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Research Article

# COMPARATIVE STUDY OF ALCOHOLIC AND AQUEOUS EXTRACTS OFTRIBULUS TERRESTRIS ON SPECIFIC AND NON SPECIFIC IMMUNE RESPONSES IN WISTAR RATS: AN IN VIVO STUDY

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**ABSTRACT**

The present study was aimed to study the effect of alcoholic and aqueous extracts of fruits of *Tribulus terrestris, commonly known as Gokhru, (belongs to family,* Zygophyllaceae) on specific and non specific immune functions in Wistar rats. The immunomodulatory activity was investigated by phagocytic carbon clearance, antibody titre, and Delayed type hypersensitivity test.

Control group received 0.1% Carboxyl methyl cellulose and treated groups received different concentrations (0.5, 1, 2 mg/kg b.w.) of alcoholic and aqueous extracts of fruits of *Tribulus terrestris* intraperitoneally.

The study revealed that alcoholic and aqueous extracts of fruits of *Tribulus terrestris* has exhibited the immunostimulatory activity by increasing humoral antibody titre and delayed hypersensitivity reaction as indicated by increase in footpad thickness, it also shown significant increase in phagocytic activity in wistar rats at different concentrations which reveals increase in the specific and non specific immune responses.

The present investigation reveals that both the aqueous and alcoholic extract of the plant possess the potent immunomodulatory activity; further investigation is required to isolate the active compound to see its mechanism of action and to develop an immunostimulating agent among herbal origin.

**Keywords:** Immunostimulant, Phagocytic index, Humoral immunity, cell mediated immunity.

## INTRODUCTION

Medicinal plants are rich source of secondary metabolites and are used for the treatment of many diseases. Many synthetic drugs are developed as immunomodulators but the major drawback of these drugs is many side effects such as myelosuppression, which is undesirable. Immunomodulator of herbal origin appears to be a better alternative to overcome the above problem [1,2,3]. The function and efficacy of the immune system may be influenced by many exogenous factors like food and pharmaceuticals, physical and psychological stress and hormones etc. resulting in their immunostimulation or immunosupression. The healthy state believed to be based on a sophisticated fine tuning of immunoregulatory mechanism [4,5]. Immunostimulators have been known to support T-cell function, activate macrophages and granulocytes, and complement natural killer cells apart from affecting the production of various effector molecules generated by activated cells (Paraimmunity) [6]. 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 [7]. *Ayurveda,* the oldest medicinal system in the world, provides leads to find therapeutically useful compounds from plants. Therefore, *ayurvedic* knowledge supported by modern science is necessary to isolate, characterise, and standardise the active constituents from herbal source. Recent screening with plants has revealed many compounds like flavonoids, alkaloids, saponins, terpenoids, monoterpenoids (linalool), glycoproteins, polysaccharides, tannins, essential fatty acids, phenolics compounds and vitamins having pronounced antioxidant, antineoplastic, antiulcer, anti-inflammatory and immunostimulating potential [8]. This combination of traditional and modern knowledge can produce better antiulcer, anticancer and immunomodulatory drugs with very few side effects.

*Tribulus terrestris***,** commonly known as Gokhru, puncture vine and goathead, etc. is a shrub belonging to the family Zygophyllaceae. Historically, *Tribulus terrestris* was used by the cultures of India and Greece as a rejuvenation tonic [9]. It was also used as a therapy for a variety of health conditions affecting the liver, kidney, cardiovascular and immune systems [10]. Today, *Tribulus terrestris* in combination with a variety of herbal products is used in headaches, eye conditions such as itching, conjunctivitis and weak vision, and nervousness[11]. This herb has also been used in connection with high blood pressure and rib pain[12]. The inhibitory effect of saponins from *Tribulus terestris* on Bcap-37 breast cancer cell line in vitro were also studied [13]. Pande and Vijaykumar [14] and Rao et al [15] successfully demonstrated the immunomodulating activity of a combination of extracts of these plants. It has tonic and aphrodisiac properties [16]. The therapeutic properties of *Tribulus terrestris* have been attributed to the presence of active compounds saponins, alkaloids and flavonoids etc [17].

Thus in our present study, we have attempted to evaluate the immunomodulatory potency of alcoholic and aqueous extracts of fruits of *Tribulus terrestris* on specific and non specific immune responses.

## MATERIALS AND METHODS

### Animals

Inbred Wistar rats of either sex, 3-4 weeks old were obtained from the National Institute of Nutrition, Hyderabad and were acclimatized for 3-4 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 200g animals were selected for the experiments. 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 *Tribulus terrestris* (voucher specimen no.13424), were collected from the suburbs of Bhopal and identified at the State Forest Research Institute, Jabalpur, M.P. 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. The

extracts were prepared with two different types of solvent, i.e. water and alcohol. Thoroughly shade dried, coarsely powdered parts of plants were extracted with Soxhlet apparatus using absolute alcohol for 48 hrs or till 12 cycles are completed for alcoholic extract and water extract was obtained by the method of Suffness and Douros [18]. Briefly, 100 g of the powder was extracted with 1 liter of double distilled water at 80$^0$C in a water bath for 72 hours. The cooled extract was filtered with muslin cloth. The extracts were then concentrated on a rotatory evaporator below 40$^0$ C, stored in an airtight container in refrigerator (-20 $^0$C) for further experimental studies.

**Preparation of the Test sample**

Suspension was made in 0.1% sodium carboxy methylcellulose and administered to animals to give a concentration of 1mg/ml. CMC was used as a vehicle.

**Sheep red blood cells (Antigen) preparation**

Fresh sheep blood was collected from the local slaughterhouse in sterile vials under sterile condition in sterile Alsevere's solution in 1:1 proportion of Alsevere's solution (Freshly prepared). Blood was kept in refrigerator and processed for the preparation of SRBC's batch, by centrifugating at 2000 rpm for 10 minutes and washing with physiological saline 4-5 times and then suspending into buffered saline for further use [19] and adjusted to a concentration of 1x10$^8$ cells/ml for immunization and challenge.

**Carbon ink suspension**

Commercially available camel brand black ink suspension was purchased from the local market and diluted in a ratio 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 7 groups of 8 each.

Group I control: Received 0.4 ml of 0.1% Carboxyl methyl cellulose (i.p.) for 7 consecutive days

Group II: Received 500 µg/kg (0.1 ml) b.w. of aqueous extract of fruit of TE, i.p. for 7 consecutive days.

Group III: Received 1 mg /kg b.w. (0.2 ml) of aqueous extract of *TE*, i.p. for 7 consecutive days.

Group-IV: Received and 2mg /kg b.w. (0.4 ml) of aqueous extract of *TE*, i.p. for 7 consecutive days

Group-V: Received 500 µg/kg b.w. (0.1 ml) of alcoholic extract *TE*, i.p. for 7 consecutive days.

Group VI: Received 1 mg /kg b.w. (0.2 ml) of alcoholic *TE* , i.p. for 7 consecutive days.

Group-VII: Received 2mg /kg b.w. (0.4 ml) of alcoholic extract *TE*, i.p. for 7 consecutive days.

**Parameters studied**

**Humoral antibody response to SRBC's**

On 8$^{th}$ and 13$^{th}$ 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 (25µl of 1% SRBC prepared in normal saline) were added to each of these dilutions and the plates were incubated at 37$^o$C for 1 hr and then examined for haemagglutination as described by Puri et. al.[20].

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

On 8$^{th}$ 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 13$^{th}$ 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 response in rats was determined by measuring the footpad thickness after 24 hrs of challenge, as described by Barcotti et.al. [21]

**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 [22]. On 8$^{th}$ day, immediate after the last dose administered to all the animals of each group control as well as treated 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 and 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:

$K = (\text{In OD}_{12\,min}) - (\text{In OD}_{0min}) / (t_{12min} - t_{0min})$

Where, $OD_{12\ min}$ and $OD_0$ are the optical densities at time $t_{12}$ and $t_0$ respectively

**Statistical Evaluation**

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

**RESULTS**

**Effect of Extracts on Humoral antibody titre (HA response):**

Humoral antibody response was significantly increased in the animals treated with aqueous extract of TE at 0.5mg/kg compared to control group. While at higher doses (1 mg/kg b.w. and 2mg/kg b.w.) the titer value was extremely significant as compared to control group. When the inter group comparison was done, there was no significant difference in titre value between 0.5mg/kg, b.w. and 1mg/kg ,b.w., while a significant two- fold increase in titre value was observed at 2 mg/kg, b.w. as compared to 0.5mg/kg, b.w (fig.-1).

Daily administration of alcoholic extract at lower dose produced no significant antibody response but at 1mg/kg and 2 mg/kg b.w. the response increased in a dose dependent manner. When compared with each other no significant difference was observed between the different dose groups. (fig- 1).

**Effect on Delayed type hypersensitivity (DTH) response**

Daily administration of the aqueous extract of TE for 7 days (0.5mg/kg, 1mg/kg and 2mg/kg b.w, i.p) resulted in a significant increase in the DTH response of rats to sheep RBC .It was observed that even lower doses shows significant effect and as the dose increased the response also increased significantly. When the groups were compared with each other the highest thickness was achieved at 2mg/kg b.w, which was significant compared to the other two doses. No significant difference was observed between 0.5mg/kg and 1mg/kg, b.w. (fig.2).

Daily administration of alcoholic extract of TE resulted in significant increase in paw thickness at doses of 0.5 and 1 mg/kg b. wt. and extremely significant increase was observed at higher dose (2 mg/kg b.wt.) when compared with control. The highest value was obtained at 2mg/kg b.wt., which was significant when compared with 0.5 mg/kg b.wt., while no significant difference was observed between 0.5mg/kg and 1mg/kg or between 1mg/kg and 2mg/kg b.wt. (Fig.2)

**Effect on Phagocytic Index**

The phagocytic index in all the groups treated with aqueous extract was found to be extremely significant as compared to control. No significant increase in phagocytic index was observed between 0.5mg/kg and with 1mg/kg b.w., while significant increase was observed when 2mg/kg b.w. was compared with 0.5 mg/kg,b.w (fig.3).

In the alcoholic extract treated groups the phagocytic index increased in a dose dependent manner as compared to control. The carbon clearance was highest at 2mg/kg, b.w., which was significant when compared with 0.5mg/kg, b.w. but not significant when compared with 1mg/kg, b.w. (fig.3).

**DISCUSSION**

Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating the specific and non specific immune system. However, recently there is an enthusiasm towards exploration of a novel group of compounds from natural sources that modulate the immune response of living systems and influence the disease process [23]. In the previous study we compared five medicinal plants and found that the Tribulus terrestris is the one which shows highest Immunomodulatory potential [24, 25]

In the present study, both alcoholic and aqueous extracts of *Tribulus terrestris* exhibited immunostimulatory activity in Wistar rats. But the aqueous extract showed a higher effect than alcoholic extract. The results indicate that the extracts not only potentiate non-specific immune response, but are also effective in improving specific immunity. *Tribulus terrestris* contains different types of active components such as saponins, flavonoids, alkaloids and glycosides which can be responsible for the immunomodulatory activity of this plant. The idea of the present study was originated with the report of Dua et al [26] for the extracts obtained from *Indian panax pseudoginseng*, which is a potent immunostimulant; it exhibits the immunomodulatory activity by augmentation of IgG, increasing the antibody titre against SRBC and increase in macrophage migration activity. The earlier reports on cell mediated immunity by Panax ginseng show that saponins enhances the immunity by increasing the proliferation of cells by con A, increasing humoral response to sheep RBC and macrophage migration inhibition [27].

The haemagglutination antibody titre was used to assess humoral immune response. The augmentation of the humoral immune response to SRBCs by extracts of *Tribulus terrestris* evidenced by increase in the antibody titres in the blood of rats. The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cell. 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 [28]. IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc [29].

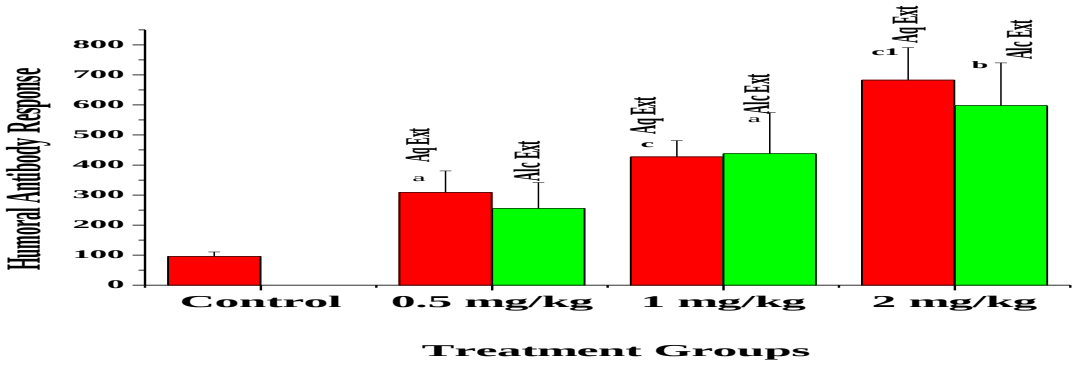


**Fig. 1: Humoral antibody response of aqueous and alcoholic extract of Tribulus *terrestris* compared to control**

Values are expressed as mean ± S.E.M. n = 8,

a = p<0.05, b = p<0.005, c p<0.001 as compared to control group. 1p<0.05, 2p<0.001, as compared to 0.5 mg/kg extract. * p<0. 05, **p<0.05 compared to 1 mg/kg b.w. of extract.

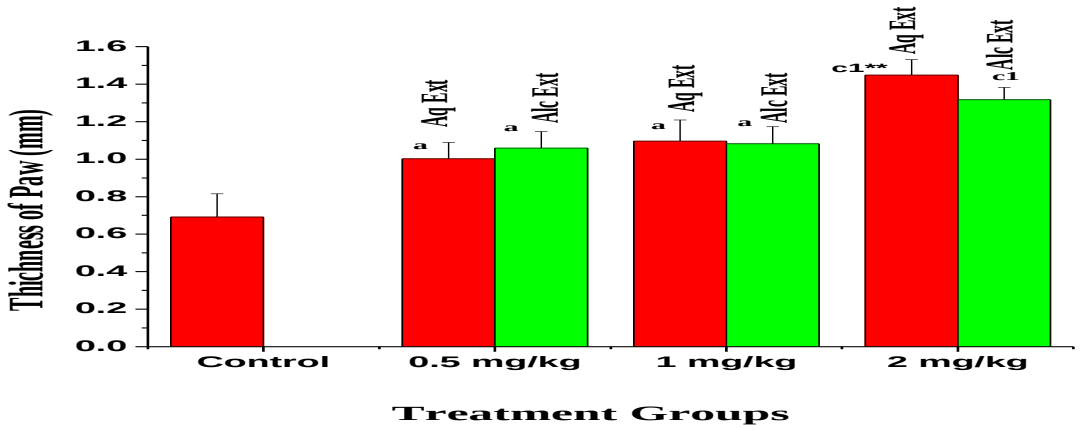


**Fig. 2: DTH Response of aqueous and alcoholic extract of Tribulus *terrestris* compared to control**

Values are expressed as mean ± S.E.M. n = 8

a = p<0.05, b = p<0.005, c p<0.001 as compared to control group.1p<0.05, 2p<0.001, as compared to 0.5 mg/kg extract.* p<0. 05, **p<0.05 compared to 1 mg/kg b.w. of extract.

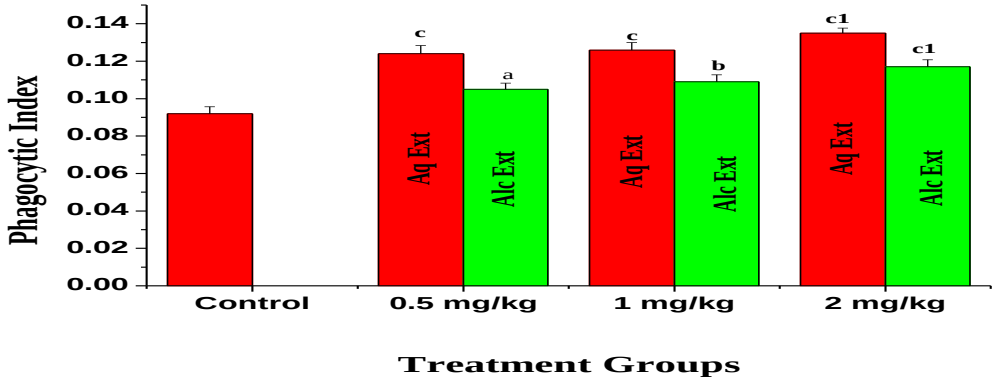


**Fig. 3: Phagocytic Index of aqueous and alcoholic extract of Tribulus *terrestris* compared to control**

Values are expressed as mean ± S.E.M. n = 8

a = p<0.05, b = p<0.005, c p<0.001 as compared to control group

1p<0.05, 2p<0.001, as compared to 0.5 mg/kg extract. * p<0. 05, **p<0.05 compared to 1 mg/kg b.w. of extract.

In the present investigation, SRBC-induced delayed type hypersensitivity was used to assess the effect of the extracts on cell-mediated immunity. Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defence against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions [29]. Therefore, increase in DTH reaction in rats response to T cell dependent antigen revealed the stimulatory effect of Tribulus terrestris on T cells. Increased carbon clearance is an indicator of enhanced in vivo phagocytic activity and competency of granulopectic system in removal of foreign particles, therefore, an indicator of enhanced immunological response against foreign particles or antigens. The process of phagocytosis by macrophages 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 [30]. Jin and Kurashige [31] reported that macrophages also serve as effector cells to provide immune surveillance against tumor cells. This seems to be the general way in which inert particulate matter is cleared from the blood. This study demonstrates that *Tribulus terrestris* treatment is potentiated more the phagocytosis of reticulo endothelial system.

Recent reports indicate that many plant products used in traditional medicine have been reported to have immunomodulating activities. While some of these stimulate both humoral and cell-mediated immunity (CMI), others activate only the cellular components of the immune system, i.e. phagocytic function without affecting the humoral or CMI.

In the present study, statistically significant rise in HA titre, DTH response and phagocytic effect of the extracts suggest that active principles of fruits which are responsible for stimulation of humoral and cell mediated response can be extracted with both aqueous as well as alcoholic extract. The exact mechanism of action, however, can only be unfolded after detailed characterization of active moieties of these fractions.

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