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Bioactive Immunomodulatory Fraction from *Tridax procumbens*

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Abstract: *Tridax procumbens* (Compositae) have been extensively used in Ayurvedic system of medicine for various ailments. Earlier studies on the extracts of *Tridax procumbens* revealed remarkable immunomodulatory activity of TPEIF (*Tridax procumbens* ethanol insoluble fraction) extract. In the present study, the results of the preliminary phytochemical study revealed the presence of alkaloids, phenolics, saponins and tannins in both extract and fractions so, an attempt was made to explore the phytoconstituents responsible for effect on cellular and humoral functions in mice. The *in vitro* (Phagocytosis) and *in vivo* (Haemagglutination and Delayed hypersensitivity) were used to study the effect of extract and fractions on the cellular and humoral immunity. Alcoholic extract revealed significant immunostimulation by *in vitro* phagocytosis, delayed hypersensitivity and haemagglutination model (ANOVA followed by Dunnett's Multiple Comparison test). Oral administration of EFTP (ethyl acetate fraction) and NFTP (n-butanol fraction) among the four fractions (20-40 mg kg^{-1}) significantly inhibited Sheep Red Blood Cells (SRBC) induced delayed type hypersensitivity reactions and significantly increased the *in vitro* phagocytic index. It also produced a significant, dose related decrease in sheep erythrocyte specific heamagglutination antibody titre. The results obtained indicate the ability of the flavonoidal fraction (EFTP) and saponin fraction (NFTP) fraction of *Tridax procumbens* to modulate both cell mediated and the humoral components of the immune system and explored the phytoconstituents responsible for immunomodulatory potential from *Tridax procumbens*.

Key words: Phagocytosis, delayed hypersensitivity, haemagglutination, compositae

INTRODUCTION

Herbal medicine has become an integral part of standard healthcare, based on a combination of time honored traditional usage and ongoing scientific research. Increased interest in medicinal herbs has prompted for scientific scrutiny of their therapeutic potential and safety. Some of the medicinal plants are believed to enhance the natural resistance of the body to infections (Atal *et al*., 1986). Apart from being specifically stimulatory or suppressive, certain agents normalize or modulate pathophysiological processes and are hence, called immunomodulatory agents. Indian medicinal plants are a rich source of

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substances which are claimed to induce paraimmunity, the non-specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions. The property of any substance to enhance non-specific resistance of body against pathogens is termed adaptogenic (Sainis *et al*., 1997). Ayurveda, the Indian traditional system of medicine, lays emphasis on promotion of health, a concept of strengthening host defenses against different diseases (Thatte and Dahanukar, 1986). These plants, labelled as Rasayana, have been endowed with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the psycho-neuro-immune axis (Katiyar *et al*., 1997). Most important area in which herbal medicine has not to witness any breakthrough is the development of adjuvants to be used in vaccination programs or immunosuppressant that can be safely exploited in organ transplantation and autoimmune diseases. These fundamental fields of immunomodulators are currently receiving inadequate attention (Upadhaya, 1997). So, a number of plant products are being investigated for immune response modifying activity. The modulation of immune response with the aid of various bioactives in order to alleviate certain diseases is an active area of interest (Wagner, 1986).

Tridax procumbens L. (TP) is commonly known as Coat Button or Kansari (Hindi) or Ghamara (in local language) and belongs to family compositae. It is extensively used in Ayurvedic system of medicine for various ailments and is shown to posses a number of pharmacological activities like hypotensive, insecticidal, leishmanicidal, hair growth promoting, wound healing, anti-inflammatory, hepatoprotective, immunomodulatory and antioxidant activity and phytochemical profile of this plant has shown the presence of flavonoids, terpenoids, alkaloids and phenolic compounds. Previous studies on the extracts of Tridax procumbens by Tiwari *et al*. (2004) revealed remarkable immunomodulatory activity of TPEIF (Tridax Procumbens Ethanol Insoluble Fraction) extract. In view of the scarcity of information on the constituents of *T. procumbens*, the purpose of the present study was undertaken to isolate the phytoconstituents responsible for immunomodulation.

MATERIALS AND METHODS

The study was carried out at Pharmacognosy and Pharmacology Departments of R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur Dist-Dhule, North Maharashtra University (MS), India between December 2007 and August 2009.

Reagents

Fresh blood was collected from sheep's sacrificed in the local slaughter house. Sheep Red Blood Cells (SRBC) were washed three times in normal saline and adjusted to a concentration of 0.1 mL containing 1×10^8 cells for immunization and challenge.

Plant Material and Extraction

The aerial parts of *Tridax procumbens* were collected in the month of December and January from locality of Gondia, Maharashtra State, India and authenticated by the Dr. D.A. Patil, HOD Botany Dept, SSVPS College, Dhule (MS), India. A voucher specimen (SA-01) was submitted at institute's herbarium department for future reference. Dried material was coarsely pulverized to powdered form. One kilogram of powdered plant material was extracted by maceration with 500 mL alcohol for 3 complete cycles. The alcoholic extract was dried at 30-40°C using a vacuum evaporator. The resulting dried extract was solubilized in purified water and fractionated in to chloroform fraction (CFTP), ethyl acetate fraction (EFTP), n-Butanol fraction (NFTP) and remnant water soluble fraction (RWSFTP), by

liquid-liquid partitioning. These fractions were investigated for immunomodulatory activity, by dissolving in 1% sodium carboxy methyl cellulose and filtered through 0.22 μm membrane filter.

Experimental Animals

Swiss Albino Mice, strain C57BL6 weighing between 20-40 g were used in the study with prior approval and scrutinization from the Institutional Animal Ethical Committee (RCPIPER/IAEC/2008-09/30). The animals were housed in clean and spacious cages provided with net and feeding bottle, at ambient temperature of 25±2°C with 12 h light and 12 h dark cycles and provided free access to standard laboratory chow mixture provided water *ad libitum* for fixed period so as to acclimatize all animals and to achieve normal constant basal food intake in all.

Effect on *in vitro* Phagocytosis of *Candida albicans* by Human PMN Cells

The extract and all the fractions obtained there of were evaluated for immunomodulatory activity, using the PMN function test. Peripheral venous blood, 10 mL, was collected from volunteers in a sterile heparinised tube. Neutrophils were isolated by Ficoll Hypaque density gradient sedimentation (Boyum, 1968, 1976). The RBC-PMN pellet was then subjected to dextran sedimentation. The supernatants, containing more than 90% of PMN cells, were collected and the cell density adjusted to 1×10^6 cells mL^{-1} using MEM.

Candida albicans (cell density adjusted to 1×10^6 cells mL^{-1} using MEM) was used as the test microorganism. The PMN cells (cell density adjusted to 1×10^6 cells mL^{-1} using MEM) were mixed with 1×10^6 cells mL^{-1} of *Candida albicans* and incubated at 37°C for 1 h in 5% CO_2 atmosphere, in the presence of the test fractions. The control was the identical solution minus the test fraction. Cytosmears were prepared after incubation. The smear was fixed with methanol, stained with Giemsa and studied under 100X oil immersion objective to determine the phagocytic activity of PMN cells. Neutrophils (100 numbers) were scanned and the cells with ingested microorganisms were counted (Lehrer and Cline, 1969; Gabhe *et al*., 2006).

Treatment of Mice

Twelve groups of mice each consisting of six animals received all extract (0.1-1.0 g kg^{-1}) and fractions (0.02 and 0.04 g kg^{-1}) Body Weight (BW) for 7 consecutive days intraperitoneally. The control group was treated with 1% sodium carboxy methyl cellulose solution, whereas native group was devoid of any treatment. No mortality or any toxic effects were observed in the above mentioned doses of these all fractions, administered intraperitoneally.

Heamagglutination Antibody Titre

Mice were intraperitoneally immunized with 1×10^8 SRBC on day 0. Blood samples were collected from individual animals from the orbital plexus on day 7. Antibody levels were determined by the haemagglutination technique (Ray *et al*., 1991). Two-fold diluted sera in saline (0.025 mL) were mixed with 0.025 mL of 0.1% v/v SRBC suspension in microtitre plates. The plates were incubated at 37°C for 1 h and then inspected for haemagglutination. The antibody titer was determined by a two-fold serial dilution of one volume (100 μL) of serum and one volume (100 μL) of 0.1% Bovine Serum Albumin (BSA) in saline. One volume (100 μL) of 0.1% SRBCs in BSA in saline was added and the tubes were mixed thoroughly. They were allowed to settle at room temperature for about 60-90 min until the control tube showed, a negative pattern (a small button formation). The value of the highest serum dilution showing visible haemagglutination was taken as the antibody titer (Puri *et al*., 1993).

Delayed Type Hypersensitivity

Mice were primed with 0.1 mL of SRBC suspension containing 1×10^8 cells intraperitoneally on day 0 and challenged on day 7 with 1×10^8 SRBC in right-hind foot pad. The contra lateral paw received saline alone. The thickness of foot pad was measured at 24 h after challenge using a Vernier Caliper. The difference in the thickness of right hind paw and left hind paw was used as a measure of DTH reaction (Ghazanfari *et al*., 2002; Fulzele *et al*., 2003).

Statistical Analysis

The data was analysed using One-Way Analysis of Variance (ANOVA), followed by Dunnett's test. The $p<0.05$ was considered significant.

RESULTS

Effects of Extract and all Fractions Obtained thereof on PMN Cell in Phagocytic Activity

Methanol extract and fractions obtained from the extract of *Tridax procumbens* were evaluated for their phagocytic activity. In this assay, ingested and associated *C. albicans* per PMN cell were measured. The results have been expressed as *Candida* per cell, the average number of *Candida* cells associated with PMN cells. Methanol extract exhibited significant phagocytic activity in dose of 200 μg mL^{-1} (Table 1). Among all the groups of fractions studied, EFTP and NBTP groups shows the significant engulfment of the *C. albicans* per PMN cell in a dose of 25 μg mL^{-1} (Table 2), as compared to the negative control.

Heamagglutination Antibody Titre

A dose-related increase in humoral antibody titer was observed in mice treated with the methanol extract in a dose of 200 mg kg^{-1} and EFTP and NFTP of *T. procumbens* in a dose of 20-40 mg kg^{-1}, per oral (Table 3, 4).

Table 1: Effect of alcoholic extract on *in vitro* phagocytosis of *C. albicans* by PMN cells

-C	+C	25 μg mL^{-1} ME	50 μg mL^{-1} ME	100 μg mL^{-1} ME	200 μg mL^{-1} ME
3.10	4.25	5.64	4.11	5.96	4.62
3.16	4.40	5.10	3.20	5.26	4.02
2.96	4.21	5.80	4.63	5.51	4.06
3.13	4.28	5.67	4.04	4.99	3.65
3.19	4.43	5.13	5.03	5.29	4.05
2.99	4.24	5.83	4.66	5.54	4.09

Table 2: Effect of fractions on *in vitro* phagocytosis of *C. albicans* by PMN cells

-C	+C	25 μg mL^{-1} EFTP	25 μg mL^{-1} CFTP	25 μg mL^{-1} NFTP	25 μg mL^{-1} RWSTP
3.10	4.25	4.24	5.31	3.96	5.62
3.16	4.40	4.70	5.28	3.98	5.82
2.96	4.21	4.10	5.63	4.21	6.06
3.13	4.28	4.72	4.84	4.29	5.65
3.19	4.43	4.21	5.53	4.30	5.66
2.99	4.24	4.13	5.46	4.04	6.19

Table 3: Effect of alcoholic extract on SRBC induced delayed type hypersensitivity and haemagglutination titer in mice

Groups	Treatment	Dose mg kg^{-1} p.o., for 7 days	HA titre (Mean±SD)	DTH response (mm) paw oedema±SD
I	Control	-----	8.7±5.9	0.29±0.13
II	ME	100	12.7±10.6	0.34±0.21
III	ME	200	22.7±10.6	0.41±0.14 *
IV	ME	400	85.3±7.1**	0.68±0.12**
V	ME	1000	106.8±14.1**	0.73±0.21 **

Control: 1% Sodium carboxy methyl cellulose; n = 6 per group. p-values are *p<0.05, **p<0.01, ***p<0.001 as compared with control were considered

Table 4: Effect of fractions on SRBC induced delayed type hypersensitivity and haemagglutination titer in mice

Groups	Treatment	Dose mg kg^{-1} p.o., for 7 days	HA titre (Mean±SD)	DTH response (mm) paw oedema±SD
I	Control	-----	8.7±5.9	0.29±0.13
II	CFTP	20	12.7±10.6	0.34±0.21
III	CFTP	40	22.7±10.6	0.41±0.14*
IV	EFTP	20	85.3±7.1**	0.68±0.12**
V	EFTP	40	106.8±14.1**	0.73±0.21**
VI	NFTP	20	92.7±10.6**	0.72±0.17**
VII	NFTP	40	150.2±4.9**	0.80±0.19**
VIII	RWSTP	20	42.7±10.6*	0.41±0.28*
IX	RWSTP	40	68.5±12.3*	0.53±0.12*

Control: 1% Sodium carboxy methyl cellulose; n = 6 per group. p-values are *p<0.05, **p<0.01, ***p<0.001 as compared with control were considered

Table 5: Effect of fractions on thymus and spleen weight using SRBC as an antigen in mice

Groups	Thymus weight	Spleen weight
Control	35.0±0.82	156.0±15
CFTP (20 mg kg^{-1})	34.0±1.1	141.0±2.3*
CFTP (40 mg kg^{-1})	31.0±1.2*	148.0±2.1
EFTP (20 mg kg^{-1})	24.0±2.0**	136.0±4.7*
EFTP (40 mg kg^{-1})	22.0±1.3**	101.0±10**
NFTP (20 mg kg^{-1})	20.0±1.5**	116.0±1.8**
NFTP (20 mg kg^{-1})	16.6±0.9**	96.4±1.2**
RWSTP (20 mg kg^{-1})	30.0±3.1	140.0±3.2*
RWSTP (40 mg kg^{-1})	28.6±4.6	134.0±2.8

Control: 1% Sodium carboxy methyl cellulose; n = 6 per group. p-values are *p<0.05, **p< 0.01, ***p<0.001 as compared with control were considered

Delayed Type Hypersensitivity Reaction

In the present study, SRBC-induced delayed-type hypersensitivity was used to assess the effect of the extract and fractions on cell-mediated immunity. In the control animals, the +48 h and +72 h response was either equal or slightly more than the 0 h response, therefore, the peak edema at +24 h was taken as a parameter for evaluating the reaction. The methanol extract (200 mg kg^{-1}) and fraction EFTP and NFTP of *Tridax procumbens* (20-40 mg kg^{-1}, p.o.) produced a significant, dose-related decrease from DTH reactivity in mice (Table 3, 4).

Effect of Plant Extract and Fractions on Body Weight, Lymphoid Organ Weight and Cellularity

No effect was observed in the spleen weight at any dose when compared with control (normal saline treated) animals (group I). At doses of 20 and 40 mg kg^{-1} a significant decrease ($p<0.05$) in relative organ weight of thymus was observed with EFTP and NFTP but, there was no effect at a dose of 20 and 40 mg kg^{-1} in its weight for CFTP and RWSTP. Lymphoid organ cellularity (Table 5) data indicate no significant decrease in the spleen cellularity at any of the doses. A significant increase was also recorded in the cellularity of thymus at doses of 50 and 100 mg kg^{-1} as compared with control animals (group I).

DISCUSSION

The present study not only proved the immunomodulatory activity of the alcoholic extract of *Tridax procumbens*, but also showed that EFTP and BFTP were more active than other fractions. Alcoholic extract and EFTP and NFTP treatment improved the heamagglutination antibody titer reflecting an overall elevation of humoral immune response

supporting the role of flavonoids and saponins in the immunostimulation. Haemagglutination antibody titer was determined to establish the humoral response against SRBC as antigen. At neutral pH, red blood cells possess negative ions cloud that makes the cells repel from one another, this repulsive force is referred to as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and get cross-link to red blood cells, leading subsequent agglutination. The smaller size and bivalency of IgG, however, makes them less capable to overcome the electric barrier. This characteristic may accounted for, IgM being more effective than IgG in agglutinating red blood cells (Kuby, 1997; Manosroi *et al*., 2003).

Delayed type hypersensitivity reaction is characterized by large influxes of non specific inflammatory cells, in which the macrophage is a major participant. In DTH, circulating T cells sensitized to the antigen from prior contact reacts with the antigen and induces specific immune response, which includes mitosis (blastogenesis) and the release of soluble mediators. This promotes phagocytosis activity and increases the concentration of lytic enzymes for more effective killing. The overall effects of these cytokines are to recruit macrophages into the area and activate them, promoting increased phagocytic activity vis-a-vis increased concentration of lytic enzymes for more effective killing. Several lines of evidence suggest that DTH reaction is important in host defense against parasites and bacteria that can live and proliferate intracellularly (Dash *et al*., 2006; Smith *et al*., 2000). Treatment of extract and EFTP and NFTP enhanced DTH reaction, which is reflected from the increased footpad thickness compared to control group suggesting heightened infiltration of macrophages to the inflammatory site. This study may be supporting a possible role of EFTP and NFTP in assisting cell-mediated immune response.

The increase in thymus weight was accompanied by increase in its cell counts. This may be partly due to stimulatory effect of plant extract and fractions on the lymphocytes and bone marrow haematopoietic cells, which ultimately home in the thymus. However, this homing may be temporary and in due course of time normalcy may ensue (Bin-Hafeez *et al*., 2003; Gayatri *et al*., 2005).

Through chemical analysis, EFTP and BFTP mainly consist of flavonoids and triterpenoidal saponins which are probably responsible for its immunomodulatory activities. Therefore, EFTP and BFTP could be an effective and useful candidate in the development of immunomodulatory drug.

CONCLUSIONS

The present study has shown the immunostimulatory activity of *Tridax procumbens* and suggests its therapeutic usefulness. The EFTP and NFTP have stimulated both humoral as well as cellular arms of immune system. Further detailed studies are required which might establish a possible use of hydro-alcoholic extract of *Tridax procumbens* in immunocompromised patients and as an adjuvant during vaccination programs in order to reduce number of non-responders to vaccines. However, detailed studies of phytoconstituents responsible and mechanisms of immunomodulation as well as probable use in immunocompromised individuals are still to be investigated.

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REFERENCES

Atal, C.K., M.L. Sharma, A. Kaul and A. Khajuria, 1986. Immunomodulatory agents of plant origin I: Preliminary screening. J. Ethnopharmacol., 18: 133-141.

Bin-Hafeez, B., R. Haque, S. Parvez, S. Pandey, I. Sayeed and S. Raisuddin, 2003. Immunomodulatory effects of fenugreek (*Trigonella foenum graecum* L.) extract in mice. Int. Immunopharmacol., 3: 257-265.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest., 97: 77-89.

Boyum, A., 1976. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol., 5: 9-15.

Dash, S., L.K. Nath, S. Bhise, P. Kar and S. Bhattacharya, 2006. Stimulation of the immune function activity by the alcoholic root extract of *Heracleum nepalense* D. Don. Indian. J. Pharmacol., 38: 336-340.

Fulzele, S.V., P.M. Satturwar, S.B. Joshi and A.K. Dorle, 2003. Study of the immunomodulatory activity of Haridradi ghrita in rats. Indian J. Pharmacol., 35: 51-54.

Gabhe, S.Y., P.A. Tatke and T.A. Khan, 2006. Evaluation of the Immunomodulatory activity of methanol extract of *Ficus benghalensis* roots in rats. Indian. J. pharmacol., 38: 271-275.

Gayatri, V., V.V. Asha and A. Subramoniam, 2005. Preliminary studies on the immunomodulatory and antioxidant properties of *Selaginella* species. Indian J. Pharmacol., 37: 381-385.

Ghazanfari, T., Z. Hassan and M. Ebrahimi, 2002. Immunomodulatory activity of a protein isolated from garlic extract on delayed type hypersensitivity. Int. Immonpharmacol., 2: 1541-1549.

Katiyar, C.K., N.B. Brindavanam, P. Tiwari and D.B.A. Narayana, 1997. Immunomodulator Products from Ayurveda: Current Status and Future Perspectives. In: Immunomodulation, Upadhyay, S.N. (Ed.). Narosa Publishing House, New Delhi, India, pp: 123.

Kuby, J., 1997. Immunology. 3rd Edn., WH Freeman and Company, New York, pp: 436-448.

Lehrer, R.I. and M.J. Cline, 1969. Interaction of *Candida albicans* with human leucocytes and serum. J. Bacteriol., 98: 996-1004.

Manosroi, A., A. Saraphanchotiwitthaya and J. Manosroi, 2003. Immunomodulatory activities of *Clausena excavate* Burm. f. wood extracts. J. Ethnopharmacol., 89: 155-160.

Puri, A., R. Saxena, R.P. Saxena, K.C. Saxena, V. Srivastav and J.S. Tandon, 1993. Immunostimulant agents from *Andrographis paniculata*. J. Nat. Prod., 58: 995-999.

Ray, A., P.K. Mediratta, S. Puri and P. Sen, 1991. Effect of stress on immune reponsiveness, gastric ulcerogenesis and plasma corticosterone in rats: Modulation by diazepam and naltrexone. Indian. J. Exp. Biol., 29: 233-236.

Sainis, K.B., P.F. Sumariwalla, A. Goel, G.J. Chintalwar, A.T. Sipahimalani and A. Banerji, 1997. Immunomodulatory Properties of Stem Extracts of *Tinospora Cordifolia*: Cell Targets and Active Principles. In: Immunomodulation, Upadhyay, S.N. (Ed.). Narosa Publishing House, New Delhi, India, pp: 95.

Smith, H.F., B.H. Kroes, A.J.J. Berg vanden, D. Wal vander and E. van den Worm *et al*., 2000. Immunomodulatory and anti-inflammatory activity of *Picrorhiza scrophulariiflora*. J. Ethnopharmacol., 73: 101-109.

Thatte, U.M. and S.A. Dahanukar, 1986. Ayurveda and contemporary scientific thought. Trends Pharmacol. Sci., 7: 247-249.

Tiwari, U., B. Rastogi, P. Singh, D.K. Saraf and S.P. Vyas, 2004. Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals. J. Ethnopharmacol., 92: 113-119.

Upadhaya, S.N., 1997. Therapeutic Potential of Immunomodulatory Agents from Plant Products. 1st Edn., Narosa Publishing House, New Delhi, pp: 149-150.

Wagner, H., 1986. Economic and Medicinal Plants Research. Academic Press, London, pp: 113-153.