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Full Length Research Paper

Effect of five medicinal plants used in Indian system of medicines on immune function in Wistar rats

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Today, the use (which we can say 'return to nature') of traditional herbal medicines, herbal health products, pharmaceuticals food supplement, cosmetics, etc. is increasing due to the growing recognition that natural products are safe; have either no or negotiable side effects. The objective of this study was to investigate the immunomodulatory properties of five different medicinal plants used in Indian system of medicines on the Wistar rats. The activity was investigated by phagocytic carbon clearance, antibody titre and delayed type hypersensitivity test. The control group received 0.1% carboxyl methyl cellulose and other groups received the different doses of all the five plants extracts such as *Tribulus terrestris*, *Cassia tora*, *Achyranthes aspera*, *Mucuna prurians* and *Abrus precatorius* intraperitoneally for 7 consecutive days. All the five plants exhibited immunostimulatory activity and out of the five plants, *T. terrestris* showed significant dose dependent increase in the 'humoral antibody titre and DTH response' as indicated by increase in footpad thickness. It also showed significant increase in the 'phagocytic index' in rats. The investigation revealed that all the mentioned plants have immunomodulatory activities.

Key words: Medicinal plants, antibody titre, delayed type hypersensitivity, phagocytosis.

INTRODUCTION

Many plants products have been exploited for modulation of immune system in number of ayurvedic formulations either alone or in group (Carrasco et al., 2009; Latorre et al., 2009; Chen et al., 2009; Sudha et al., 2010). Some of the plant exhibiting the immunostimulatory activity has been reported as *Withania somnifera* (Davis and Kuttan, 2000), *Argyreia 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* (Siddhiqui et al., 2008). The modulation of immune response by using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations (Patwardhan and Vaidya, 2004). Instead of random screening of a large number of plant extracts, traditional systems of medicine provide an extremely vast body of source material for the development of new drugs

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(Holland, 1994). Some of these plant products are believed to enhance the natural resistance of the body to infection (Atal et al., 1986), on the basis of their constituents like polysaccharides, lectins, saponins and flavonoids etc. Some of these stimulate both 'humoral and cell mediated immunity', while others activate only the cellular components of the immune system. They also suppress both humoral and cell mediated immunity (Atal et al., 1986; Sharma et al., 1994). It is expected that these non-specific effects offer protection against different 'pathogens' including bacteria fungi viruses and so on and constitute an alternative to 'conventional chemotherapy' (Atal et al., 1986) .

Mucuna prurians Linn (Fabaceae), is commonly known as 'cowhage' plant or 'kapikacho' or 'kevach' in Hindi. *Mucuna prurians* seeds are well known to contain levodopa and possess anabolic, androgenic, analgesic (pain-relieving), anti-inflammatory, anti- Parkinson's, antispasmodic, antivenin, aphrodisiac, febrifuge (fever reducing), hormonal, hypocholesterolemic (cholesterol lowering), hypoglycemic, immunomodulator, nervine (nerve control balancing), neurasthenic (nerve pain relieving), antilithic (kidney stones preventing or eliminating), and antiparasitic properties. It is used for the management of several free radical-mediated diseases such as ageing, rheumatoid arthritis, diabetes, atherosclerosis, male infertility and nervous disorders', etc (Chopra et al., 1956; Tempe and Geole, 2009; Anusha et al., 2008). The seeds are astringent, laxative, anthelmintic, alexipharmic and tonic agents (Sathiyanarayanan and Arulmozhi, 2007). *Achyranthes aspera* commonly known as Apamarg (family: Amaranthaceae), is used as antiarthritic, antifertility, laxative, antihelminthic, aphrodisiac, antiviral, antispasmodic, antihypertensive, anticoagulant, diuretic and as an antitumor agent (Ratra and Misra, 1970; Workiehneh et al., 2006; Vetrichelvan and Jagadeesan, 2003). It is also used to treat children for 'colic' with 'hydrophobic' 'hypoglycemic' thyroid-stimulating brain tonic and 'antiperoxidative properties' (Misra and Singh, 1991; Batta and Rangaswami, 1973; Akhtar and Iqbal, 1991; Tahiliani and Kar, 2000). *Tribulus terrestris* commonly known as 'Gokhru', 'puncture vine', 'goathead', etc is a shrub belonging to the family Zygophyllaceae used as rejuvenation tonic and for variety of health conditions affecting the liver kidney, cardiovascular, immune systems and for management of male erectile dysfunction (wwwhttp:// tribulusterrestriscom; Adaikan et al., 2000; Rao et al., 1996; Stuart, 1990; Gauthaaman and Ganesan, 2008). *Cassia tora* commonly known as 'Charota' belonging to family Caesalpiniaceae leaves and seeds are used as acrid laxative, antiperiodic, anthelmintic, ophthalmic liver tonic cardiotonic and expectorant agent (Lassak and McCarthy, 1996; Jang et al., 2007). The therapeutic properties of the plant have been attributed to the presence of saponins and flavonoids (Wealth of India, 1976). *Abrus precatorius* commonly known as 'Indian liquorice' 'Jequirity', belongs to the family Leguminosae. The seeds have haemagglutinating, mitogenic and tumoricidal activities and are used for construction of immunotoxins. It also induces antitumor immunity against Meth-A tumor cells (Praveen kumar et al., 1994; Bhutia et al., 2009a; 2009b).

As we know, the human population is in direct exposure to different risk factors such as pathogenic agents and mycotoxins which impair immune function and this is controlled by the use of immunomodulators. The aim of this study was to evaluate the effects of five plants on different aspects of immunity such as phagocytic index, humoral antibody titre and delayed type of hypersensitivity on animal model.

MATERIALS AND METHODS

Animals

Inbred Wistar rats of either sex of three to four weeks old were obtained from the National Institute of Nutrition Hyderabad and were acclimatized for three to four weeks in the animal house of Jawaharlal Nehru Cancer Hospital and Research Centre Bhopal under standard conditions of temperature (23 ± 2°C), relative humidity (50 ± 5%) and light (10:14 h of light and dark, respectively). The animals were housed in polypropylene cages containing sterile paddy husk (procured locally) as bedding and fed with standard animal feed and filtered acidified water *ad libitum.* Seven to eight weeks old and weighing 150 to 200 g animals were selected for the experiments. The research was conducted in accordance with the guidelines of internationally accepted principles for laboratory animal use and cares (CPCSEA) as approved by the Institutional Animal Ethics Committee.

Plant material and extract preparation

Fruits of *T. terrestris* (voucher specimen no. 13424), seeds of *C. tora* (voucher specimen no. 17477), seeds of *A. precatorius* (voucher specimen no. 18367), seeds of *M. prurians* (voucher specimen no. 14448) and seeds of *A. aspera* (voucher specimen no. 17775) were collected from the suburbs of Bhopal in the month of August to October and identified at the State Forest Research Institute Jabalpur MP India, where a voucher specimen has been preserved for future identification. The plant parts were thoroughly washed with tap water and dried on filter paper sheets under shade at room temperature for more than one month. Thoroughly shade dried coarsely powdered parts of the plants were extracted with soxhlet apparatus using 50% alcohol for 48 h or till 12 cycles were completed. The extracts were then concentrated on a rotatory evaporator below 40°C and were stored in an airtight container in the refrigerator for further experimental studies.

The extracts were not soluble in water, therefore, suspensions of all the extracts was made in 0.1% sodium carboxy methylcellulose (CMC) solution in distill water prior to intraperitoneal administration to the animals. It was used within 7 days and stored at 8°C; for further use, freshly prepared solution was used. The vehicle alone served as the control.

Preliminary phytochemical screening

Extracts of all the plants were subjected to preliminary phytochemical screening using the methods described by Kokate (2001) and Trease and Evans (1983) for the detection of various plants constituents and Lowry et al. (1951) for protein test.

Test for flavonoids

To 1 ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow colour was produced in the plant extract, which became colorless on addition of a few drops of dilute acid; indicates the presence of flavonoids.

Test for alkaloids

It was carried out by adding 0.5 g extract in 5 ml 1% HCl, boiled, filtered and Mayer's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

Saponins

The extract was subjected to frothing test for the identification of saponin. The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 min. The formation of 1cm layer of foam showed the presence of saponins.

Phenol/tannins

The test for tannins was carried out by adding 3 g of each plant extract to 6 ml of distilled water, filtered and ferric chloride reagents was added to the filtrate. Blue black precipitate indicates the presence of tannins and phenols.

Carbohydrates: Molisch's test

3 ml of each of the plant extract was added to 2 ml of Molisch's reagent and the resulting mixture was shaken properly. 2 ml of concentrated H_2SO_4 was then poured carefully down the side of the test tube. A violet ring at the interphase indicates the presence of carbohydrate.

Protein

5 ml of copper reagent was added to the tubes containing 1 ml of the plant extract, mixed well and incubated for 10 min. 1 ml of folin's reagent was mixed. Tubes were incubated for 30 min at room temperature and absorbance was taken at 700 nm. Standard curve was prepared using 50 mg % BSA.

Glycosides

Killer-Kiliani test was adopted. 2 ml of each extract was dissolved in 2 ml of glacial acetic acid containing one drop of $FeCl_3$ solution. The mixture was then poured into a test tube containing 1 ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of glycoside.

Preparation of sheep red blood cells (antigen)

Fresh sheep blood was collected from the local slaughterhouse in the vials under sterile conditions in sterile Alsevere's solution in 1:1 proportion of Alsevere's solution (freshly prepared). Blood was kept in the refrigerator and processed for the preparation of SRBC's batch by centrifugating at 2000 rpm for 10 min, washing with physiological saline 4 to 5 times, suspending into buffered saline for further use (Dash et al., 2006) and adjusted to a concentration of $1x10^8$ cells/ml for immunization and challenge.

Preparation of carbon ink suspension

Commercially available Camel brand black ink suspension was purchased from the local market and diluted in a ratio of 1:50 with normal saline and used for carbon clearance test in a dose of 1 ml/200 g body weight of rat.

Experimental design

Animals were divided into 11 groups consisting of 6 animals each. Group control : received (0.2 ml) 0.1% carboxyl methyl cellulose intraperitoneally (ip) for 7 consecutive days; Group I: received 1 mg/kg bw of *T. Terrestris* fruits (TTE) extract (0.2 ml ip) for 7 consecutive days; Group II: received 2 mg/kg bw of *TE* (0.4 ml ip) for 7 consecutive days; Group III: received 200 mg/kg bw of *C. tora* extract (0.4 ml ip) for 7 consecutive days; Group IV: received 400 mg/kg bw of *C. tora* extract (0.8 ml ip) for 7 consecutive days; Group V: received 500 µg/kg bw of *A. aspera* extract (0.1 ml ip) for 7 consecutive days; Group VI: received 1 mg/kg bw of *A. aspera* extract (0.2 ml ip) for 7 consecutive days; Group VII: received 100 µg/kg bw of *M. prurians* extract (0.02 ml ip) for 7 consecutive days; Group VIII: received 200 µg/kg bw of *M. prurians* extract (0.04 ml ip) for 7 consecutive days; Group IX: received 125µg/kg bw of *A. precatorius* extract (0.25 µl ip) for 7 consecutive days; Group X: received 250 µg/kg bw of *A. precatorius* extract (0.50 µl ip) for 7 consecutive days.

Humoral antibody response to SRBC's

On the 8th and 13th day of the study, the rats from all the groups were immunized and challenged respectively with SRBCs in normal saline (0.1 ml of suspension containing $1x10^8$ SRBC) intraperitoneally. Blood was withdrawn from the retro orbital plexus from all antigenically sensitized and challenged rats on day 14 and centrifuged to get serum. Serial two fold dilution of serum was made in normal saline in microtitre plates and sheep RBC (25 µl of 1% SRBC prepared in normal saline) was added to each of these dilutions. The plates were incubated at 37°C for 1 h and then examined for haemagglutination as described by Puri et al. (1994).

Delayed type hypersensitivity (DTH) response using SRBC's as an antigen

On the 8th day of the study, all the groups of rats were primed by injecting 0.1 ml of suspension containing $1x10^8$ SRBC into the right hind footpad subcutaneously. The contra lateral paw received an equal volume of 0.1% CMC similarly. On the 13th day, the animals were challenged by injecting 0.1 ml of $1 x10^8$ SRBC's into the left hind footpad of the rats subcutaneously. The extent of DTH

Table 1. Phytochemical screening of hydroalcoholic extract of five medicinal plants (*A. precatorius, T. terrestris, M. prurians, A. aspera* and *C. tora*).

Bioactive compound	*A. precatorius*	*T. terrestris*	*M. prurians*	*A. aspera*	*C. tora*
Flavonoids	+	+	-	+	+
Alkaloids	+	+	+	-	-
Saponins	-	+++	+	++	+
Phenols / tannins	+	+	+	-	-
Carbohydrates	-	+	-	+	+
Proteins	+	-	+	+	-
Glycosides	-	+	-	+	+

+, Trace; ++, +++ , excess (indicates the presence of phytochemical constituents).

response in rats was determined by measuring the footpad thickness after 24 h of challenge as described by Barcotti et al. (1984).

Macrophage phagocytosis by carbon clearance method

Phagocytic activity of the 'reticulo endothelial' system *in vivo* was determined by carbon clearance test. After completion of the extract treatment (Biozzi et al., 1953), on 8th day, immediately after the last dose was administered to all the animals of each group, the control as well as the treated groups received an intravenous injection of carbon suspension (1:50 dilution of Indian ink camel) in a dose of 1 ml/200 g body weight. Blood was withdrawn from the retro orbital venous plexus before injection (0 min) and 12 min after injection of the carbon suspension, 50 µl of blood was lysed with 4 ml of 0.1% sodium carbonate solution (Na_2CO_3). The optical density was measured spectrophotometrically at 650 nm wavelength. The results were expressed as phagocytic index:

$$K = (\ln OD_{12\ min}) - (\ln OD_{0min}) / (t_{12min} - t_{0min}) \quad (2)$$

and OD_0 are the optical densities at time t_{12} and t_0,

Where, $OD_{12\ min}$ respectively.

Statistical evaluation

All the results were expressed as the mean ± standard error (SEM) and data were analyzed using Student's "t" test.

RESULTS

Preliminary phytochemical screening

The presences of various phytoconstituents of the extracts were detected by phytochemical screening. The results were presented in Table 1.

Effect of extracts on humoral antibody titre (HA response)

Daily administration of 50% alcoholic extract of *A. precatorius* for 7 consecutive days produced a significant ($p < 005$) increase in humoral antibody titre at 1.25 µg, but no significant effect was observed at the higher dose of 2.5 µg/kg bw. *A. aspera* (500 µg and 1 mg/kg, ip) produced a dose related increase ($p < 005$) in antibody titre compared to the control rats but no significant difference was observed between the two doses when compared with each other. *M. prurians* (200 µg/kg, ip) and *C. tora* (400 mg/kg, ip) showed significant ($p < 005$) effect compared with control, but no significant effect was observed at the lower doses. Humoral antibody response was significantly increased ($p < 001$) in the animals treated with *T. terrestris* at doses 1 and 2 mg/kg bw in a dose dependent manner compared when the control group. At higher dose (2 mg/kg, bw), the titer value was extremely significant when compared with the control group. When the doses were compared with each other, no significant difference was found. Maximum titer value observed at the 14th day at 1 mg/kg and 2 mg/kg bw was found to be 59733 ± 14279 and 78933 ± 157, respectively, while the control group showed maximum titre value of 8533 ± 1349 (Figure 1).

Effect of extracts on delayed type hypersensitivity (DTH) response

Daily administration of 50% alcoholic extract of *Abrus precatorius* for 7 consecutive days produced a significant ($p < 005$) increase in DTH response of rats to sheep RBC at 125 µg, but the effect at the higher dose was not significant from the control. *A. aspera* (500 µg and 1 mg/kg, ip) produced a dose related increase ($p < 005$) in DTH response in rats compared with the control, but no significant difference was observed between the two doses when compared with each other. *M. prurians* (100 µg and 200 µg/kg, ip) and *C. tora* (200 mg and 400 mg/Kg, ip) showed significant ($p < 005$) effect only at the higher doses. *T. terrestris (*1 mg and 2 mg/Kg, ip*)* for 7 consecutive days produced a dose related increase in the

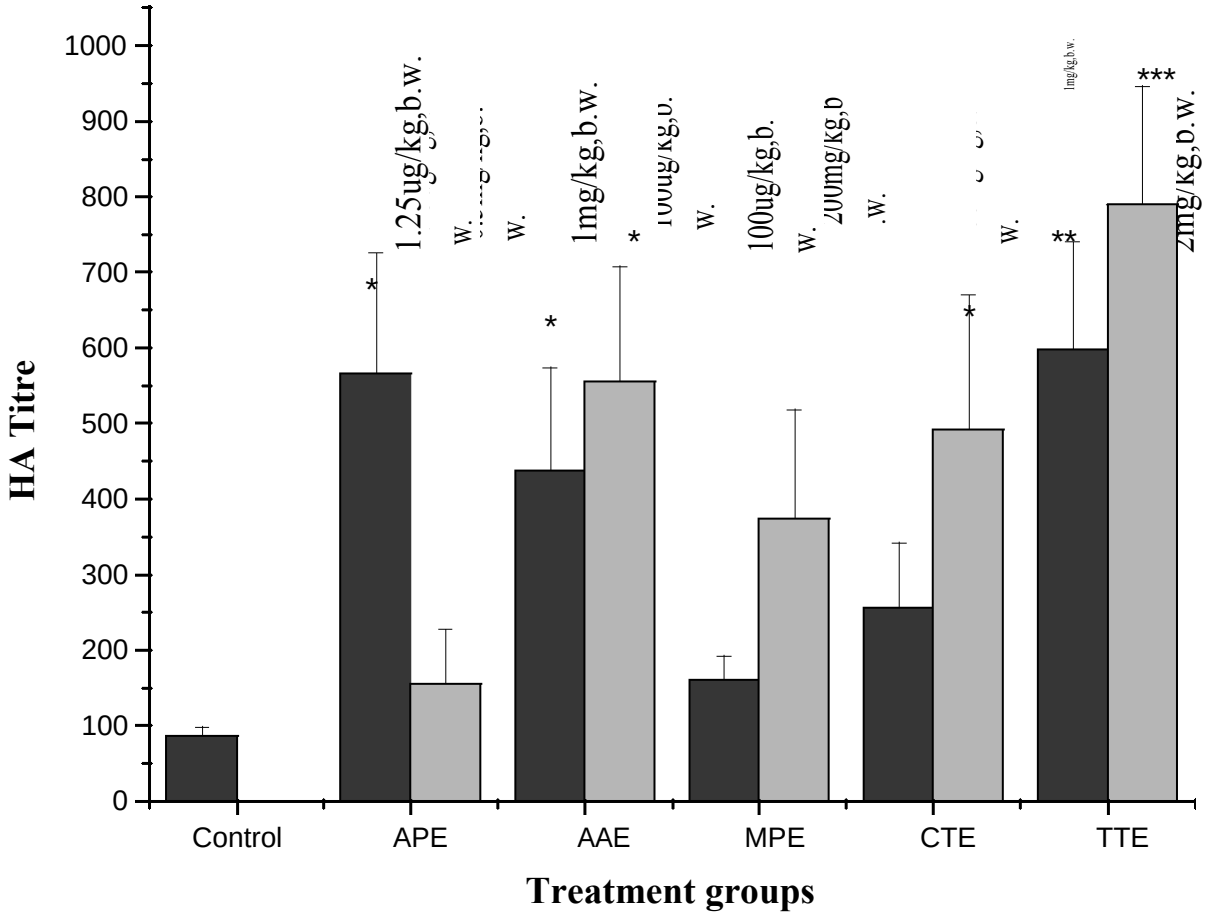


Figure 1. Effect of 50% alcoholic extract of *A. precatorius* (APE), *A. aspera* (AAE), *M. prurians* (MPE*)*, *C. tora* (CTE) and *T. terrestris* (TTE) on humoral antibody response in Wistar rats.

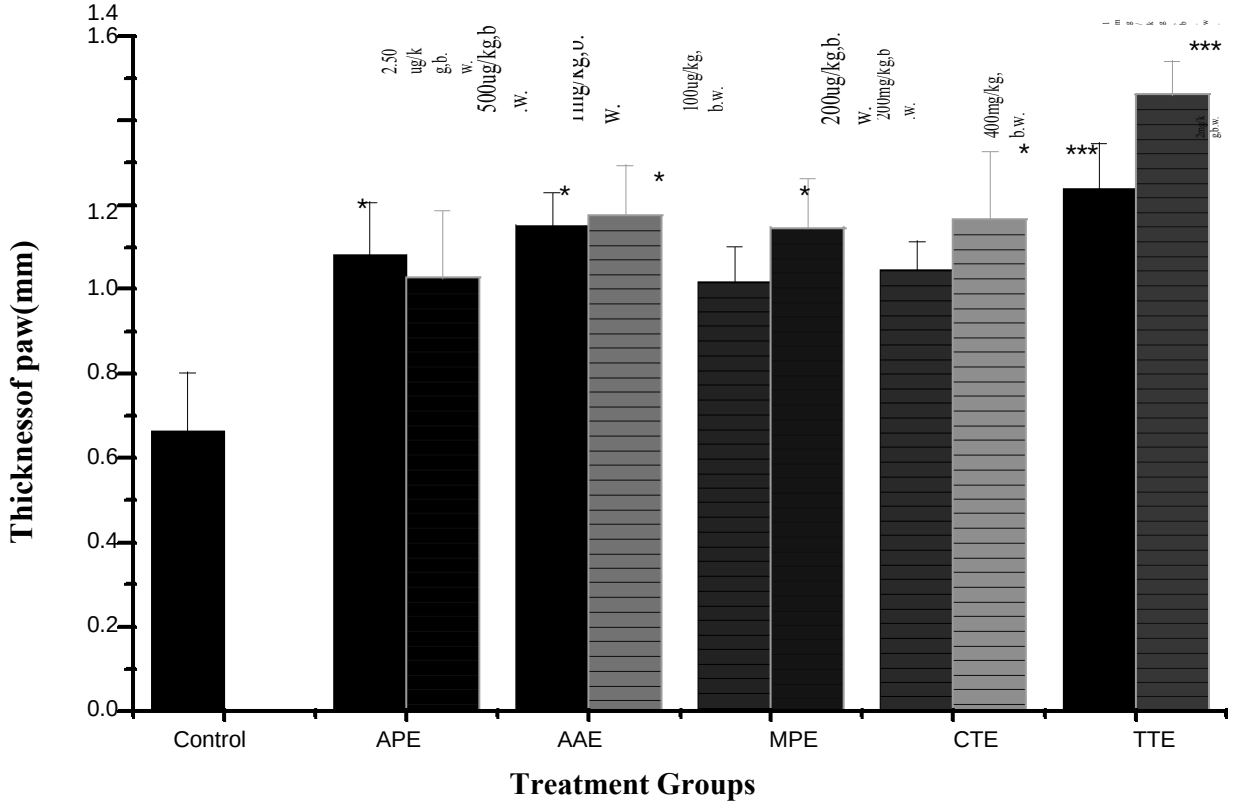
delayed type hypersensitivity reaction to sheep RBC in rats when compared with the control. *T. terrestris* at 1 mg/kg bw resulted in the significant increase ($p < 001$) of DTH response. The thickness of the hind paw challenged with sheep RBC in TTE treated group was almost 54% more (1237 ± 0108) than that in the control group (0663 ± 0139) while at 2 mg/kg, the thickness was 66% more (1462 ± 0079) than that in the control (Figure 2).

Effect of extracts on phagocytic index

The phagocytic index in all the groups treated with *A. precatorius, A. aspera, M. prurians, T. terrestris* and *C. tora* showed significant phagocytic index when compared with the control group. Maximum phagocytic index was observed in *T. terrestris* when compared with the control and as well as other groups. The phagocytic index was increased in the dose dependent manner (Figure 3).

DISCUSSION

The use of medicinal plants to cure human illnesses has been practiced from the time immemorial. Some of these drugs are believed to have enhanced the natural resistance of the body to infection (Atal et al., 1986). 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). Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its



*= p<0.05, ** = p<0.01, ***= p< 0.001 compared to control group

Figure 2. Effect of 50% alcoholic extract of *A. precatorius* (APE), *A. aspera* (AAE), *M. prurians* (MPE), *C. tora* (CTE) and *T. terrestris* (TTE) on DTH response in Wistar rats.

functions; if it results in an enhancement of immune reactions, it is named as an immunostimulative drug which primarily implies stimulation of specific and non specific system that is, granulocytes, macrophages, complement, certain T lymphocytes and different effector substances (Galindo-Villegas and Hosokawa, 2004; Ardo et al., 2008). Immunosupression implies mainly to reduce resistance against infections stress and may occur on account of environment or chemotherapeutic factor (Makare et al., 2001). It was evident from this study that intraperitoneal administration of hydroalcoholic extracts of all the five plant exhibited enhanced immunomodulatory activity in wistar rats.

The alcoholic extract of all the plants at different doses showed almost a threefold increase in DTH response compared with the untreated controls. The highest DTH response was found in *T. terrestris,* when these plants were compared with each other. An increase in DTH response indicates the stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction (Luster et al., 1982; Dean et al., 1979). DTH is a part of the process of graft rejection tumor immunity and most important immunity to many intracellular infectious microorganisms, especially those causing chronic diseases such as tuberculosis (Elgert, 1996). The mechanism behind this elevated DTH during the CMI response involves effector mechanisms carried out by T lymphocytes and their products (lymphokines) (Miller et al., 1991). DTH requires the specific recognition of a given antigen by the activated T lymphocytes which subsequently proliferates and releases cytokines. These in turn increase vascular permeability, induce vasodilatation macrophage accumulation and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated, TH1 cells encounter certain antigens

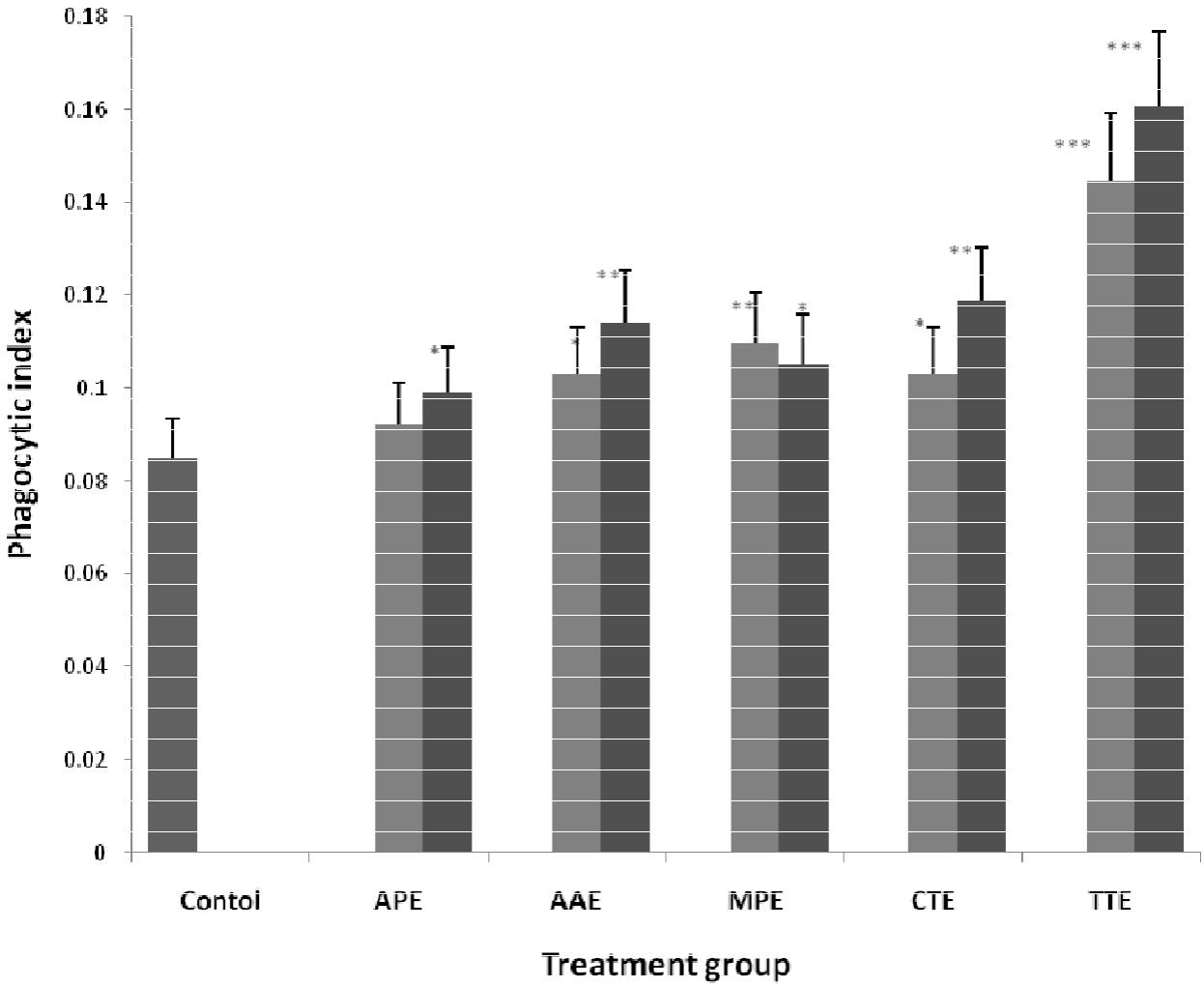


Figure 3. Effect of 50% alcoholic extract of *A. precatorius* (APE), *A. aspera* (AAE), *M. prurians* (MPE), *C. tora* (CTE) and *T. terrestris* (TTE) on phagocytic index in Wistar rats. *p < 0.05; **p < 0.01; ***p < 0.001 compared with the control group.

namely SRBCs (Bafna and Mishra, 2004).

The increase in antibody titre may be due to the stimulation of macrophages and B-lymphocytes subsets involved in antibody synthesis (Makare et al., 2001; Gokhale et al., 2003; Sehar et al., 2008). The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. The antibody response is the culmination of a series of cellular and molecular interactions occurring in an orderly sequence between a B cell and a variety of other cells of the immune system (Roitt et al., 2001). The cellular and humoral immunity is regulated by distinct subsets of helper T-cells (Th); the former is regulated by Th1 cells and the latter is controlled by Th2 cells (Marinko and Dragutin, 2001).

The role of phagocytosis is primarily the removal of microorganisms and foreign bodies but also the elimination of dead or injured cells. The intraperitoneal injection of all these plants enhanced the rate of carbon clearance (more than a two fold increase), when compared with the control group. The results are in allegiance to the mechanism related to phagocytosis by macrophages (Ziauddin et al., 1996). 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 the

host defense the ability to counter the infectious diseases and pathological conditions in human (Furthvan and Bergvanden, 1991). The process of phagocytosis by macrophages also includes opsonization of the foreign particulate matter with antibodies and complement C3b leading to a more rapid clearance of foreign particulate matter from the blood (White and Gallin, 1986; Soehnlein et al., 2008). In this study, all the plants showed the significant increase in the phagocytic activity of the macrophages as evident by an increase in the rate of carbon clearance.

Further, our preliminary phytochemical studies showed the presence of alkaloids, carbohydrates, tannins, phenolic compounds and saponins glycosides which in other plants have been reported to have immunomodulatory activities (Rudi, 1993). The phenolic compounds and saponins 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. The results of this study were consistent with the work of other investigators in determining the effectiveness of selected natural plant 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* possess immunostimulating properties (Zhang et al., 2007).

The different types of immune responses fall into two categories; specific or adaptive immune response and nonadaptive or nonspecific immune response. Specific immune response responds to the challenge with a high degree of specificity as well as the remarkable property of "memory". Typically, there is an adaptive immune response against an antigen within five to seven days after the initial exposure to a particular antigen. Exposure to the same antigen sometimes in the future will result in a memory response. The immune response to the second challenge occurs more quickly than the first and is stronger often more effective in neutralizing and clearing the pathogen/antigen. Memory response generates a life-long immunity following an infection. The two key features of the adaptive immune response are thus, specificity and memory. From the results observed in this investigation, it may definitely be concluded that alcoholic extract of all the five plants possessed immunomodulatory activity by stimulating both cell mediated and humoral immune responses. These findings provide scientific evidence to the ethnomedicinal use of these plants by the tribal population that thrived by them for ages. All the plants possess therapeutic applications for the future.

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