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Research Article

Scrutinizing the role of aqueous extract of *Trapa bispinosa* as an immunomodulator in experimental animals

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ABSTRACT

The objective of the present study was to scrutinize the immunomodulatory potential of aqueous extract of fruits of *T. bispinosa* (TBAE) in experimental animals. The immunomodulatory effect was assessed in rats against sheep red blood cells (SRBC) as antigen by studying cell-mediated delayed type hypersensitivity reaction (DTH), humoral immunity response and percent change in neutrophil count. Macrophage phagocytosis assay was carried out by carbon clearance method in mice. Oral administration of TBAE dose dependently increased immunostimulatory response. Delayed type hypersensitivity reaction was found to be augmented significantly ($p < 0.05$) by increasing the mean foot pad thickness at 48 hr and production of circulatory antibody titre (humoral antibody response) was significantly ($p < 0.05$) increased in response to SRBC as an antigen. In addition, immunostimulation was counteracted by up regulating macrophage phagocytosis in response to carbon particles. Immunostimulatory property of TBAE further confirmed by elevated neutrophil counts significantly ($p < 0.01$) compared to control values. The result of present study suggests that aqueous extract of fruits of *T. bispinosa* could stimulate the cellular and humoral response in animals and it deserves further researches to develop an immunostimulating agent among herbal origin.

Keywords: *Trapa bispinosa*, Immunomodulation, Neutrophils, Haemagglutination titre.

INTRODUCTION

The immune system is involved in the etiology as well as pathophysiological mechanism of many diseases (Liu, et al., 2009). Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles (Sharma, 1983). Further, immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases especially when host defense mechanism has to be acquired under the conditions of impaired immune responsiveness (Lily, et al., 2005). Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity, the non-specific immunomodulation of especially granulocytes, macrophages, natural killer cells and competent functions (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 & Dahanukar, 1986).

Immunostimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. Therefore, stimulatory or suppressive agents have been shown to possess activity to normalize or modulate pathophysiological processes and are hence called 'immunomodulatory agents' (Wagner, 1983). Among the suppressive synthetic substances, cyclophosphamide has been extensively studied (Shand & Howard, 1979). However, the major drawback of this drug is myelosuppression, which is undesirable (Hardman, et al., 1996). Moreover, natural adjuvants, synthetic agents, antibody reagents are used as immunomodulatory agents. Nevertheless, there are major limitations to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system (Makare, et al., 2001). As an upshot, there is high prevalence of usage of herbal plants to treat diseases of immune system for hundreds of years. Besides, compared to synthetic drugs, herbal drugs are frequently considered to be less toxic with fewer side effects (Momin, 1987). Therefore, the search for more effective and safer agents exerting immunomodulatory ac-

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tivity is becoming a field of major interest all over the world (Patwardhan, et al., 1990).

A number of plants used in Indian traditional system of medicines for upgrading therapy and chronic diseases have been shown to stimulate immune responses and several active substances have also been isolated. In recent years, immunostimulatory activity has been reported in a number of Ayurvedic plants like *Withania somnifera* (Davis & Kuttan, 2000), *Argyrea speciosa* (Gokhale, et al., 2003), *Tridax procumbens* (Tiwari, et al., 2004), *Ficus benghalensis* (Gabhe, et al., 2006), *Actinidia macrosperma* (Lu, et al., 2007) and *Tinospora cordifolia* (Siddiqui, et al., 2008).

Trapa bispinosa is an aquatic floating herb belongs to the family trapaceae (George & Lawrence, 1951; Kirtikar & Basu, 1993). It is grown through out Asia and tropical Africa in lakes and ponds and is often cultivated for its edible fruit. The medicinal values of the whole herb and fruit have long been recognized in folklore medicine as a cure for various diseases (Rahman, et al., 2001). The whole herb has been reported for hepatoprotective activity (Kar, et al., 2004), antimicrobial activity (Rahman, et al., 2000), antibacterial activity (Rahman, et al., 2001), antitumor activity (Irikura, et al., 1972), antioxidant activity (Song, et al., 2007) and free radical scavenging activity (Kim, et al., 1997). Further, the fruits have been used as intestinal astringent, aphrodisiac, anti-inflammatory, in leprosy, urinary discharges, fractures, sore throat, bronchitis and anemia (Kirtikar & Basu, 1993; Nadkarni, 1976). In addition to this, the juice of fruit has been used for diarrhea and dysentery (Vhotracharcho, 1987). Despite many therapeutic effects, so far no data is available on the immunomodulatory effects of *Trapa bispinosa*. In this context, the objective of the present study was to scrutinize the immunomodulatory potential of aqueous extract of fruits of *T. bispinosa* in experimental animals.

MATERIALS AND METHODS

Collection and identification of plant

The fruits of *T. bispinosa* were collected from Banaskantha, (Gujarat), India in the month of August and September, 2008. The fruits were identified and authenticated by Botanist, Dr. Siddapa, Head of Botany Department, Sree Siddaganga boy's College, B. H. Road; Tumkur (Karnataka), India. The voucher specimen (MSA1) for the same has been preserved for future reference.

Extract preparation

The fruits of *T. bispinosa* were collected and shade dried. The dried fruits were reduced to a coarse powder. The powder (500g) was extracted exhaustively using a Soxhlet apparatus with 500 ml distilled water at 60-80 °C for 18hr. The extract was concentrated at controlled temperature (60-70°C) to obtain a semisolid mass (40g). The final obtained aqueous extract was weighed; percentage yield was calculated and stored in

a cool place. The freshly prepared solution of extract (dissolved in distilled water) was utilized for the further treatment of animals.

Phytochemical analysis

Preliminary phytochemical studies of the aqueous extract of *T. bispinosa* (TBAE) was performed for major classes of constituents like alkaloids, saponins, glycosides, steroids, tannins and phenolic compounds according to published standard methods (Paech and Tracey, 1955).

Experimental animals and feeds

Swiss albino rats (150-250g) and mice (25-35g) of either sex were obtained from animal house of Sree Siddaganga College of Pharmacy, Tumkur, India. All the animals were housed in a room maintained at $22 \pm 1^\circ\text{C}$ with a relative humidity of $60 \pm 5\%$ and a 12-hr light-dark cycle. They were allowed to acclimatize for a week prior to experiment and had free access to standard pellet diet (Hindustan Lever Pellets, Bangalore, India), water was provided *ad libitum*. All experiments were carried out with strict adherence to ethical guidelines and were conducted as per approved protocol by the Institutional Animal Ethics Committee (IAEC) and as per Indian norms laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, vide approval number SSCPT / IAEC / 40 / 06-07.

Antigen

Fresh blood was collected from sheep's sacrificed in the local slaughter house in a sterile bottle containing Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride). Sheep red blood cells (SRBC) were washed three times in normal saline to adjust to a concentration of 0.1ml contains 5×10^8 cells and 0.25×10^8 cells for immunization and challenge respectively.

Delayed type hypersensitivity (DTH) reaction using SRBC as an antigen

The method described by Doherty (1981, pp.237-242) was used. Rats were divided into four groups of six each. Group-I: control animals received normal saline for 7 days. Group-II, -III and -IV: received TBAE 50, 100 and 200mg/kg respectively for 7 days. All the rats were immunized (i.p) on day 0 with 0.8 ml SRBC suspension containing 5×10^8 cells/0.1 ml. The edema was induced in the right hind paw of rats by challenging with 0.1 ml SRBC suspension containing 0.25×10^8 cells in the sub-plantar region on day 7. The contra-lateral paw received equal volume of phosphate buffer and was served as a control. The increase in the paw volume at 0 and 48 hr was assessed using a plethysmometer (UGO, Basil, Italy). The difference in the thickness of the right hind paw and left hind paw was used as a measure of delayed type hypersensitivity reaction.

Humoral immune response to SRBC as an antigen

The method performed by Rao (1994, pp.553-558) was used in the present study. Rats were divided into five groups of six each. Group-I and -II: received normal saline for 7 days. Group-III, -IV and -V: received TBAE 50, 100 and 200 mg/kg respectively for 7 days. All rats were immunized (i.p) on day 0 with 0.8 ml SRBC suspension containing 5×10^8 cells/0.1 ml. In order to induce immunosuppression, cyclophosphamide (50 mg/kg) was administered orally on 5th day of the experiment (except group-I). On 7th day blood was withdrawn from the retro-orbital plexus (under light anaesthesia) of all antigenically challenged rats. 25 μ l of serum was serially diluted with 25 μ l of phosphate buffered saline. 0.1 ml SRBC suspension containing 0.25×10^8 cells was added to each of these dilutions and incubated at 37°C for 1hr. The value of highest serum dilution carrying visible haemagglutination was taken as the antibody titre expressed in terms of number of wells. The dilution of different groups was statistically compared.

Carbon clearance assay in mice

This assay was carried out according to the method of Tiwari, et al. (2004). Mice were divided into four different groups of six each. Group I: received normal saline for 7 days and served as a control. Group-II, -III and -V: received TBAE 50, 100 and 200 mg/kg respectively for 7 days. All mice were injected with 0.25 ml of Indian ink [which was diluted with PBS (pH 7.4) to eight times before use] into the tail vein on 7th day after a warm up period of 15 min at 37°C. Two drops of blood was collected at different time interval of 5, 10, 15 & 20 minutes by tail nipping. Blood was lysed with 3 ml distilled water and centrifuged (2000 \times g, 10 min, 10°C). Absorbance was recorded at 650 nm by spectrophotometer [UV 1601, Shimadzu (Asia Pacific) Pvt Ltd., Sydney, Australia] using pre-injected blood sample as a blank. The phagocytic index, *K*, was calculated by using equation:

$$K = \frac{\ln OD_1 - \ln OD_2}{t_2 - t_1}$$

Where, OD1 and OD2 depict the optical densities at times t1 and t2 respectively.

Neutrophil adhesion test using rats

This test was carried out according to the method of Fulzele, et al. (2003). Rats were divided into four groups of six each. The treatment schedule was similar to that of SRBC-induced DTH reaction. On the 7th day of drug treatment, blood samples were collected from the retro-orbital plexus (under light anaesthesia) of all rats into heparinized vials and analyzed for total leucocyte count (TLC) and differential leucocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubat-

ed with 80 mg/ml of nylon fibers for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophils gives Neutrophil index (NI) of blood sample. Percent neutrophil adhesion was calculated using formula:

$$\text{Neutrophil adhesion (\%)} = \frac{NI_U - NI_t}{NI_U}$$

Where, NI_U = Neutrophil index of untreated blood sample and NI_t = Neutrophil index of treated blood sample

Statistical analysis

All the results were reported as mean \pm standard error of the mean (SEM). Statistical analysis was performed using a one-way analysis of variance (ANOVA). If the overall p-value was found to be statistically significant (i.e., $p < 0.05$), further comparisons among groups were made by employing Dunnett's test. All statistical analyses were performed using Graph Pad software (San Diego, CA).

RESULTS

In the present study, preliminary phytochemical studies show the presence of alkaloids, carbohydrates, starch, tannins, phenolic compounds and saponins glycosides.

The effect of TBAE administration on T-cell mediated DTH reaction is shown in Table. 1.

Table 1. Effect of TBAE on Delayed type hypersensitivity reaction in rats

Group	Treatment	DTH response (% increase in paw volume)
I	Control (normal saline)	23.53 \pm 0.15
II	TBAE (50 mg/kg)	32.00 \pm 0.14*
III	TBAE (100 mg/kg)	40.94 \pm 0.48*
IV	TBAE (200 mg/kg)	52.23 \pm 0.71**

Values are expressed as mean \pm SEM (n=6). Significantly different from control * $p < 0.05$; ** $p < 0.01$.

TBAE (50, 100 and 200 mg/kg) produced a significant ($p < 0.01$) dose dependent increase in DTH response in rats at 48 hr compared to control group. Further, TBAE at a higher dose (200 mg/kg) showed significant ($p < 0.01$) enhancement of immune response to SRBC with increase in percentage edema of (52.23 \pm 0.71) compared to control group (23.53 \pm 0.15).

The influence of TBAE administration on humoral immune response is shown in Table 2. Administration of test extract (50, 100 and 200 mg/kg) produced dose

related increase in humoral antibody titre as evident by haemagglutination at that dilution. The antibody titre

increased in percentage of neutrophil adhesion.

Table 2. Effect of TBAE on Humoral immune response

Group	Treatment	Humoral immune response \pm SEM (Mean antibody titre levels)
I	Control (normal saline)	6.40 \pm 0.981
II	Cyclophosphamide (50 mg/kg)	3.20 \pm 0.491*
III	TBAE (50 mg/kg)	3.60 \pm 0.400
IV	TBAE (100 mg/kg)	8.60 \pm 0.98*, @
V	TBAE (200 mg/kg)	11.22 \pm 1.96*, @

Values are expressed as mean \pm SEM (n=6). Significantly different from control *p<0.05; significantly different from control cyclophosphamide group @p<0.01.

values were increased significantly (p<0.05; p<0.01) compared with the values obtained from control and cyclophosphamide treated animals respectively. The antibody titre level was found to be significant (p<0.01) high in animals treated with higher dose (200 mg/kg) of TBAE compared to the control and cyclophosphamide treated group.

The results of carbon clearance assay are demonstrated in Fig 1. To assess the functional changes in macrophages in response to TBAE treatment, their phagocytic ability was determined. Phagocytic index was significantly (p<0.05; p<0.01) increased after the administration of TBAE (100 and 200 mg/kg) compared to control group. However, TBAE failed to produce any significant effect at a lower dose (50 mg/kg).

Table 3 depicts the effect of TBAE on neutrophil adhesion of rats. The TBAE at a dose of 200 mg/kg showed a significant (p<0.05) increased in percentage of neutrophil adhesion (22.18 \pm 1.148) compared to control value (15.25 \pm 0.198). However, the extract at both the doses (50 and 100 mg/kg) did not produce any significant

DISCUSSION

Many of the disorders today are based on the imbalances of immunological processes like DTH (cell mediated) reactions and humoral responses (Kanjiwani, et al., 2008). DTH is a part of the process of graft rejection, tumour immunity and most important immunity to many intracellular infectious micro-organisms, especially those causing chronic diseases viz tuberculosis (Elgert, 1996). Further, DTH requires the specific recognitions of a given antigen by activated T-lymphocytes which subsequently proliferate and release cytokines. These in turn, increase vascular permeability, induce vasodilation, macrophage accumulation (Descotes, 1999) and activations, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing (Kuby, 1997). In the present, DTH reaction is measured by foot-pad thickness, after 48hr of antigenic challenge and subsequent immunization with SRBC, the animal showed significant increase in volume of paw edema due to production of antibodies in response to the antigen. This potentiation of DTH response indicates that TBAE has stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction and thus increases cell mediated immunity.

The index of humoral immune response is the increase in antibody titre value due to increase in immune response (Sehar, et al., 2008). Cyclophosphamide is an immune suppressor, which decreases the antibody titre level; therefore it serves as an ideal standard to compare with the test drug in order to establish whether the immune response is substantial. The humoral immunity involves interaction of B-cells with the antigen and their subsequent proliferations and differentiations into antibody secreting plasma cells (Gokhale et al., 2003). Further, antibody functions as the effector of the humoral response by binding to antigen by neutralizing it or facilitating its eliminations by cross-linking to form clusters that are more readily ingested by phagocytic cells. In the present study, to evaluate the effect of TBAE on humoral response, its influence was tested on SRBC specific haemagglutination antibody titre in rats. Cyclophosphamide (50mg/kg, p.o.) showed significant inhibition in antibody titre response, whereas TBAE showed an en-

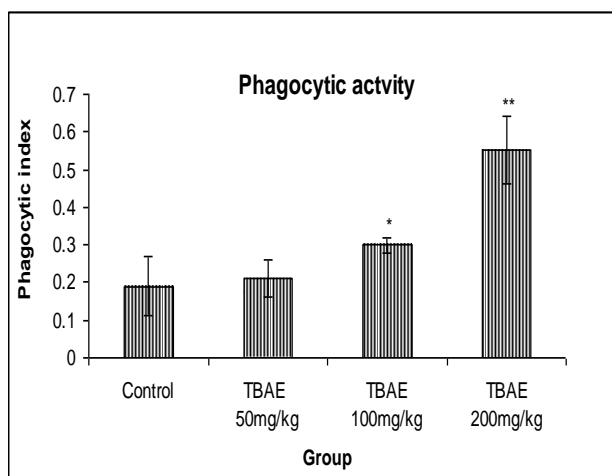
Table 3. Effect of TBAE on Neutrophil adhesion test

Group	Treatment	Neutrophil index		Neutrophil Adhesion (%)
		Untreated blood sample	Fiber treated blood sample	
I	Control	3.330 \pm 0.087	2.824 \pm 0.092	15.25 \pm 0.918
II	TBAE (50 mg/kg)	3.754 \pm 0.058**	3.162 \pm 0.075*	15.81 \pm 0.857
III	TBAE (100 mg/kg)	5.212 \pm 0.136**	4.204 \pm 0.050**	19.22 \pm 1.135
IV	TBAE (200 mg/kg)	4.221 \pm 0.103**	3.281 \pm 0.048**	22.18 \pm 1.148*

Values are expressed as mean \pm SEM (n=6). Significantly different from control *p<0.05; **P<0.01.

hanced production of circulating antibody titre. This augmentation of the humoral response to SRBC antigen by increase in haemagglutination antibody titre

Figure 1. Effect of TBAE on carbon clearance assay



Values are expressed as mean \pm SEM (n=6). Significantly different from control * $p < 0.05$; ** $p < 0.01$.

indicated the enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis (Benacerraf, 1978).

Phagocytic index generally increases whenever there is an increase in immune response and its effects are associated with varied pathologic conditions in humans (White & Gallin, 1986). Besides, phagocytosis represents an important innate defense mechanism against ingested particulates including whole pathogenic microorganisms (Atal, et al, 1986). The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. In a view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to B-cells, TBAE was evaluated for its effect on macrophage phagocytic activity. When the carbon particles are injected intravenously, the rate of clearance of carbon from blood by macrophage is governed by an exponential equation. This seems to be the general way in which inert particulate matter is cleared from the blood. In this study, the increase in phagocytic index as seen from the carbon clearance test after treatment with TBAE advocate effect of extract on macrophages and therefore on the subsequent stimulation of the immune system.

Neutrophils represent a multi-functional cell type in innate immunity that contributes to bacterial clearance by recognition, phagocytosis and killing whereas leucocytes are responsible for the production of antibodies leading to enhancement of immunity (Soehnlein, et al., 2008). Further, Neutrophil granules contain a variety of toxic substances that kill or inhibit growth of bacteria, fungi and mediators of this cell will be increased only when the immune system is stimulated. Therefore, in neutrophil adhesion test, the TLC and DLC were ana-

lyzed. In this test, a significant increase in total and differential count was observed when TBAE was administered orally. Macrophages are polymorphonuclear lymphocytes which play an important role in modulation of the immune system. These cells then secrete number of cytokines likes CSF and IL-1 which in turn stimulates neutrophils and increases neutrophil index (Stanford, et al., 2002). This gives host defense the ability to counter the infectious diseases. Our results demonstrated that the rise in neutrophil index as shown by enhanced adhesion of neutrophil to nylon fiber further suggests that *T. bispinosa* may be useful in promoting the protection of body by phagocytosis, even in diseased conditions where immunity is depressed.

The plant is rich source of phenolic compounds which in other plants have been reported to have immunomodulatory activities (Rudi, 1993). The phenolic compounds can stimulates or suppress the immune system due to the hydroxyl groups in the structure. These groups can affect the enzyme or electron transferring system regulating an immunomodulatory property especially phagocytic activity. Some of the chemical constituents isolated from *T. bispinosa* include ascorbic acids, amylase and amylopectin and antibacterial compounds (Rahman, et al., 2001). Further, our preliminary phytochemical studies show the presence of alkaloids, carbohydrates, starch, tannins, phenolic compounds and saponins glycosides. The results of this study were consistent with the work of other investigators in determining the effectiveness of selected natural products against allergen induced inflammation. For example Mungantiwar, et al. (1999) demonstrated the immunomodulatory effects of alkaloidal fraction of *Boerhaavia diffusa* in mice. Furthermore, it has been reported that, diosgenyl saponins isolated from *Paris polyphylla* possesses immuno-stimulating properties (Zhang, et al., 2007).

CONCLUSION

Trapa bispinosa is a promising immunostimulatory agent as per designed models of immunostimulatory activity. It influenced T-cell production, enhanced neutrophil count, phagocytosis by cells and humoral response at a higher dose against SRBC in the absence as well as in the presence of immunosuppressant agent; cyclophosphamide. Although, the present study did not give the complete view of the mechanism whereby the extract modulates the immune system however, the extract contains compounds which had immunomodulatory activity. Besides, to isolate the active constituents and clarify its mechanism of action will be our auxiliary objective.

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