

Regular intake of *Terminalia chebula* can reduce the risk of getting typhoid fever

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Abstract

Typhoid fever is a life-threatening illness caused by the bacterium *Salmonella typhi*. It is characterized by a persistent high fever, inflammation, ileal perforation, liver abscess, diarrhea, profuse sweating, rose colored spot and gastroenteritis. Multi drug resistance has increased worldwide. Keeping in view of the limited scope of available vaccine against salmonella infection and increasing resistant of this disease to antibiotics, the need of the day is to evaluate the efficacy of the natural plant products which can be used against this disease. In the present study aqueous extract of the fruit of *Terminalia chebula* (T) was evaluated for its anti salmonellae activities *in vitro* and *in vivo*. T exhibit anti salmonellae activity against *S. typhi* and *S. typhimurium* showing a clear zone of inhibition *in vitro*. T at a concentration of 10,12 and 15 mg/ml was bacteriostatic and above it was highly bactericidal. Mice pretreated with T at a dose of 100 (T100), 200 (T200) and 500 (T500) mg/kg body weight for a period of 30 days and challenged with 100000CFU of *S. typhimurium* exhibit a protection of 83.4%, 83.4% and 100% respectively. This study was further confirmed by studying the clearance of bacteria from liver and estimating the enzymes alanine aminotransferase and aspartate aminotransferase. The results indicated that regular intake of the above fruit can prevent salmonellae infection and can reduce the risk of getting typhoid.

Key words: *Terminalia chebula*, *salmonella typhi*, *salmonella typhimurium*, typhoid.

Introduction

Typhoid is a human-restricted and highly adapted invasive disease caused by *Salmonella typhi* (Gordon, 2008). It remains a serious problem in Zimbabwe, Australia, Western French Guiana, Thailand, Ivory coast, India, Florida, Turkey, Spain and Nigeria. A number of reports regarding the epidemiology of this disease have been made (Perera et al., 2007; McGovern et al., 2007; Paris et al., 2008).

It is characterized by a persistent high fever (as high as 40 °C or 104 °F), inflammation (Godinez et al., 2008), ileal perforation, liver abscess, diarrhea profuse sweating, rose colored spot and gastroenteritis. *Salmonella* is also reported to cause oxidative stress (Farr and Kogoma, 1991). A number of other symptoms were reported by Khan et al. (2008). Treatment against this disease was antibiotics and vaccination.

Antibiotics, such as chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole, amoxicillin and ciprofloxacin, have been commonly used for the treatment of typhoid fever in developed countries. Prompt treatment of the disease with antibiotics decreases the case-fatality rate to approximately 1%. When untreated, the fever persists for three weeks to a month. Death occurs in between 10% and 30% of untreated typhoid fever.

Resistance to ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole and streptomycin is now common, and these agents have not been used as first line treatment now for almost 20 years. Typhoid that is resistant to these agents is known as multidrug-resistant typhoid (MDR typhoid).

Two vaccines currently recommended by the WHO for the prevention of typhoid (WHO, 2008) are the live oral Ty21a vaccine and the injectable Vi capsular polysaccharide vaccine. They provide 50 to 80% protection and are recommended for travelers to areas where typhoid is endemic. There exists an older killed whole-cell vaccine that is still used in countries where the newer preparations are not available, but this vaccine is no longer recommended for use, because it has a higher rate of side effects like pain and inflammation at the site of injection.

Keeping in view of the limited scope of available vaccine against salmonella infection (Mastroeni and Menager, 2003) and increasing resistant of this disease to antibiotics (Hazir et al., 2002; Prabha et al., 2002; Hassan et al., 2001; Gupta et al., 2001), the need of the day is to evaluate the efficacy of the natural plant products which can be used against this disease.

Multi drug resistance has increased worldwide. The decision on antimicrobial therapy must take such resistance into account (Crum, 2003). According to the symptoms displayed by typhoid only those plants or plants product will be beneficial which posse's antioxidant, immunomodulatory, hepatoprotective, anti inflammatory, analgesic, antipyretic and antimicrobial activities.

Terminalia chebula Gertn. (TC) is an important herbal drug in Ayurvedic pharmacopeia. It is called the "king of medicines". It is always listed first in the Ayurvedic materia medica because of its extraordinary potential of healing. In Ayurveda it is thought to destroy all diseases and remove all waste from the body. At the same time, it is known to help tissue growth and health. It is known by its local name as Haritaki, Harar, Harida, Black myroblan, Chebulic myroblan and Harada. It is found all over India from eastern to western region.

TC is reported to be antimicrobial (Sato et al., 1997; Ahmad et al., 1998; Malekzadeh et al., 2001; Bonjar, 2004; Aqil and Ahmad, 2007), hepatoprotective (Tasaduq et al., 2003; Tasaduq et al., 2006), anti-inflammatory (Pratibha et al., 2004), immunomodulatory (Srikumar et al., 2005), antioxidant (Lee et al., 2007; Tejesvi et al., 2008, Lee et al., 2005; Cheng et al., 2003) and adaptogenic (Rege et al., 1999).

Salmonella typhimurium (*S.typhimurium*) causes an invasive disease in mice that has similarity with human typhoid. Swiss albino mice were taken as an animal model to study the above said disease against the fruit of this plant in vivo.

2. Materials and methods

2.1 Plant material

The fruit of TC comes under family Combetraceae. It was purchased from Okhla market of New Delhi. It was then authenticated by Dr. M.P. Sharma, Department of Botany, Jamia Hamdard, New Delhi-110062, India.

2.2 Extract preparation

The dried fruits were first washed with water to remove the impurities. After thorough washing the fruits were dried in the shade. Dried fruits were then powdered by continuously grinding and sieving. The powdered form was soaked in distilled water for overnight. It was then centrifuged at 3000 rpm for 15 minutes, filtered in sterile condition and then lyophilized to get the sterile powder (T).

2.3 Microorganisms

S. typhimurium (wild) and *S. typhi* (wild) were used in this experiment. The above standard strains were obtained from National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India. Both the bacterial strains were further characterized and authenticated in the Department of Microbiology, Majeedia Hospital, New Delhi, India.

2.4 Animals

Swiss albino mice were used as an animal model. Research was conducted according to the internationally accepted principles for laboratory animal use. Mice weighing 20-25 gm were used for the study. 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 °C. Mice were provided with pellet diet (Lipton, India) and water ad libitum.

2.5 Antisalmonellae activity invitro

Agar well diffusion method (Perez et al., 1990) with slight modification was used to determine antisalmonellae activities of T against *S.typhi* and *S. typhimurium*. Sterile nutrient agar was used as a medium. Overnight cultures of *S. typhi* and *S.typhimurium* were obtained. The plates were swabbed with culture of respective microorganisms. Wells were then made aseptically with cork borer having the diameter of 6 mm. The solution of the drug was prepared in autoclave water. About 200 µl of test solution (500 mg/ml of drug) was poured into the well with the help of dropping pipette under aseptic condition. Well containing autoclave

distilled water acts as a control. The plate was then placed in a refrigerator for 2 hrs as a period of pre-incubation diffusion followed by incubation at 37±0.5°C. The zone of inhibition of microbial growth was measured after incubation for 18 hrs. Each experiment was carried out in three replicates and the mean results were recorded.

2.6 Bactericidal kinetic assay invitro against *S.typhimurium*

Time killing curves of the drug against *S. typhimurium* were obtained by performing the experiment of bactericidal kinetic assay. The protocol of bactericidal kinetic assay (Gadhi et al., 2001) was slightly modified. A series of tubes having drugs (T) at a concentration of 10, 12 and 15 mg/ml in nutrient broth were taken. About 100000 CFU of *S. typhimurium* was added in each tube. Drugs free broth containing the same inocula was used as a control. All the tubes were then placed in an incubator at 37± °C. Bacterial inoculums from incubated tubes were then taken out at an interval of 0, 1, 2, 3, 4, 6, 8, 10, 12 hr and then plated on TSI plate followed by incubation at 37± °C. The number of viable cells were then counted and expressed in terms of log₁₀ CFU/ml. The absence of drug carry over effect was verified. Tests were performed in triplicate and the mean effect of T on *S. typhimurium* was calculated.

2.7 Study of protective effect of drug against *S. typhimurium* invivo

A number of Swiss albino mice were taken to test the efficacy of drug (T) against *S. typhimurium*. The above animals were divided into three sets. Each set contains four groups. Each group has six animals. Three groups of animals were pretreated orally with (T) at a dose of 100 (T100), 200 (T200) and 500 (T500) mg/kg body weight and fourth group which received saline act as a control. After 10 days of pretreatment, the animals were exposed to a challenge dose (100000 CFU) of *S. typhimurium* (wild) intraperitoneally. For the second set of animals same experiment was repeated but the pretreatment was done for a period of 20 days. The last set of animals were pretreated in the same way for a period of 30 days and exposed to a challenge dose (100000 CFU) of *S. typhimurium* (wild). The animals were observed for 14 days and the percent survival rate was recorded.

Two groups of animals were further taken. One group was fed with T500 and other with saline which act as a control. They were then subjected to a challenge dose of 200000 CFU of *S.typhimurium* and then the survival rate was again recorded.

2.8 *S. typhimurium* clearance study invivo

S. typhimurium is a pathogenic bacterium. As the mice were exposed to this bacterium, the bacterium gains entrance to the reticulo-endothelial system, especially liver and spleen. The number of *S.typhimurium* (CFUs) increases slowly up to one week in the liver and then attains a constant value. The increase in number of bacteria in liver indicates the infection and decrease in number of bacteria indicates lowering of infection.

To determine the effect of T the mice were divided into three sets. Each set has four groups. Every group contains six animals. Three groups of mice in first set were pretreated orally with (T) at a dose of 100 (T100), 200 (T200) and 500 (T500) mg/kg body weight and fourth group of this set which received saline act as a control. These mice were pretreated for 10 days and then exposed to a challenge dose (50000 CFU) of

S. typhimurium (wild) intraperitoneally. In the same way the second set of animals were pretreated for a period of 20 days with same doses of T and then exposed to 50000 CFU of *S. typhimurium*. The last set of animals were pretreated again in the similar way but for a period of 30 days and exposed to same doses of bacteria. The animals were observed and then sacrificed at day 7 post infection (PI). The liver of the infected mice were aseptically removed and washed with sterilized PBS. It was then weighed from each group and was homogenized separately in PBS at room temperature. A small aliquot from each homogenate was cultured on nutrient agar plates. After overnight incubation of the culture plates at 37°C, bacterial colonies were obtained, which were then screened for *S. typhimurium* by standard dabbing method on triple-sugar-iron-agar (TSI-agar) plates and finally counted. The bacterium imparts a blackish hue to pink colored plates. The results of the experiments were expressed as number of viable bacteria (log₁₀ CFU gm⁻¹ tissue) in liver tissue.

2.9 Estimation of SGPT and SGOT from blood serum of mice infected with *S.typhimurium*.

Alanine aminotransferase (SGPT or ALT) and aspartate aminotransferase (SGOT or AST) are the marker enzymes of liver injury. The rise in the level of these enzymes indicate the rise in infection and the fall in the level of enzymes marks a sign of relief from *S. typhimurium* infection. Blood was obtained separately from each of infected mice mentioned above in dried centrifuge tubes separately. It was then kept for clot formation and then centrifuged for five minutes at 800 x g in a fixed rotor centrifuge at room temperature. Serum gets separated by centrifugation. It was then carefully collected using a pipette and was finally kept at 4°C for experimental purpose.

SGPT and SGOT were determined by the kit supplied by span diagnostics Ltd, New Delhi, India. The enzyme activity was expressed in U/ml. Here one unit is defined as one imole of the pyruvate formed under defined conditions per ml of serum. The assay consisted of 0.1 ml of serum diluted to 1.0 ml with -ketoglutarate-alanine buffer substrate (pH 7.4) for ALT and with -ketoglutarate-aspartate substrate (pH 7.4) for AST determination. The procedure of estimation was based on the method described by Reitmann and Frankel (Reitmann and Frankel, 1956).

3. Results

Aqueous extract obtained from 100 grams of dried fresh fruit of the above mentioned plant on lyophilisation yields 46 gm of drugs (T).

T exhibit antisalmonellae activities against *S.typhi* and *S. typhimurium* showing a clear zone of inhibition. The diameter of zone of inhibition of T against *S.typhi* and *S. typhimurium* were same that is 21mm. Thus T exhibit a high antimicrobial activity against the above pathogens *invitro*.

T at a concentration of 10,12 and 15 mg/ml was bacteriostatic and at a concentrations more than 15mg/ml were highly bactericidal (Fig1). Both the above experiments claimed the drugs to be used against *S.typhimurium* for *invivo* studies.

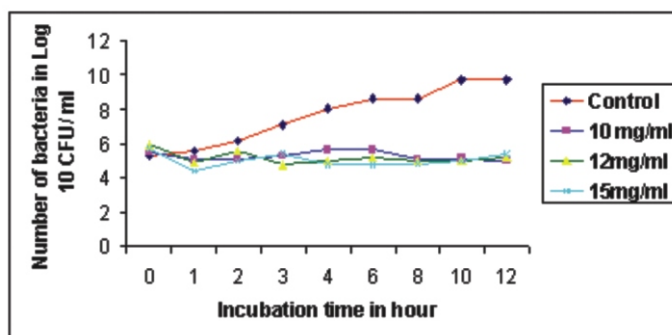


Fig 1: Bactericidal kinetic assay of T against *S.typhimurium*, Concentration of T was 10 mg/ml, 12 mg/ml and 15 mg/ml. Control was without drug.

Animals pretreated with T orally at a dose of 100, 200 and 500 mg/kg body wt for a period of 10 days exhibit 66.7%, 50% and 66.7% survival against 100000CFU dose of *S. typhimurium* respectively. Pretreatment with same doses of T for a period of 20 days followed by challenge with same dose of bacteria exhibit 66.7%, 66.7% and 66.7% survival respectively. Animals pretreated for a period of 30 days with same doses of extract exhibit 83.4%, 83.4% and 100% survival when exposed to 100000 CFU dose of *S. typhimurium* respectively. Animals pretreated for 30 days and challenged with 200000 CFU exhibit the protection as shown in the table 1.

CFU exhibit the protection as shown in the table 1.

Dose (mg/kg body wt).	No of days for which pretreatment was done	No of Swiss albino mice taken initially	Bacterial (<i>S.typhimurium</i>) doses			
			100000 CFU	200000 CFU	100000 CFU	200000 CFU
T 100	10	6		4	66.7	
T 200	10	6		3	50	
T 500	10	6		4	66.7	
T 100	20	6		4	66.7	
T 200	20	6		4	66.7	
T 500	20	6		4	66.7	
T 100	30	6		5	83.4	
T 200	30	6		5	83.4	
T 500	30	6		6	100	
T 500	30	6	3			50

Table1: Protection study shown by different doses of T against *S typhimurium* *invivo* study. T100 = *Terminalia chebula* (100 mg/kg body wt), T200 = *Terminalia chebula* (200 mg/kg body wt), T500 = *Terminalia chebula* (500 mg/kg body wt), Pretreatment was done for a period of 10, 20 and 30 days.

Mice pretreated with T at a dose of 100, 200 and 500 mg/kg body wt for 10 days subjected to exposure of 50000 CFU dose of bacteria exhibit a clearance of 0.11 log₁₀, 0.48 log₁₀ and 0.97 log₁₀ CFU of bacteria per gram of liver tissue (Fig 2). Pretreatment for 20 days with same doses of T and challenge with same dose of *S.typhimurium* exhibit a clearance of 0.73 log₁₀, 1.13 log₁₀ and 1.63 log₁₀ CFU of bacteria per gram of liver tissue respectively (Fig 3). Pretreatment with T for 30 days followed by challenge with 50000 CFU dose of bacteria showed a clearance of 0.83

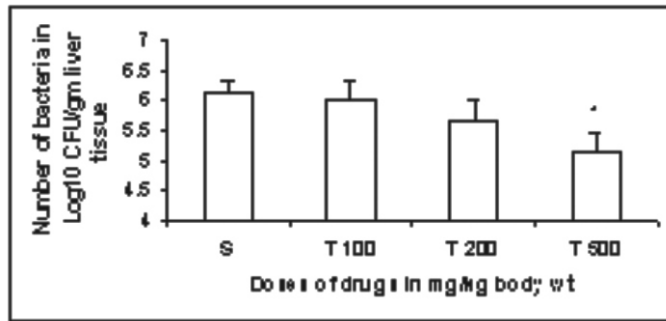


Fig 2: *S.typhimurium* clearance from liver when the pretreatment of mice was done for a period of 10 days with water extract of *Terminalia chebula* (T). S = Saline, T100= *Terminalia chebula* (100 mg/kg body wt), T200 = *Terminalia chebula* (200 mg/kg body wt), T500 = *Terminalia chebula* (500 mg/kg body wt). All the above treatments were exposed to a challenge dose of *S. typhimurium* (50000 CFU). Values are significantly different. *P<0.05, **P<0.01.

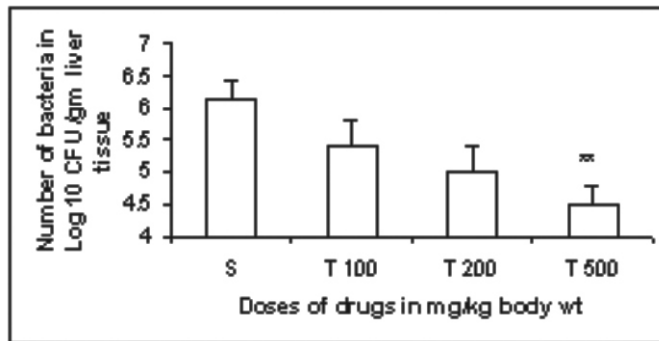


Fig 3: *S.typhimurium* clearance from liver when the pretreatment of mice was done for a period of 20 days with water extract of *Terminalia chebula* (T). S = Saline, T100= *Terminalia chebula* (100 mg/kg body wt), T200 = *Terminalia chebula* (200 mg/kg body wt), T500 = *Terminalia chebula* (500 mg/kg body wt). All the above treatments were exposed to a challenge dose of 50000 CFU of *S. typhimurium*. Values are significantly different. *P<0.05, **P<0.01.

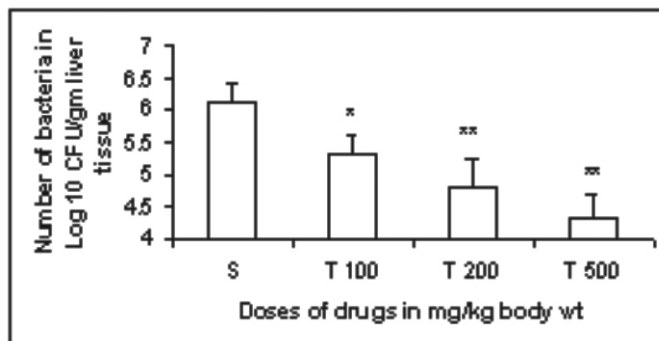


Fig 4: *S.typhimurium* clearance from liver when the pretreatment of mice was done for a period of 30 days with water extract of *Terminalia chebula* (T). S = Saline, T100= *Terminalia chebula* (100 mg/kg body wt), T200 = *Terminalia chebula* (200 mg/kg body wt), T500 = *Terminalia chebula* (500 mg/kg body wt). All the above treatments were exposed to a challenge dose of 50000 CFU of *S.typhimurium*. Values are significantly different. *P<0.05, **P<0.01.

log₁₀, 1.33 log₁₀ and 1.83 log₁₀ CFU per gram liver tissue respectively (Fig 4).

Animals pretreated with T500 for a period of 10 days followed by challenge with 50000 CFU of *S.typhimurium* exhibit a decrease of 45%

in the level of SGPT and 18.15% in the level of SGOT as compared to control infected with *S. typhimurium* (Fig 5 & 6). Mice pretreated with T500 for a period of 20 days followed by challenge with 50000 CFU exhibit a decrease of 50% in the level of SGPT and 20 % decrease in the level of SGOT as compared to infected control (Fig 7&8). Animals pretreated with T500 for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium* exhibit a decrease of 57% in the level of SGPT and 21% in the level of SGOT as compared to control infected with bacteria (Fig 9 & 10). 4. Discussion

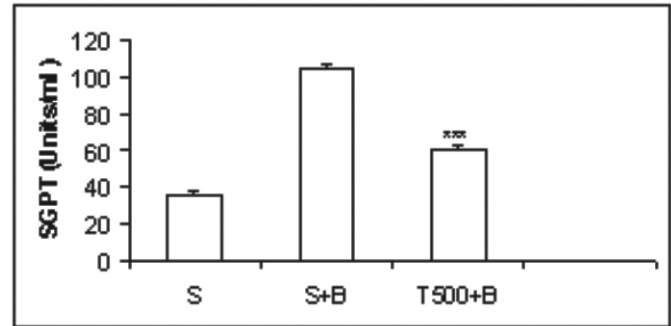


Fig 5 SGPT induced by *S. typhimurium* in mice pretreated with drug for a period of 10 days. S = Saline, S+B = saline+ 50000 CFU bacteria, T 500+B = Water extract of *Terminalia chebula* (500 mg/kg body wt) + 50000 CFU bacteria. Values are significantly different. ***P<0.001.

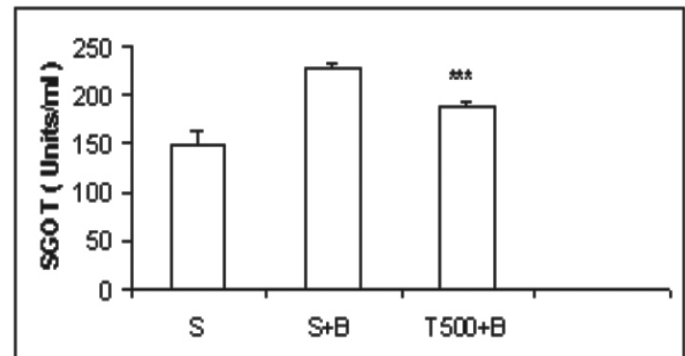


Fig 6: SGOT induced by *S typhimurium* in mice pretreated with drug for a period of 10 days. S = Saline, S+B = saline+ 50000 CFU bacteria, T 500+B = Water extract of *Terminalia chebula* (500 mg/kg body wt) + 50000 CFU bacteria. Values are significantly different. ***P<0.001.

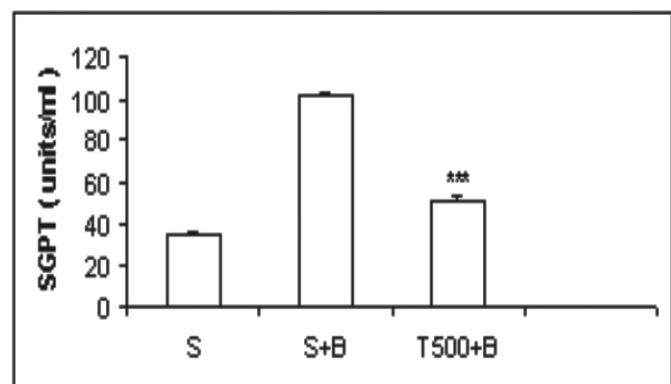


Fig 7: SGPT induced by *S typhimurium* in mice pretreated with drug for a period of 20 days. S = Saline, S+B = saline+ 50000 CFU bacteria, T 500+B = Water extract of *Terminalia chebula* (500 mg/kg body wt) + 50000 CFU bacteria. Values are significantly different. ***P<0.001.

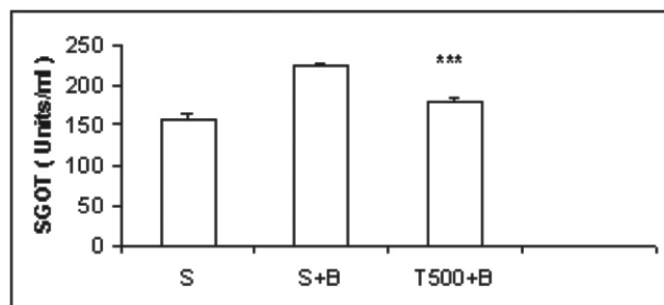


Fig 8: SGOT induced by *S typhimurium* in mice pretreated with drug for a period of 20 days. S = Saline, S+B = saline+ 50000 CFU bacteria, T 500+B = Water extract of *Terminalia chebula* (500 mg/kg body wt) + 50000 CFU bacteria. Values are significantly different. ***P<0.001.

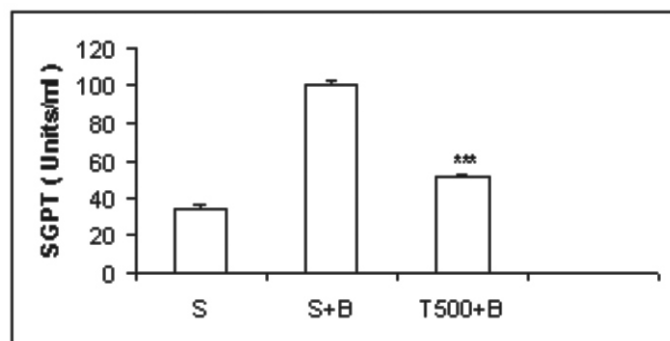


Fig 9: SGPT induced by *S typhimurium* in mice pretreated with drug for a period of 30 days. S = Saline, S+B = saline+ 50000 CFU bacteria, T 500+B = Water extract of *Terminalia chebula* (500 mg/kg body wt) + 50000 CFU bacteria. Values are significantly different. ***P<0.001.

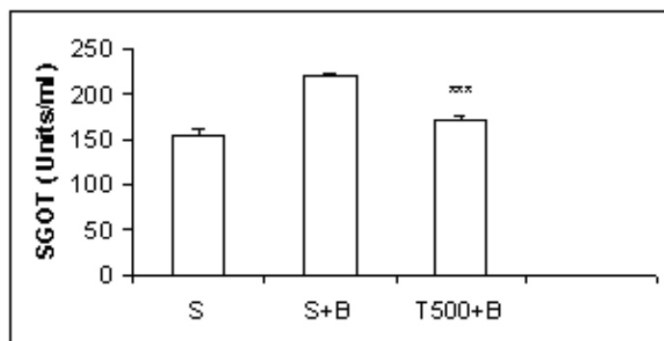


Fig 10: SGOT induced by *S typhimurium* in mice pretreated with drug for a period of 30 days. S = Saline, S+B = saline+ 50000 CFU bacteria, T 500+B = Water extract of *Terminalia chebula* (500 mg/kg body wt) + 50000 CFU bacteria. Values are significantly different. ***P<0.001.

Discussion

Reports are available that indicated tannic acid to be bacteriostatic or bactericidal to some Gram (+)ve and Gram (-) ve pathogens (Kau, 1980). Antimicrobial activities have been reported against the above plant. This plant also contains tannic acid and gallic acid as their chemical constituent. There have been reports of antimicrobial properties in gallic acid (Panizzi *et al.*, 2002). The above reason supports our work regarding the antisalmonellae activities of this extract.

Rasayana plants prevent ageing, reestablish youth, strengthen life and brain power and prevent diseases (Sharma, 1983). They increase the

resistance of the body against any onslaught. TC is also one of the Rasayana plant. Chyawanprash is an ancient Ayurvedic preparation, which has been claimed to have health-promoting effects and have been advocated for degenerative diseases. It contains TC as one of its constituents. Triphala, which is used in fever, cough, asthma, rheumatism, and inflammation of the lungs, contains *Emblica officinalis*, TC and *Terminalia bellarica* in equal proportion. The above properties of the herbs support its protective effects against salmonellosis also.

Liver involvement is commonly observed in patients with typhoid fever and elevated serum alanine transaminase levels have been reported (Shetty *et al.*, 1999). An increase in SGOT have been also reported in typhoid fever (Klotz *et al.*, 1984). This indicated the accumulation of bacteria and thus damage of liver. TC is reported to protect liver (Tasaduq *et al.*, 2003). Chebulic acid present in the fruit is reported to be hepatoprotective (Lee *et al.*, 2007). It is also reported to prevent liver toxicity (Tasduq *et al.*, 2006). Animals pretreated with T500 for a period of 30 days cleared maximum bacteria from reticuloendothelial system. Since in typhoid the multiplication of bacteria take place in liver causing liver damage. The clearance of bacteria from liver clearly indicates the effectiveness of drug to be used against experimentally induced salmonellosis. Further this study was supported by observing the serum enzymes in animals treated with T followed by challenge with *S.typhimurium*.

Thus the study confirmed that pretreatment with T reduces the infection of *S.typhimurium* in mice. As *S.typhimurium* causes an invasive disease in mice that has similarity with human typhoid. It is therefore concluded that regular intake of *Terminalia chebula* in the the diet can reduce the risk of having typhoid fever. Efforts are therefore required to explore the efficacy of this drug at molecular level against typhoid.

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