytes and accessory cells types which are required for the expression of this reaction (Mitra *et al*., 1999).

HPTLC analysis of AEHrs depicts four spots corresponding to the RF 0.02, 0.06, 0.14 and 0.93, respectively (Fig. 3) indicating the chief phytoconstituents of this plant are flavonoids, alkaloid. Flavonoids were identified on the basis of previous reports of Rf value 0.02 (Krishna *et al*., 2011), 0.06 (Rajeswari and Krishnakumari, 2010) and quercetin at Rf 0.93 (Kumar *et al*., 2010). Rf values 0.14 (Bai *et al*., 2006) (Petasinine) were corresponding to alkaloids.

CONCLUSION

It is hereby, suggested that the plant extract used in the present study i.e., blooms of *H. rosa-sinensis* have potentiality to stimulate humoral immune response more efficiently then the cellular immune response from day 5 onward of the treatment. This property may be due to the presence of flavonoids and alkaloids in the extract of this plant.

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