Protection against experimental salmonellosis by *Terminalia belerica* and *Punica granatum* extracts: immunological evaluation

A. Madani and S. K. Jain*  
Department of Biotechnology, Hamdard University, New Delhi, India.  
sk608@rediffmail.com*

Abstract: Typhoid fever (TY) is an acute systemic infection caused by the bacterium *Salmonella enterica* serovar Typhi and is transmitted by the fecal oral route mainly via contaminated food and water. While the developing countries have high rate of morbidity and mortality due to TF, epidemics take place in developed world also. There are increased incidences of multi drug resistant in *S. typhi* strains that has further complicated its management and only a few antibiotics are now effective in treatment of typhoid. We report that the aqueous extracts of *Terminalia belerica* and *Punica granatum*, confer protection against experimentally induced salmonellosis. Clearance of bacteria from reticuloendothelial system is increased in a time dependent manner. Drugs enhance the delayed-type hypersensitivity reaction against *S. typhimurium*. Parallel to the activation of delayed-type hypersensitivity reaction, drugs reduced the amount of persistent bacteria in livers and provide cytoprotection against ill effect of bacteria. The cytokines act as communication molecules between host cells in the defense against the enteric pathogen, especially in *Salmonella*. A significant decrease was observed in the IL-1α concentration in serum of *S. typhimurium* infected mice that were pretreated with drugs. These results correlate with the fact that both Tb and Pg have the capacity in involvement of the induction of immune response as a mechanism of protection against typhoid.

Key words: Typhoid fever, *T. belerica*, *P. granatum*, anti-*Salmonella* activity, survival, DTH and cytokine.

Introduction

Typhoid fever (TF) caused by *Salmonella enterica* serotype *Typhi* (*S. typhi*) is a major public health problem, particularly in developing countries. TF caused 21,650,974 illnesses and 216,510 deaths annually (Crump, 2004). In Western countries, the disease has been brought very close to eradication levels. Multi-drug-resistant (MDR) *S. typhi* strains have been reported from different parts of India (Lakshmi, 2006; Achla, 2005; Dutta, 2005; Walia, 2005; Madhulika, 2004; Safdar, 2004; Chande, 2002) that have created a significant therapeutic problem. Consequently, the murine model of salmonellosis has been used extensively to explain potentially clinical relevant mechanisms of anti-*Salmonella* host-defence (Yrild et al., 2000). The treatment used against this disease is antibiotic while vaccination is used for prevention. In view of the increasing resistant to the antibiotics (Ahmad et al., 2002; Hazbir et al., 2002; Lee et al., 2002; Prabha et al., 2002; Atoba et al., 2001; Gupta et al., 2001; Hassan et al., 2001; Launay et al., 1997; Swaddiwudhipong et al., 2000) and less than desired efficacy of currently available vaccines, it is pertinent to evaluate the efficacy of the natural plant products in the treatment of typhoid by screening the local medicinal plants for anti-*Salmonella* activity. The approaches for the management of infectious diseases include either to destroy the causative organism, or to enhance the immunity of an individual by the administration of immunostimulants.

**Terminalia belerica** Roxb (N.O Combretaceae), commonly known as "bahera" is distributed throughout the forests of India. It has been reported to have hepatoprotective effect, antioxidant activities and inhibitor of lipid peroxidation. Different constituents identified in its fruit include gallic acid (3,4,5, trihydroxy benzoic acid), ellagic acid, chebulagic acid (Row & Murty, 1970) etc. **Punica granatum** Rox (N.O Punicaceae) commonly known as ‘Anar’ is a shrub that is cultivated throughout the India. It contains ascorbic acid, citric acid, ellagic acid, ellagittannin, flavogallol, gallic acid and tannins. The extracts of the fruit rind powder exhibited antibacterial activity against many bacteria especially *Salmonella para typhi* (Trivedi & Kazmi, 1979). The main components present in this plant is phenolics and tannins (Panizzi et al., 2002) and flavonoids (Tsuchiya et al., 1996) that show high antimicrobial activity.

**Material and methods**

**Plants material**

Plant materials were procured from local market of New Delhi with their identity confirmed and only the authenticated material were used for experiments. Prior to use, it was insured that the herbs were free from contamination, and had no microbial growth.

**Animals**

Swiss albino mice (22-30g) of 5-8 weeks were used for all the experiments. The animals obtained from the Central Animal House Facility, Hamdard University were maintained on a standard laboratory feed (Amrut Laboratory, Navmiharashtra Chakan Oil Mills Ltd, Pune) and water *ad libitum*. All the studies were conducted...
according to ethical guidelines of the Jamia Hamdard Animal Ethics committee and "Committee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA).

2.3 Bacteria

The standard strains of *S. typhi* (wild) and *S. typhimurium* (wild) were obtained from National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, and characterized at the Microbiology Laboratory, Majeedia Hospital, Hamdard University, to confirm their identities. They were grown overnight in nutrient broth at 37°C. Dilution and pour plating onto TSI agar plate was done to check the viability of bacteria using standard Dabbing methods. Both *S. typhi* and *S. typhimurium* were used for *in vitro* studies while *S. typhimurium* was used for all the *in vivo* studies.

2.4 Preparation of plants extract

Plants extract were prepared by the method described by Ahmad et al. (2001) with minor modifications. Briefly, 100 g powdered plant material was soaked in 200 ml of petroleum benzene for 72 h, with stirring every 24 h with a sterile glass rod. At the end of extraction period, it was centrifuged and the supernatant was filtered through Whatmann No. 1 paper. Extraction was repeated three times. The filtrates were pooled and evaporated to dryness in a Buchi Rotavapor (Labortechnik, Switzerland) and stored at -20°C until further use. Residue left after petroleum benzene extraction was sequentially extracted with chloroform, acetone and ethanol as above. Aqueous extract was made by extracting the dried fruit powder directly with doubled distilled water that was then centrifuged, filtered, lyophilized and stored as above.

**Doses and Dosage**

Animals were divided into different groups, each group contains six animals. The study comprised of following treatment schedules: **Group S:** Normal saline; **Group SB:** Normal saline + (0.5xLD$_{50}$) of *S. typhimurium* (wild); **Group Tb500:** Aqueous extract of *Terminalia belerica* (Tb) (500mg per kg)+ (0.5xLD$_{50}$) of *S. typhimurium* (wild); **Group Pg500:** Aqueous extract of *Punica granatum* (Pg) (500mg per kg)+ (0.5xLD$_{50}$) of *S. typhimurium* (wild).

**Survival study**

Above doses and design were used for this study. Animals were pretreated with respective extracts as mentioned in dose and design. In each case, the mice were observed for 15 days of post bacterial infection and the results were expressed as percent survival of mice in each group.

**Bacterial clearance study**

The mice were pretreated with the drugs, challenged with *S. typhimurium* and killed after 7 days of challenge. The liver of the animals was aseptically removed, washed with PBS and homogenized (10%w/v) in PBS containing 1.15% KCl at room temperature. An aliquot from each homogenate was cultured on TSI agar plates. Bacterial colonies obtained after overnight incubation at 37°C were screened for *S. typhimurium* growth on above plates and counted individually. The *Salmonella* impart a blackish hue in TSI agar plates. The results of the experiment were expressed as number of viable bacteria in log$_{10}$ CFU per gm tissue.

**Protein estimation**

Protein estimation was performed using the method of Lowry et al. (1951).

**Delayed-type Hypersensitivity (DTH)**

**Sonicated antigen preparation of *S. typhimurium***: The sonicated antigen was made as described by Tiwari and Kamat, (1986). Briefly, *S. typhimurium* were grown at 37°C on nutrient agar, suspended in phosphate buffered saline (PBS), pH 7.2, harvested and washed with PBS. The suspended cells were disrupted by sonication (Ultrasonic Processor, Heat system Ultrasonic, Inc, USA), and centrifuged at 10,000xrpm for 1 h. The supernatant was lyophilized and the protein contents of the lyophilized material were estimated.

**Immunization**: To analyze the DTH response were carried out by standard footpad swelling method as described by Collins and Mackaness (1968).

**T-cells proliferation assay**

Blood was collected from mice using heparanised capillaries and collected it in heparinised tubes. Lymphocytes were isolated from the blood by density gradient centrifugation on Histopaque-1077 (Sigma), and assayed for proliferation according to standard method using 96-well plates (Luster et al., 1982). To summarize, 5 ml blood was diluted with an equal volume of PBS and carefully layered on 3 ml of Histopaque-1077 solution in a sterile conical centrifuge tube. It was then centrifuged at 600xg for 30 minutes at room temperature. The mononuclear cells as a white ring at the interface get separated. The mononuclear cells were carefully taken out with a sterile glass pasteur pipette, pelleted at 400xg for 10 min and the cells pellet was suspended in 1 ml of the RPMI 1640 (growth media; containing 100units/ml penicillin G, 100mg/ml streptomycin and 10% Fetal Bovine Serum). The viability of the cells was checked by the trypsin blue dye exclusion method and final concentration of viable cells adjusted to a cell density of 1x10$^6$cells/ml. 100µl of these cells were pipetted into each well of 96 U
The mice were pretreated with Tb & Pg for 15 days followed by a challenge with sublethal dose of *S. typhimurium*. Liver homogenate was plated on TSI agar, allowed to grow and the bacterial colonies was counted. The results have been expressed as a number of viable bacteria per gram liver. S+B=Saline+0.5xLD50 of *S. typhimurium*, Tb+B= *T. belerica* (500mg per kg)+0.5xLD50 *S. typhimurium* and Pg=P. granatum (500mg per kg)+0.5xLD50 *S. typhimurium*. Values are significantly different **p<0.01**.

**Cytokine (IL-1α) estimation**

IL-1α activity was measured in the serum using IL-1α Enzyme Linked Immuno Sorbent Assay (ELISA) kit for mouse IL-1α (Cytimmune Sciences Inc, 8075 Greenmead Drive College Park, Maryland). Results have been expressed as IL-1α pg/ml.

**Bacterial agglutination test**

Antigen preparation and agglutination test: The bacterial agglutination test was performed by the method of Ascencio *et al.* (1990) with minor modifications. Bacterial cells at mid log phase were centrifuged at 3000rpm at 4°C for 5 minutes and the pellet was suspended in saline (5x10^8 bacteria/ml). In negative control PBS was taken instead of bacteria. The cell preparation was incubated with antisera for 1hr at 37°C. The agglutination is characterized by a coarse granular bacterial clumping with antibodies and is scored on a scale of +1 (weak agglutination) to +4 (strong reaction). Results were recorded after 3-5 minutes.

**Results**

**Survival study**

Protective role of these drugs can be understood from the Table 1. Animals were pretreated orally with aqueous extract of Tb and Pg at the doses of 250 and 500mg per kg body weight for a period of 30 days. Control group received saline only and were challenged with 2xLD50 dose of *S. typhimurium* on day 31. All the animals in control group started to show the symptoms of salmonellosis, such as ruffled fur, lethargic movements, slow responsiveness to external stimuli and diminished desire to consume food at the end of day 1 post challenge and all the animals died within 2-3 days. However, the Tb pretreated animals exhibited 83.3% and 100% survival respectively. Similarly, when the animals were pretreated with aqueous extract of Pg at the above doses for same period, the survival rates were 66.6% and 83.4% respectively. The survival of mice suggested that the aqueous extracts of Tb and Pg have conferred significant protection against experimentally induced salmonellosis.

**Bacterial clearance study**

When control animals were challenged with sublethal dose of *S. typhimurium* and analyzed after 7 days, the livers of animals in the control group were highly infected with *S. typhimurium* and exhibited rapid clearance of bacteria. The bacterial clearance study indicated that during normal infection, the bacterium reach the liver and rapidly colonize.

Pretreatment with Tb and Pg for 15 days followed by challenge with sublethal dose of *S. typhimurium* inhibited the bacterial translocation to liver and exhibited rapid clearance of bacteria. The results were recorded after 3-5 minutes.

The mice were pretreated with Tb & Pg for 15 days followed by a challenge with sublethal dose of *S. typhimurium*. Liver homogenate was plated on TSI agar, allowed to grow and the number of bacterial colonies was counted. The results have been expressed as a number of viable bacteria per gram liver. S+B=Saline+0.5xLD50 of *S. typhimurium*, Tb+B= *T. belerica* (500mg per kg)+0.5xLD50 *S. typhimurium* and Pg=P. granatum (500mg per kg)+0.5xLD50 *S. typhimurium*. Values are significantly different ***p<0.001***.
The animals were pretreated with Tb. Control received saline only. After 30 days of treatment, mice were immunized with 0.5xLD$_{50}$ of S. typhimurium and observed for 7 days. On day 8, PI, the bacterial sonicate of 50µg of protein was injected into the right hind footpad in all the experimental groups. The left hind footpad received an equal volume of saline, which served as a control. The thickness induced in saline injected (left) foot was substituted from the values obtained from swelling induced in right hind footpad by sonicated S. typhimurium cell lysate.

Bacterial burden in liver reduced by 78.59% and 74.29% respectively. Further, 30 days pretreatment with above drugs, it exhibited a maximum clearance (86.81% and 84.12% respectively). Thus bacterial clearance result showed the minimum ill effect of infection (Fig. 1 & 2).

Cell mediated immune (CMI) responses

**Delayed Type Hypersensitivity (DTH):** To analyze the effect of drugs on DTH response in mice, the animals were pretreated with the above dose of aqueous extracts of Tb and Pg. Control mice received saline only. After 30 days, animals were challenged with sub-lethal (0.5xLD$_{50}$) dose of S. typhimurium and were observed for 7 days. On day 8 PI, the right hind footpad was injected with S. typhimurium cell lysate. The left hind foot was injected with saline and served as reference. Results have been summarized in Fig. 3 & 4. Tb was more effective in enhancing the DTH response than Pg. The potentiation of immunological response may provide bases for the protection conferred by these drugs against salmonellosis.

T-cell proliferation: T-cells play an important role in immune response. The effect of various treatments in T cell population is shown in Fig. 5. When the control animals were infected with bacteria, there was a marked inhibition (63.85%) in T cell population. Pretreatment of animals with Tb and Pg not only negated the detrimental effect of bacterial infection but helped in proliferation of T cells. With the pretreatment of Tb and Pg, the bacterially infected animals showed an increase of 7.4% and 3.11% in T cell population as compared to control animals. ConA is known proliferator of T-cells. It causes stimulation of T cell population (B vs A). However, bacterial infection resulted in decrease in T cells and the T cell population in infected animals remained unaltered irrespective of ConA stimulation (D vs C). However, the pretreatment with the drugs enabled the animals to resist the effect of bacterial infection which was revealed by the fact that the population of T cell remained enhanced to ConA stimulated level even after bacterial infection (F & H vs B). These results show that the drugs cause a stimulation of T cells and also protect the animals against the ill effect of bacterial infection.

**Effect of drugs on cytokine (IL-1α) concentration:** The stimulatory effect of drugs on the cytokine (IL-1α) concentration in serum has been summarized in Fig. 6. When control animals were infected with bacteria, there was a marked increase in IL-1α concentration (82.96% increase). However, no such increase was seen in drug pretreated animals.

Humoral immune response

**Bacterial agglutination assay:** The effect of above drugs on bacterial agglutination has been summarized in Table 2. The animals were pretreated with Tb and Pg. Control mice received only saline. After 30 days, animals were sensitized with S. typhimurium and observed for 7 days. On day 8, mice were immunized with heat-killed bacteria to raise the antibodies.
animals were bled on day 21 post immunization.

**Table 1. Survival study**

<table>
<thead>
<tr>
<th>Dose (in mg/kg) and (µg per kg)</th>
<th>No. of days</th>
<th>No. of animals</th>
<th>Bacterial doses</th>
<th>2xLD$_{50}$</th>
<th>Animals survived</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
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<td>0.0</td>
<td>00.00</td>
<td></td>
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</tr>
<tr>
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<td>83.30</td>
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</tr>
<tr>
<td>Tb500</td>
<td>30</td>
<td>6</td>
<td>6.0</td>
<td>100.00</td>
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</tr>
<tr>
<td>Pg250</td>
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<td>6</td>
<td>4.0</td>
<td>66.60</td>
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</tr>
<tr>
<td>Pg500</td>
<td>30</td>
<td>6</td>
<td>5.0</td>
<td>83.30</td>
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<tr>
<td>Chloramph100</td>
<td>30</td>
<td>6</td>
<td>6.0</td>
<td>100.00</td>
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</table>

The mice were pretreated (orally) with different doses of Terminalia belerica and Punica granatum for 30 days. The animals were challenged with S. typhimurium (2xLD$_{50}$), followed for 15 days after challenge. Chloramphenicol was used as standard (100µg per kg). Tb250=T. belerica (250mg per kg), Tb500=T. belerica (500mg per kg), Pg250=P. granatum (250mg per kg) and Pg500 = P. granatum (500mg per kg).

The serum was used for the agglutination assay. Interaction of bacteria (pre-coated in the plate) with anti-serum, showed positive bacterial agglutination clumping at the mean dilution of 57.60 as observed in control animals. The Tb and Pg treated animals on the other hands, showed agglutination at mean dilution of 140.8 and 115.2. Thus treatment of drugs showed immunostimulatory capacity and enhanced production of antibodies.

**Discussion**

In our search for herbal remedy for typhoid, a large number of plants were screened for anti-Salmonella activity. This screening revealed that a few plants have strong anti-Salmonella activities (Madani, 2007). In order to understand the molecular basis of action of these drugs, their immunological role was studied. When mice were pretreated for 30 days at the doses of 250 and 500 mg per kg body weight of two drugs, namely Tb and Pg, followed by a challenge with lethal doses of bacteria, protection of animals against salmonellosis was seen. Tb pretreatment exhibited 83.4% and 100% survival, whereas Pg pretreatment exhibited 66.6% and 83.3% survival respectively (Table 1). Amongst other chemicals, tannin present in studied plants may have capacity to prevent the growth of bacteria by precipitating bacterial proteins and making nutritional proteins unavailable for them (Fluck, 1973).

Salmonella infection begins with bacterial penetration of the intestinal epithelium followed by their dissemination throughout the reticuloendothelial system especially in liver, where they multiply (Nnalue, 1992). Salmonella infection in mice increases the incidence of bacterial translocation into liver that serves as major site of salmonellosis.

However, the bacterial burdens in the liver of S. typhimurium infected mice were significantly lower in drugs treated mice than the untreated mice. This fact supports the results shown in the Fig. 1 & 2. The fast clearance of bacteria from reticuloendothelial system by these herbs is due to their bactericidal property.

The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic organisms. The plants extracts caused an increase in footpad thickness 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 (Fig. 3 & 4). 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.

LPS, an endotoxin from cell wall of bacteria is a T-cell independent antigen and stimulates selectively B-lymphocytes whereas, concavalin A selectively stimulates T-lymphocytes. In the present study, it was observed that S. typhimurium inhibited lymphocyte proliferation stimulated by concavalin A (T-lymphocytes) significantly over control, indicating that S. typhimurium inhibits immune response. Interestingly, the drugs were able to arrest the S. typhimurium induced inhibition of lymphocyte proliferation (Fig. 5).

IL-1α is a highly potent pro-inflammatory cytokine that is produced by macrophages in...
response to Salmonella infection. The increased IL-1α concentration in blood has been observed during infections (Reddy & Grieco, 1989; Dinarello & Wolff, 1993). The significant increased in the cytokine (IL-1α) concentration in serum of infected mice with S. typhimurium was overcome in drugs pretreated mice (Fig. 6).

The augmentation of humoral immune response against S. typhimurium by drugs is evidenced by increase in the level of antibody in mice (Table 2). The drugs thus confer protection against experimental salmonellosis by stimulating phagocytosis and immunostimulation.

Acknowledgements

These studies were supported by CCRUM in form of an Adhoc research project to SKJ. AM is a SRF of ICMR.

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