OPEM
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Oriental Pharmacy and Experimental Medicine 2008 **8(4)**, 380-385
DOI 10.3742/OPEM.2008.8.4.380

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Immunomodulatory effect of *Tinospora cordifolia* and *Centella asiatica* and its modulation on cyclophosphamide challenge

NA Siddiqui[1,*], Mohd Ali[2] and Shobhna Singh[3]

[1]Department of Pharmacy, I.E.T., M.J.P.Rohilkhand University, Bareilly (U.P.), India; [2]Department of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India; [3]Department of Pharmacy, I.E.T., M.J.P.Rohilkhand University, Bareilly (U.P.), India

Received for publication September 10, 2007; accepted March 18, 2008

SUMMARY

Ethanolic extracts of *T. cordifolia* and *C. asiatica* were evaluated for immunostimulatory effect in mice against sheep RBCs as antigen by three models viz. delayed type hypersensitivity reaction, percent change in neutrophil count and haemagglutination titre. Immunostimulatory effect in the presence of immunosuppressant agent, cyclophosphamide (100 mg/kg, i.p.) was also investigated. *T. cordifolia* and *C. asiatica* significantly ($p < 0.001$, $p < 0.05$ respectively) enhanced foot pad thickness when measured after 24 hours of sheep RBC antigen challenge. Both the plant materials increased foot pad thickness even after being subjected to immunosuppressant exposure. *T. cordifolia* revealed enhanced neutrophil counts, while *C. asiatica* had no significant effect on neutrophil counts. *T. cordifolia* exhibited significantly ($P < 0.01$) elevated neutrophil levels even in the presence of cyclophosphamide administration. Both the plants exhibited humoral antibody response, as haemagglutination titre values were significantly high as compared to control. *T. cordifolia* and *C. asiatica* could combat immunosuppressant effect of cyclophosphamide ($P < 0.01$). This suggests that *T. cordifolia* and *C. asiatica* can be regarded as biological response modifiers and can be utilized for the development of immunostimulating agent among plant sources.

Key words: Immunomodulation; Neutrophils; Haemagglutination titre; Cyclophosphamide

INTRODUCTION

From time immemorial, plants have been explored as medicines by human beings and insurgence to plant based products has increased. Plant products, in the form of whole extracts, have become increasingly respectable for consumers as well as physicians. Some of the medicinal plants are believed to promote positive health and maintain organic resistance against infection by re-establishing body equilibrium and conditioning of the body tissues (Bhagwan, 1978). *T. cordifolia* has been reported to enhance host resistance and reduce side effects of some toxic agents (Dhuley, 1997). Methanolic extract of stem of *T. cordifolia* has been reported to increase WBCs and it increased bone marrow cellularity. This extract also increased plaque forming cells in the spleen, circulating antibody titre and increased the macrophage activation (Mathew and Kuttan, 1999). The aqueous and

*Correspondence: NA Siddiqui, Department of Pharmacy, I.E.T., M.J.P.Rohilkhand University, Bareilly (U.P.), India. Tel: +0091-9456244935, 0091-581-2315192; E-mail: nasirpharm@yahoo.co.in

ethanolic extract of *T. cordifolia* inhibited the immunosupression produced by cyclophosphamide (Manjrekar *et al*., 2000). While *C. asiatica* have been proved to be useful strong adaptogenic agent. It has been included in the formulation of herbal preparations, CIHP I and II, which are adaptogenic preparations (Grover *et al*., 1995).

The restorative and rejuvenating power of these plants might be due to their action on immune system, which is responsible for the protection against various diseases. Looking into the beneficial effects of drug extracts and their herbal formulations, the present study is an attempt to explore the immunomodulatory effects of *T. cordifolia* and *C. asiatica* against sheep RBC antigen challenge in mice via three models viz. delayed type hyper sensitivity reaction, neutrophil count, and humoral antibody response in mice. Immunostimulant activity in the presence of immunosuppressant, cyclophosphamide has also been investigated.

MATERIALS AND METHODS

Plant materials and chemicals

The dried aerial parts of *C. asiatica* commonly known as 'Brahmi' and stem cuttings of *T. cordifolia* commonly known as 'Giloya' were purchased from "Natural Drugs and Botanicals", Sahibabad, Ghaziabad (U.P.) and identified by a taxonomist. A voucher specimen of all the crude drugs has been deposited in the "Phytochemistry Research Laboratory", Faculty of Pharmacy, Jamia Hamdard, New Delhi.

Cyclophosphamide (Ledoxan) was purchased from Dabur India Ltd., Ghaziabad, (U.P.), India and used as an immunosuppressant. For dosing, the drug was dissolved in water for injection i.p.

Carboxy methyl cellulose (CMC) and sodium chloride were purchased from s.d. fine Chemicals Ltd, Mumbai, India.

Extract Preparation

The air dried drug was coarsely powdered and exhaustively extracted in a Soxhlet apparatus with ethanol (95%) for 72 h. The extract was concentrated and dried on rotary flash evaporator to get a dark brown mass. The dried extract was used for the pharmacological investigations.

Animals

Swiss albino mice of either sex, weighing 20 - 25 g, were housed in standard conditions of temperature, humidity, 12 h/12 h light/dark cycles and fed with standard pellet diet (Hindustan Lever Pellets, Bangalore, India) and tap water ad libitum. The animals were obtained from the Laboratory Animal Resource Section, Division of Animal Genetics, Indian Veterinary Research Institute, Izat Nagar, Bareilly (U.P.), India and experimentation was carried out with prior approval of the ethical Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), vide approval number 453/01/a/CPCSEA.

Antigen

Sheep red blood cells (SRBC) were obtained from Division of Pathology, Indian Veterinary Research Institute, Izat Nagar, Bareilly (U.P.), collected in Alsevier's solution and washed three times in large volumes of pyrogen-free, sterile saline and adjusted to a concentration of 0.1 ml containing 1×10^8 cells.

Methodology

The 95% ethanolic extracts of *T. cordifolia* and *C. asiatica* were administered as 0.1% suspension of CMC intraperitoneally at a dose of 100 mg/kg body weight/day, from day 1 to day 14.

Cyclophosphamide was dissolved in water for injection i.p. and injected at a dose of 100 mg/kg body weight, i.p. on day 12 to the animals of group IA, IIA and IIIA. Sheep RBCs were adjusted to a concentration of 0.1 ml containing 1×10^8 cells administered intraperitoneally on day 7 as sensitizing dose and 0.05 ml (containing 2×10^8 cells) on day 14 as challenging dose given subcutaneously in right hind foot paw in all groups.

Delayed Type Hypersensitivity (DTH) Reaction

The method described by Doherty (1981) was used in the present study. Mice of either sex were divided into six groups of six each. The mice of all groups were primed with 0.1 ml of SRBC suspension containing 1×10^8 cells, i.p., on day 7 and challenged on day 14 with 0.05 ml of 2×10^8 SRBC in the right hind foot pad. The contralateral paw received equal volume of saline. During this period, the ethanolic extracts of test drugs were fed from day 1 to day 14 in the dose 100 mg/kg body weight, i.p. to the animals of group II, III, IIA and IIIA while the animals of control groups I and IA received 0.1% CMC intraperitoneally. On day 12, one group for each test drug (group IIA and IIIA) and a control group (group IA) were administered with cyclophosphamide (100 mg/kg body weight, i.p.). On day 14, the thickness of the foot pads of all the groups was measured at 24 and 48 h after challenging. It was measured by plethysmometer (Ugo Basil, Italy). The difference in the thickness of the right hind paw and the left hind paw was used as a measure of delayed type hypersensitivity (DTH) reaction.

Neutrophil Count

The method for neutrophil count was described by Ziauddin *et al.* (1996). Mice of either sex were divided into six groups of six each. The treatment schedule was similar to that of SRBC-induced DTH reaction. After sensitization with SRBC on day 7, blood samples were collected from the retro-orbital plexus of individual animals on day 10 and then on day 14 in all the groups. The differential leukocyte count was performed by fixing the blood smears and staining with Field Stains A and B, and percent neutrophil in each sample was determined.

Humoral Antibody (HA) Response

Mice of either sex were divided into six groups of six each. The treatment schedule was similar to that of SRBC-induced DTH reaction. After sensitization with SRBC on day 7, blood samples were collected from the retro-orbital plexus of individual animals on day 10 and then on day 14 in all groups. The antibody titres were determined using the method described by Puri *et al.* (1994). Briefly, an aliquot (25 µl) of two fold diluted sera in saline was challenged with 25 µl of 0.1% v/v SRBC suspension in microtitreplates (Laxbro). The plates were incubated at 37 °C for 1 h and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre. The antibody titres were expressed in a graded manner, the minimum dilution may be represented as ½. The mean dilutions of different groups were statistically compared.

Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M.) and statistical analysis was carried out by employing one- analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered to be statistically significant.

RESULTS

The effect of both the plant material *T. cordifolia* and *C. asiatica* on experimentally induced DTH reaction has been shown in table. Both the plant materials significantly enhanced the foot pad thickness when measured after 24 h of challenge. The foot pad thickness normalized within forty eight hours in both the groups. Thus the results of DTH study indicate that the ethanolic extracts of both the plant materials function as mediators of ensuring hypersensitivity response particularly by attracting and activating macrophages. Cyclophosphamide challenge decreased delayed type hypersensitivity reaction in mice as compared to the control group without cyclophosphamide treatment (group I Vs group IA). Pretreatment with *T. cordifolia* and *C. asiatica* could combat the immunosuppressant effect of cyclophosphamide as in both the groups IIA and IIIA, there was significant increase in foot pad thickness after 24 h of day 14 challenge by

Table 1. Immunological activity profiles of ethanolic extract of *T. cordifolia* and *C. asiatica* against sheep RBCs in presence and absence of cyclophosphamide

Groups	Treatment	DTH Activity		Neutrophil counts (%)	HA Titre
		24 h	48 h		
I	Control (0.1% CMC)	0.28 ± 0.01	0.20 ± 0.01	70 ± 0.58	16.33 ± 0.33
II	*T. cordifolia*	0.37 ± 0.01***	0.18 ± 0.01	78 ± 1.07**	117.33 ± 10.67**
III	*C. asiatica*	0.30 ± 0.01*	0.21 ± 0.01	68 ± 1.65	58.67 ± 5.33*
I A	Control (0.1% CMC + CP)	0.16 ± 0.01	0.25 ± 0.13	55 ± 1.65	14.67 ± 4.34
II A	*T. cordifolia* + CP	0.21 ± 0.01**	0.14 ± 0.01	63 ± 0.73**	29.33 ± 2.67**
III A	*C. asiatica* + CP	0.21 ± 0.01***	0.13 ± 0.01	54 ± 1.27	29.33 ± 2.67**

N = 6 mice per group, tabular value represents mean ± S.E., dose: 100 mg/kg, for all the substances. *P < 0.05; **P < 0.01; ***P < 0.001.

SRBC. The foot pad thickness normalized within 48 h of challenge in the animals of all groups.

Blood was collected on day 1 and analysed for percent neutrophil count. The animals were sensitized with sheep RBC on day 07, blood samples were collected from retro-orbital plexus of each animal on day 10 and day 14, and percent neutrophil count was made for each sample. The percent neutrophil count on day 10 was found to be slightly more as compared to day 01 (P = NS) in all groups. The count of day 14 was found to be significantly enhanced by *T. cordifolia* (P < 0.01). The neutrophil count remained unchanged in *C. asiatica* treated group. Thus *T. cordifolia* significantly augmented neutrophil count while *C. asiatica* had no effect on the same. Cyclophosphamide administration caused reduction in neutrophil counts in control group IA, as compared to control group I. Pretreatment with *T. cordifolia* significantly (P < 0.01) enhanced the neutrophil count as compared to control group IA (0.1% CMC + Cyclophosphamide) even in the presence of cyclophosphamide. Ethanolic extract of *C. asiatica* had no significant effect on neutrophil count in cyclophosphamide treated group.

After sensitization with 0.1 ml (1×10^8) sheep R.B.C. on day 07, the blood samples were taken from retro- orbital plexus from each animal on day 10 and day 14. The antibody titre was determined for all groups, utilising the blood sample collected. After 10 days of treatment by the test materials i.e. extract of *T. cordifolia* and *C. asiatica*, the results reveal no change in haemagglutination (group II and III). However the samples of day 14 exhibited significant humoral antibody response. Increased HA titre values in the *T. cordifolia* and *C. asiatica* treated groups showed significant humoral antibody response as compared to control group I (P < 0.01. P < 0.05 respectively). The myelosuppressant effect of cyclophosphamide has been revealed by the observance of slight decrease in HA titre value in group IA as compared to control group I. The HA titre value in cyclophosphamide treated groups reveals that *T. cordifolia* and *C. asiatica* treatment could combat the myelosupressant effect of cyclophosphamide (P < 0.01) as they showed increased HA titre value as compared to control group IA.

DISCUSSION

Delayed type hypersensitivity reaction is characterized by large influxes of non specific inflammatory cells in which macrophage are a major participant. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized T_{DTH} cells. Activation of T_{DTH} cells in the presence of the antigen through appropriate antigen action leads to secretion of various cytokines including interlukin-2, interferon-γ, macrophage migration inhibition factor and tumor necrosis factor-β (Askenase and Van Loveren, 1983). Secreted

cytokines recruit macrophages into the area and activate them, promoting enhanced phagocytic activity, vis-a-vis augmented concentration of lytic enzymes for more effective killing of foreign entities. Thus DTH reaction is important in host defense against parasite and bacteria that can live and proliferate within the cells. In the present study, *T. cordifolia* and *C. asiatica* showed significant increase in foot pad thickness when measured after 24 h of sheep RBC challenge. The foot pad thickness normalized within 48 h. Administration of cyclophosphamide in animal reduced mean foot pad thickness in control group IA as compared to control without cyclophosphamide exposure. Both the drugs significantly enhanced the foot pad thickness even in the presence of cyclophosphamide, which reveals that the test materials have effect on T-cells and may play a role in providing immunity against parasite and bacterial intracellular proliferation.

The role of phagocytosis is primarily the removal of micro-organisms and foreign bodies, and also the elimination of dead or injured cells. Phagocytic defects are associated with varied pathological conditions in humans. Neutrophils are capable of phagocytosis and their enhancement may provide immunity through phagocytosis. In view of the pivotal role played by neutrophils the test extracts were evaluated for their effect on neutrophil counts. Pretreatment of mice with the extract of *T. cordifolia* enhanced total neutrophil count significantly ($P < 0.01$) when sensitized with antigen, sheep RBCs. The neutrophil levels decreased in the presence of immunosuppressant agent, cyclophosphamide in the control group IA as compared to control group I. Extract of *T. cordifolia* increased neutrophil count even in the presence of immunosuppressant cyclophophamide ($P < 0.01$) hence *T. cordifolia* may be useful in promoting the protection of body by phagocytosis, even in diseased conditions where immunity is depressed. *C. asiatica* revealed no significant effect on neutrophil count. However, the levels remain within the normal range of mice.

T. cordifolia was found to be more effective ($P < 0.01$) than *C. asiatica* ($P < 0.05$) for HA titre activity. Both the plants enhanced the antibody production even when animals were exposed to immuno-suppressant treatment with cyclophosphamide thus both the drugs could combat the myelosupressant effect of cyclophosphamide. The present study establishes the immunostimulant status of *T. cordifolia* and *C. asiatica* as both of them played a role in the activation of T-lymphocytes and the augmentation of circulating antibodies. *T. cordifolia* was found to be more beneficial in terms of immunostimulant effect as it could enhance neutrophil count in the presence as well as in the absence of cyclophosphamide. Authors have also reported immunomodulatory effect of *T. cordifolia*'s polysaccharide on natural killer cells, T-cells and B-cells in the dose 100 μg/ml (Nair *et al*., 2004). Thus, the results indicate that *T. cordifolia* is a promising immunostimulatory agent as per designed models of immunostimulatory activity. It influenced T-cells production, enhanced neutrophil counts and produced significant humoral response against sheep RBCs in the absence as well as in the presence of immunosuppressing agent, cyclophosphamide. *C. asiatica* was next to *T. cordifolia* as an immunos-timulating agent as it could enhance phagocytosis by T-cells and have good humoral response in the presence as well as in the absence of cyclophos-phamide but had no effect on neutrophil count. The immunostimulant properties of *C. asiatica* have been reported to be comparable to recombinant interferon α-2b injection (Patil *et al*., 1998). Authors have reported its immunomodulating activity due to non specific cellular and humoral immune responses. The data available till date, suggest that it may have chemopreventive or anticancer potential (Punturee *et al*., 2005). Further in depth study on the parameters investigated in the present experimentation, need to be designed and explored for immunomodulatory activity. Both the test materials can further be utilized for development of immunostimulant agents against various diseases

because *T. cordifolia* and *C. asiatica* extracts have been found to have influenced several different functions of immune system.

The findings of the present study establish the immunostimulant status of the drugs *T. cordifolia* and *C. asiatica* and suggest their therapeutic usefulness in a variety of diseases susceptible to immunomodulation.

ACKNOWLEDGEMENTS

The authors are grateful to the Laboratory Animal Resource Section, Division of Animal Genetics, Indian Veterinary Research Institute, Izat Nagar, Bareilly, India for providing the animals and facilities for pharmacological activity. We are also thankful to "Natural drugs and Botanicals" Sahibabad, Ghaziabad (U.P.), India for supplying genuine plant materials.

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