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IMMUNOMODULATORY ACTIVITY OF *TERMINALIA CHEBULA* AGAINST *SALMONELLA TYPHIMURIUM* IN MICE

K. H. Khan[1,2*]
[1]*Centre for Biotechnology, Faculty of Science, Hamdard University, Jamia Hamdard, New Delhi-110062, India*
[2]*Medical Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India*

Abstract
Typhoid is a worldwide problem today due to the emergence of multidrug resistance *Salmonella typhi* and limited scope of vaccine against this disease. As the plant product has little side effects and is the source of many compounds having wide range of biological activities, diverted the author to study the protective role of *Terminalia chebula*, against *Salmonella typhimurium*. The author has already reported the protective effect of aqueous extract of the fruits of this plant against *S. typhimurium in vitro* as well as *in vivo* and also the antioxidant activity against these bacteria. In this study the same extract was evaluated for its immunomodulatory activity against *S. typhimurium in vivo*. Animals pretreated with the same extract at a dose 500 mg/kg body wt orally showed an increase in WBC count by $3x10^3$/cu mm and lymphocyte count by 4 % as compared to saline treated control challenged with 50000 colony forming unit of *S. typhimurium*. The drug showed the proliferation of lymphocyte by 102% and increase in food pad thickness by 28.87% as compared to infected control in delayed type of hypersensitivity test. Thus the drug showed its protective effect through its immunomodulatory activity in mice and can be used in typhoid.

Key Words: Delayed type of hypersensitivity; Lynphocyte proliferation; *Salmonella typhimurium*; *Terminalia chebula*; Typhoid.

Introduction

Typhoid is endemic in many developing countries and remains a substantial public health problem despite recent progress in water and sanitation coverage [1]. It is an infectious disease caused by Salmonella enterica serotype typhi, remains an important worldwide cause of morbidity and mortality. It is characterized by high fever, rose colored spot, bone marrow depression, diarrhea, eosinophilia and various other symptoms reported by us in a review article on typhoid [2].

A major impediment to the effective chemotherapy of typhoid is the ever increasing numbers of resistant strains of *S. typhi*. More over there is limited efficacy of vaccine to the younger population [1,3]. Due to the above reasons we diverted our study toward the use of plant products as it has less side effects and are the reservoir of various organic compounds which act as antimicrobial, antioxidant, immunomodulatory and also posses various other pharmacological properties required to combat infectious diseases. The basic approaches behind the management of infectious diseases include either to destroy the bacteria, or to booster the immune system of an individual by the administration of immunostimulants.

In humans, *S. enterica*, serovar *Typhimurium* (*S. typhimurium*) causes a self-limiting gastroenteritis, while in mice it causes a systemic typhoid-like disease, which is used as a model of human typhoid [4]. Infection of mice with *S. typhimurium* results in splenomegaly and a macrophage mediated immunosuppression. Host resistance to systemic salmonellosis is mediated by reactive oxygen and nitrogen intermediates, inflammatory cytokines, and specific CD4D and CD8D T cells, and B cells [5].

Medicinal herbs represent a rich source from which novel antibacterial chemotherapeutic agents could be obtained. A number of plants having high medicinal value have been reported by us [6]. Immunomodulatory activity of triphala on neutrophil functions was also reported [7].

Terminalia chebula Retz is antioxidant, hepatoprotective, antimicrobial, adaptogenic and anti-inflammatory as reported by this author based on literature survey [3]. The aqueous extract of this fruit was reported to have anti

* *Corresponding Author, Email: hamkishwar191@yahoo.co.in; kishwar@vit.ac.in*

Salmonella typhimurium activity *in vitro* and also *in vivo*. Moreover this author reported that the same extract given orally at a dose of 500 mg/kg body wt (T500) showed 100% protection against $1X10^5$ colony forming units (CFU) of *S. typhimurium* when injected itraperitoneally [8]. Further studies were reported by author using the same aqueous extract against the oxidative stress induced by the same dose of the same bacteria. It was found by author that the mice pretreated orally with T500 for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium* showed a reduction of catalase (CAT) and lipid peroxidation (LPO) activity and increased in reduced glutathione (GSH) activity as compared to saline control mice subjected to same dose of bacteria [9].

In another study the author reported that the same dose (T500) for same period of time following the same route showed a decrease in xanthine oxidase activity (XO), increase in glutathione peroxidase (GPX) and also glutathione reductase (GR) activity [3] against same doses of *S. typhimurium* proving to be antioxidant against the same bacteria. The author further studied the protective immunological role of the same drugs (T500) following oral route against the same doses of bacterium and tried to represent his work in this research article.

Material and Methods

Plant material

In this study dried fruits of *Terminalia chebula* Retz were used. The fruits belong to the family *Combretaceae*. It was purchased from the market of Okhla at New Delhi, India and was identified by University taxonomist.

Preparation of plant extract

The fruits were washed and dried. Aqueous extract of the fruits were used in this experiment. The extract was prepared as described by this author [3]. The dried powder of the extract was stored in sterile tube at 4 ^{o}C. The sterile powder was named as T in this manuscript.

Microorganisms

During this study wild type of *S. typhimurium* was used. The standard strain of *S. typhimurium* was obtained from National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India. This bacterial strain was further characterized and authenticated by the Department of Microbiology, Majeedia Hospital, New Delhi, India.

Animals

Swiss albino mice were used as an animal model. Mice weighing about 20-25 gm were used in this study. These animals were supplied by Central Animal House, Hamdard University, New Delhi-62 and kept under standard laboratory condition for 12 hr light dark cycle at 25$\pm$ 10 ^{o}C. The above animals were supplied with pellet diet (Lipton, India) and water ad labitum. Much precaution and care was taken so to provide approximately no pain to animals.

Research was performed according to the internationally accepted principles for laboratory animal use. All the studies were conducted at Jamia Hamdard, Hamdard University, New Delhi according to ethical guidelines of the Jamia Hamdard Animal Ethics Committee and "The Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA) in the use of animals for scientific research.

Dose and Dosages

The drug used in this experiment was aqueous extract of *T. chebula* (T) by oral route. Animals pretreated with T500 for a period of 30 days provide 100% protection against $1X10^5$ CFU of *S. typhimurium* as reported by this author earlier [8]. In determining the enzymes of oxidative stress the author used 50000 CFU of *S. typhimurium* intraperitoneally [3] [9]. As this work is the extension of previous work the author used T500 orally and 50000 CFU of *S. typhimurium* intraperitoneally. The animals were divided into following groups containing six animals in each group.

The study comprised of following treatment schedule.

Group S: Normal saline.

Group S+B: Normal saline+50000 CFU of *S. typhimurium* (wild).

Group T500: Aqueous extract of *T. chebula* (500 mg/kg body wt).

GroupT500+B: Aqueous extract of *T. chebula* (500 mg/kg body wt) + 50000 CFU of *S. typhimurium* (wild).

Counting of white blood corpuscles

The mice were divided into three groups having six mice in each group. First and second group was pretreated orally with saline for a period of 30 days and third group was pretreated with T500 for same period of time and through same route. After 30 days of pretreatment the animals in second and third group were injected with 50000 CFU of *S. typhimurium* intraperitoneally. At 7th day of post infection (PI) the blood of animals were collected and the leukocytes were counted at Majeedia Hospital, New Delhi-62, India.

Counting of lymphocytes

In this study the blood was used from the same animals. The blood was collected in dried tube and lymphocyte counts were taken at Majeedia Hospital, New Delhi-62 for authentic results.

Lymphocyte proliferation assay

Eighteen animals were used for this experiment. The animals were divided into three groups having six animals in each group. The animals of first and second groups were treated with saline orally for a period of 30 days and the third group with T500 for same period of time through the same route. After pretreatment for a period of 30 days the animals of the second as well as third groups were challenged with 50000 CFU of *S. typhimurium.* Blood was then withdrawn from retro-orbital plexus of mice. Lymphocytes were then separated from the blood by density gradient centrifugation on Histopaque-1077. The buffy coats were used to isolate mononuclear cells. Sterile pasteur pipette were used to pipette out mononuclear cells from buffy coat. The cells were pellet out at 400Xg for 10 minutes. Cells in the pellet was then suspended carefully in 1 ml of growth medium (RPMI-1640) containing 10% FBS and recommended concentration of antibiotic. The viability of cells was assessed by the trypan blue exclusion method. The cells were then plated in 96-well culture plates (Tarsons) at a cell density of $1X10^6$ cells/well. ConA was used as a mitogenic agent. The culture plate was incubated at 37 ^{o}C in CO_2 (5%) incubator (Forma Scientific,Ohio), with water-saturated air for 6 days. After 6 days the cells were pulsed with 1 μCi of [^{3}H]-methylthymidine. After 18 hr of radiaoactive pulse, cells were harvested and the amount of [^{3}H]-thymidine incorporated into DNA was determined by liquid scintillation spectroscopy [10].

Delayed-type hypersensitivity

Delayed type hypersensitivity (DTH) is a type IV hypersensitivity mediated by sensitive T_H helper cells which release various cytokines. It is one of the manifestations of cell mediated immunity. For the determination of DTH the requirement is the preparation of antigen from the organism, immunization of animals with the same organism and then finding out the DTH of the drug against the test organism in the terms of foot pad swelling.

Preparation of antigen from S. typhimurium by sonication

The sonicated antigen was made as described by Tiwari and Kamat [11]. Briefly, *S. typhimurium* were grown at 37°C on nutrient agar, suspended in phosphate buffer saline (PBS), pH 7.2, harvested and washed with PBS. The suspended cells were disrupted by sonication, and centrifuged at 10,000 rpm for 1 hr. The supernatant was lyophilized and the protein contents of the lyophilized material were estimated by Lowry's method [12].

Immunization of animals

The experiment of DTH was carried out by standard footpad swelling method as enumerated by Collins and Mackaness [13]. The animals were divided into two groups having six animals in each group. One group was pretreated with saline and other with T500. These groups were immunized with 50000 CFU of *S. typhimurium* intraperitoneally.

Analyzing the effect of drugs on DTH response against S. typhimurium

On the 7th day of immunization, the right hind footpad was injected with *S. typhimurium* cell lysate. The left hind foot was injected with saline and served as control. The food pad swellings were then recorded at an interval of 3hr, 6hr, 12hr, 24hr, 48hr and 72hr. Results have been summarized in Fig 4.

Results

Forty six grams of drug was obtained from 100 grams of dried fruit as reported by this author earlier [3]. This lyophilized aqueous extract was used in this experiment.

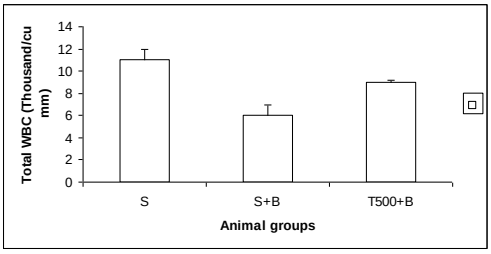

Fig. 1. Total WBC induced by *S. typhimurium* in mice when pretreated for 30 days.
S = Mice pretreated with saline for a period of 30 days. S+B = Saline treated mice (S) + 50000 CFU of *S. typhimurium*. T500+B = Mice pretreated with 500 mg/kg body wt of T for 30 days followed by challenge with 50000 CFU of *S. ryphimurium*. Values are significantly different. ***P< 0.001.

Swiss albino mice pretreated with saline for a period of 30 days orally and then subjected to 50000 CFU of *S. typhimurium* by intraperitoneal route showed a decrease of total leucocyte count by $5x10^3$ /cu mm at day 7th of PI as compared to normal uninfected saline treated control. Mice pretreated with T500 for a period of 30 days followed by challenge with same doses of *S. typhimurium* exhibits an increase in total leucocytes count by $3x10^3$/cu

mm as compared to infected saline treated control subjected to same doses of *S. typhimurium* (50000 CFU) (Fig1).

Animals pretreated with saline for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium* showed a decrease of lymphocyte count by 6% at day 7th of PI as compared to saline control mice not subjected to the *S.typhimurium*. Mice pretreated with T500 through oral route for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium* intraperitoneally showed an increase of lymphocyte count by 4% as compared the the control mice subjected to same doses of *S. typhimurium* through the same route at day 7th of PI (Fig 2).

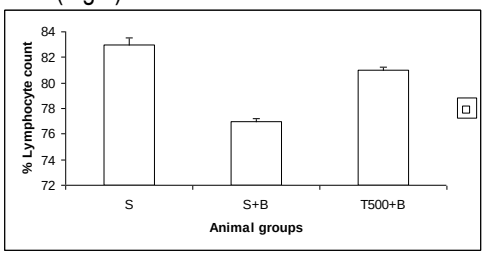

Fig. 2. Percent lymphocyte count induced by *S. typhimurium* in mice pretreated for a period of 30 days.
S = Mice pretreated with saline for a period of 30 days, S+B = Saline pretreated mice (S) challenged with 50000 CFU of *S. typhimurium.* T500+B =Mice pretreated with 500 mg/kg body wt of T for 30 days followed by challenge with 50000 CFU of *S. typhimurium*. Values are significantly different. ***P< 0.001.

Swiss albino mice pretreated with T500 through oral route subjected to 50000 CFU *of S. typhimurium* intraperitoneally showed an increase of 102% in the level of T cell proliferation in lymphocyte proliferation assay as compared to saline treated control subjected to same doses of bacteria through same route (Fig 3).

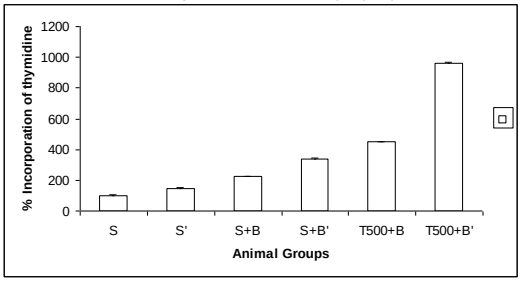

Fig. 3. Lymphocyte proliferation induced by *S typhimurium* in mice pretreated for a period of 30 days.
S = Mice treated with saline, S' = Saline (S) + ConA (5ug), SB = Saline pretreated animal + (50000 CFU of *S. typhimuriium*), SB'= Saline (S) +Bacteria (50000 CFU of *S. typhimurium*) + ConA (5ug), T500+B= Mice pretreated with 500mg/kg body wt of T + 50000 CFU of *S. typhimurium*, T500+B' = Mice pretreated with 500mg/kg body wt of T + 50000 CFU of *S. typhimurium* + ConA (5ug).
Values are significantly different. ***P< 0.001.

The drug T500 when evaluated for its DTH activity against *S. typhimurium* showed an increase of 28.87% of footpad thickness as compared to the saline treated control subjected to same doses of *S. typhimurium* (Fig 4).

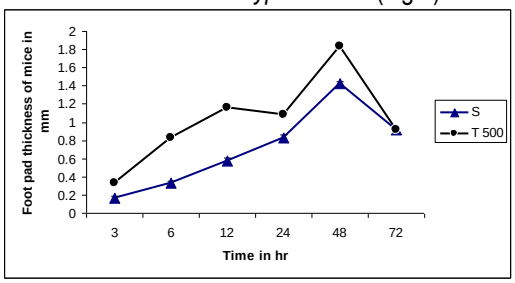

Fig. 4. DTH response induced by water extract of *Terminalia chebula* against *S. typhimurium*. *Terminalia chebula* was administered at a dose of 500mg/kg body wt. and compared with saline treated control. Foot pad thickness is the difference of thickness of treated and control foot pad. Values are significantly different. ***P< 0.001.

Discussion

Encounters with bacterial pathogens lead to subclinical infection, most of the time, with mounting of active humoral and cell-mediated immune responses culminating in the elimination of the organism [14]. Blood contains leukocytes or white blood cells that play an important role to defense the body against microorganism. The category of leukocyte includes lymphocytes, granulocytes, monocytes and macrophages. Lymphocytes are mononuclear leukocytes that mediate humoral and cell mediated immunity. The lymphocytes are of three types namely B lymphocytes, T lymphocytes and null cells. B cells bring about humoral immunity. T cells are again divided into T helper cells (T_H cells), T suppressor cells (T_s cells), T cytotoxic cells (T_c cells) or T killer cells (Tk cells) and T delayed type hypersensitivity cells (T_D cells). The activated T_H cells secrete lymphokines. The lymphokines increase the response of B cells, T killer cells and T suppressor cells. The B cells are activated to produce antibody. T_D cell is also known as T_H1 cells that secrete variety of cytokines that recruit and activate macrophages and other nonspecific inflammatory cells. Activated macrphages exhibit increased level of phagocytosis and an increased ability to kill microorganism through various cytotoxic mediators. The influx and activation of macrophages in the DTH response is important in host defense against parasites and bacteria that live within the cells, where circulating antibody cannot reach them [15].

Earlier it was reported by this author that T500 showed 100% protection against $1X10^5$ C.F.U of *S. typhimurium* when injected itraperitoneally [8]. In this study animals pretreated with T500 followed by challenge with 50000 CFU of *S. typhimurium* showed an increase in the level of WBC or leucocyte explained the encounter of

bacteria against the cells of the immune system indicating protection of the body against *S. typhimurium*. Further study showed the increase in lymphocyte counts which again claimed the protective effect of drugs against *S. typhimurium*. This was again confirmed by studying the lymphocyte proliferation assay which clearly indicated the proliferation of T-Lymphocyte and finally the protective effect of drugs.

The drug (T500) caused an increase in footpad thickness in mice at 48hr followed by a decrease of swelling at 72hr as compared to saline treated control, which indicates that the plants extract exhibited DTH response against *S. typhimurium*. As CMI plays major role in defense against salmonella infection, the induction of DTH response by drug treatment may be the mechanism of protection against salmonellosis.

Aqueous extract of *T. chebula* was reported to produce an increase in humoral antibody (HA) titer and delayed-type hypersensitivity (DTH) in mice [16] which further supports this study. As there is an increase in lymphocytes in animals pretreated with T500 followed by challenge with 50000 CFU of *S. typhimurium* it can also be concluded that the killing of the *S. typhimurium* takes place via humoral immune response also. Over all it proves that the protection to animals against *S. typhimurium* is due to the immunomodulatory activity of the drug.

ROS propagate inflammation by stimulating release of cytokines, such as interleukin-1, tumour necrosis factor-α, and interferon-β, which stimulate recruitment of additional neutrophils and macrophages. Thus, free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralisation by antioxidants and radical scavengers which can attenuate inflammation [17].

T. chebula contains a number of compounds like chebulagic acid [18], gallic acid [19], ellagic acid [20] which are highly antioxidant. The above said plant was also reported to have an antioxidant property [21]-[23]. The immunomodulatory activity of this extract may be due to its antioxidant activity.

Conclusion

The fruits of Indian medicinal plant *T. chebula* are known for their pharmacological activity and in this manuscript it has been shown that the extract can be used as an effective immunomodulator against *S. typhimurium*. Aqueous extract of *T. chebula* showed an increase in WBC and lyphocytes count against *S. typhimurium*. The extract also proliferated lymphocyte and exhibited delayed type of hypersensitivity. The result showed its immunomodulatory activities. Further study is required to test this drug on other immunological parameters to confirm its immunological activities against *S. typhimurium*.

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