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Anti-*Salmonella* activity of *Terminalia belerica*: *In vitro* and *in vivo* studies

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To search for an herbal remedy for protection against and treatment for typhoid fever, a number of plants were screened. Anti-*Salmonella* activity of *Terminalia belerica*, an ingredient of Ayurvedic preparation '*triphala*' used for treatment of digestive and liver disorders, has been reported. Fruits of *T. belerica* were extracted with petroleum ether, chloroform, acetone, alcohol and water and efficacy of extracts against *Salmonella typhi* and *Salmonella typhimurium* was evaluated. Alcoholic and water extracts of *T. belerica* showed significant anti-*Salmonella* activity and MIC was 12.5 mg/ml against *S. typhimurium.* Aqueous extracts of *Picrohiza kurroa* and *Vitits vinefera* also showed low anti-*Salmonella* activity where as aqueous extracts of *Asparagus racemosus* and *Zingiber officinale* showed no anti-*Salmonella* activity. Extracts of *T. belerica*, *Picrohiza kurroa* and *Vitits vinefera* with other solvents such as chloroform and petroleum ether showed insignificant activity. Results showed that aqueous extract of *T. belerica* was bactericidal at high concentrations where as low concentrations showed bacteriostatic property. *In vitro* cellular toxicity studies showed no cyto-toxicity associated with *T. belerica* extracts. Pretreatment of mice with aqueous extract of *T. belerica* conferred protection against experimental Salmonellosis and 100% survival of animals has been reported when challenged with lethal doses of *S. typhimurium*.

Keywords: Anti-*Salmonella* activity, Non-cytotoxic, *T. belerica,* Typhoid

Salmonellosis are wide spectrum diseases of human and animals[1]. *S. typhi*, causative organism for human typhoid, *S. typhimurium*, causative organism for rodent/murine counterpart has been extensively used as animal models to understand pathophysiology of disease. Medicinal herbs with unique chemical compounds that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered as potential candidates for developing new antimicrobial drugs. It is expected that plant extracts acting on target sites other than used by antibiotics for their action will be beneficial against drug resistant microbial pathogens. Therefore, there is a strong need to develop alternate antimicrobial drugs for the treatment of infectious diseases particularly typhoid fever. Advantages of herbal drugs include true improvement of disease conditions, little or no harmful side effects and relatively low cost than other forms of treatment. *Terminalia belerica* Roxb (N.O Combretaceae), commonly known as "*bahera*" in Hindi and "belleric myrobalan" in English, is distributed through out the forests of India at altitudes below 10,000 m except in dry and arid regions. Presence of gallic acid, ellagic acid (also present in the heartwood and bark), ethyl gallate, galloy1 glucose, chebulagic acid, mannitol, glucose, galactose, fructose, rhamnose and β-sitosterol has been reported in the fruits of *T. belerica*[3]. It has been reported to have therapeutic values for the treatment of liver and digestive disorders[4]. Aqueous and alcoholic extracts of *T. belerica* showed anti-bacterial activity. Its antimicrobial activity against different bacterial strains like *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Psudomonas aeruginosa* and *Staphylococcus aureus* has been reported[5]. However, its potential as anti-*Salmonella* agent has not been documented. *T. belerica* is one of the constituents of Ayurvedic preparation "*Triphala*" and extensively used in a number of other Ayurvedic formulations.

Materials and Methods

Plants material—Plant materials were procured from local market of New Delhi, India. The identity of these was confirmed by an expert taxonomist from the Department of Botany, Hamdard University, New Delhi. Only the authenticated material was used for experiments. Prior to use, it was ensured that the herbs are free from contamination, and have no microbial growth.

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Bacteria—The study was carried out with standard strains of *S. typhimurium* (wild) and *S. typhi* (wild), obtained from National *Salmonella* Phage Typing Centre, Lady Harding Medical College, New Delhi. The identity of the bacterial strains was further confirmed at the Microbiology Laboratory, Majeedia Hospital, Hamdard University, New Delhi.

Animals—Swiss albino mice (22-30 g) of 5-8 weeks were used for all the experiments and were obtained from the Central Animal House Facility, Hamdard University. The animals were maintained on a standard laboratory feed (Amrut Laboratory, Navmaharashtra Chakan Oil Mills Ltd, Pune) and water *ad libitum*. Animals were allowed to acclimatize for one week before the experiments under controlled light/dark cycle (14/10 hr). All the experiments were approved by the Institute's Animal Ethics Committee constituted as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

Preparation of plants extracts—Plant extracts were prepared according to the method described by Ahmad *et al*[6] with minor modifications. Briefly, 100 g of powdered plant material was soaked in 200 ml of petroleum ether for 72 hr, with stirring every 24 hr with a sterile glass rod. At the end of extraction period, it was centrifuged and supernatant was filtered through Whatmann No.1 paper. This extraction was repeated three times. 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 ether extraction was sequentially extracted with chloroform, acetone and ethanol. Aqueous extract was made by extracting the dried fruit powder directly with water that was then centrifuged, filtered, lyophilized and stored as above.

Screening of the plant extracts for anti-Salmonella activity—Anti-*Salmonella* activity was determined by agar well diffusion method as described by Perez *et al*[7] with minor modifications. Agar plates were prepared by dissolving 2.6 g Nutrient broth in 200 ml of double distilled water, supplemented with 200 mg of glucose and 4 g of agar. Autoclaved media was poured in 55 mm petri plate and allowed to solidify. Overnight cultures of *S. typhi* and *S. typhimurium* were prepared and 10^5 CFUs of bacteria (100 µl) were spread on the plates. Wells of 6 mm diameter were then punched into agar plates and test extracts (or PBS in case of controls) were applied in these wells. Plates were kept at 4°C for 6 hr followed by 37°C for 16 hr. Zones of inhibition were measured.

Minimum inhibitory concentration (MIC)—MIC of test extracts was determined by using different concentrations of extracts in triple sugar iron agar plate. Nine plates of triple sugar iron agar containing different concentration of drug (5-50 mg/ml) were used, a plate having PBS served as control. The 100 µl of *S. typhimurium* culture in nutrient broth having 10^5 CFU bacteria was poured on each plate and spread. All plates were incubated at 37°C for 16 hr.

Bactericidal kinetic assay—Bactericidal kinetic assays were performed by the method of Gadhi *et al*[8] against *S. typhimurium* with minor modifications. A series of tubes containing nutrient broth and sterile extracts at varying concentrations (5-50 mg/ml) were inoculated with 10^5 CFU of *S. typhimurium* and incubated at 37°C. After 0, 1, 2, 4, 6, 8, 10, and 12 hr of incubation, bacterial inoculums from each tube were plated on triple sugar iron agar plate. Plates were incubated overnight at 37°C and number of viable bacteria was counted.

In vitro cellular toxicity studies—Method described by Xiano-guo and Ursula[9] with minor modifications was used to study the cellular toxicity. Briefly, aliquots of 10-fold serial dilutions of the extract in PBS were taken in eppendorf tubes. A negative control tube (containing saline only) and a positive control tube (containing tap water) were also included in the analysis. Fresh sheep erythrocytes were added to each tube to give a final volume of 1.0 ml. The tubes were incubated at 37°C for 30 min, centrifuged and observed for hemolysis.

Determination of LD_{50}—The LD_{50} of the bacterium was determined by the methods of Reid and Munch[10]. Mice were divided into groups having six animals in each and *S. typhimurium* was administered intraperitoneally to experimental groups, controls received saline only. LD_{50} of bacteria was found to be 100,000 CFU (Nasser 2002)[11].

Doses and Dosage—*Animals were divided into different groups. Each group contains 6 animals. The study comprised of following treatment schedules.*

Group S – Normal saline.

Group SB–Normal saline + (2.0× LD_{50}) of *S. typhimurium* (wild).

Group TB250–Aqueous extract of *T. belerica* (250mg per kg) + (2.0X LD_{50}) of *S. typhimurium* (wild).

Group TB500–Aqueous extract of *T. belerica* (500 mg per kg) + (2.0 × LD_{50}) of *S. typhimurium* (wild).

Group Ch – Chloroamphenicol (10 mg per kg body wt) + (2.0 × LD_{50}) of *S. typhimurium* (wild).

Survival study—Above doses and design were used for this study. Aqueous extract was dried and residue was re-suspended in water to get the suspension of the crude drug. Suspension was administered orally, once daily for 30 consecutive days at a dose of 500 mg/kg body weight of the animals, while controls received normal saline. Animals were observed for 15 days of post-bacterial infection.

Preparation of sample solutions—Different samples (0.5 g each) were separately extracted with methanol (4×25 ml) under reflux (30 min each time) in a water bath, pooled, concentrated and made up to 100 ml with methanol. These solutions were used for the estimation of ellagic acid in the samples.

Calibration curve for ellagic acid—Standard solutions of ellagic acid (10 µl) was applied in duplicate (band width: 5 mm, distance between the bands: 5 mm) on separate pre-coated silica gel 60 F254 TLC plates (E. Merck, Cat. No. 1.05554.0001) (0.2 mm thickness) using a CAMAG Linomat IV Automatic Sample Spotter. Separation was done using toluene/ethyl acetate/formic acid (10.8 ml) (3:2:0.4, v/v as solvent) in a CAMAG glass twin trough chamber (20×10 cm) and allowed to run up to a distance of 8 cm (temperature 25 ± 2°C, RH 40%). Plates were then dried in air and scanned at 280 nm using CAMAG HPTLC Scanner 3 and CATS 4 software. Estimation of ellagic acid in different samples of *Terminalia belerica* was done in same manner.

Results and Discussion

Screening of plants for anti-Salmonella activity—Based on the literature, a number of plants with reported antibacterial activity viz. *Terminalia belerica*, *Asparagus recemosus*, *Zingiber officinalis*, *Vitis vinefera* and *Picrorhiza kurroa* were selected for assessing their activity against *Salmonella*. It was found that amount of material extracted by different solvents varied considerably between different plants (Table 1). Crude extracts were screened for antimicrobial activity against *S. typhi* and *S. typhimurium*. and their potency was quantitatively assessed by the presence of zone diameter and compared with chloramphenicol used as standard. Water extracts of three plants showed significant anti-*Salmonella* activity. Extracts of *T. belerica*, *P. kurroa* and *V. vinefera* exhibited activity against *S. typhi* and *S. typhimurium*. Ethanolic extract of above plants also exhibited an activity against both *S. typhi* and *S. typhimurium*. Extracts with other solvents as well as aqueous/ ethanolic extracts of other plants did not show any activity. Results *T. belerica* was most effective compared to other three plants (Table 2). MIC of water and alcoholic extract of *T. belerica* was found to be 12.5 mg/ml against *S. typhimurium*.

Bactericidal kinetic assay—Bactericidal kinetic assay against *S. typhimurium* showed that *T. belerica* at a concentration of 10 mg/ml was bacteriostatic for first 8 hr and then it started to inhibit the growth of bacteria. However, at higher concentration it was bactericidal (Fig. 1).

In vitro cellular toxicity study—Cellular toxicity of *T. belerica* extracts having significant anti-*Salmonella* activity was examined against sheep erythrocytes. Hemolysis of erythrocytes served as an indicator of toxicity. No hemolysis was observed with extracts at varying dilutions ranging from 0.1 to 100 mg/ml. Only positive control (tap water) showed strong hemolysis. Negative control (PBS) exhibited no hemolysis (Table 3).

Protection against experimental salmonellosis—Animals were pretreated (orally) with *T. belerica* extract at two different doses (250 and 500 mg per kg

Table 1—Quantitative estimation of material extracted with different solvents:
[Values are weight of dried powder (g) of the extract per 100 g of dry weight of respective plant parts]

Name of plants Common name	*A. racemosus* *Shatavar*		*Z. officinale* *Adrak*		*T. belerica* *Bahera*		*P. kurroa* *Kutki*		*V. vinefera* *Munaka*
Plants part	Root	Root	Rhizo	Rhizo	Fruit	Fruit	Root	Root	Fruit
Petroleum ether extract	0.10		1.37		57.5		0.20		
Chloroform extract	0.13		2.67		2.15		0.65		
Acetone extract	0.15		0.97		12		0.20		
Alcoholic extract	4.0		7.36		3.1		20.1		
Water extract		5.0		12		29.5		18	56

Table 2—*In vitro* susceptibility of *S. typhi* and *S. typhimurium* to drugs to different plant extracts.
[Values are mean of zone of inhibition (mm) of 3 experiments].

Name of the plants	Part Used	Zone of inhibition (diameter in mm)[a]									
		S. typhi					*S. typhimurium*				
		PE	CL	AC	ET	WA	PE	CL	AC	ET	WA
T. belerica	Fruit	-	-	17.0 +0.8	19.0+1.3	20.0+0.9	-	-	17.0 +1.2	19.0 +1.1	20.0+0.4
A. recemosus	Root	-	-	-	-	-	-	-	-	-	-
Z. officinalis	Rhizome	-	-	-	-	-	-	-	-	-	-
P. kurroa	Root	-	-	-	17.0+0.8	18.0+0.5	-	-	-	17.0 +0.5	18.0 +0.7
V. vinefera	Fruit	-	-	-	17.0+0.6	18.0+0.3	-	-	-	17.0 +1.0	18.0 +0.9

[a]All determination were done in triplicate.
(-), Indicates no activity. PE=petroleum extract, CL=chloroform extract, AC=acetone extract, ET=ethanol extract and WA=water extract.

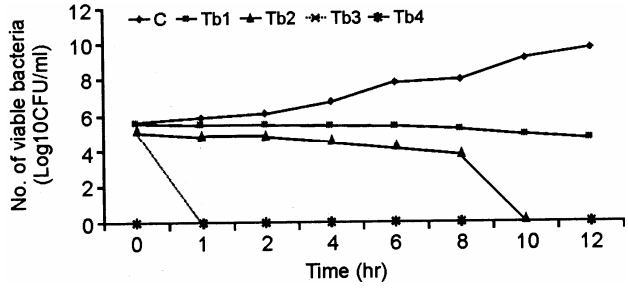

Fig. 1—Effect of *T. belerica* extract on growth of *S. typhimurium*. [Concentration of *T. belerica* was 10, 15, 20 and 25 mg per ml, which correspond as Tb1, Tb2, Tb3 and Tb4, C- control without extract].

body weight) for 30 days and were challenged with lethal doses of ($2\times LD_{50}$) of *S. typhimurium*. All animals in control group died within 7 days of infection. Pretreatment with drug conferred protection against disease in a dose dependent manner and animals exhibited 83.3% and 100% survival at concentration of 250 and 500 mg per kg body weight, respectively (Fig 2). Further survived animals did not show any disease symptoms.

HPTLC profile—HPTLC profile of fractionated methanol extracts of *T. belerica* with different solvents has been presented (Fig. 3). Ellagic acid was found in each fraction like methanol, ethyl acetate, butanol, aqueous and crude extract. Butanol fraction has much lower concentration of ellagic acid where as its concentration was high in other fractions.

Medicinal plants are used by a large proportion of Indian population. Reasons for this include (a) therapeutic potency of these drugs resulting in improvement of conditions after herbal treatment (b)

Table 3—Cellular toxicity of *T. belerica* extract against erythrocyte.

Sample (mg/ml)	Erythrocyte hemolysis
0.10	-
1.00	-
10.0	-
100.0	-
Negative control (PBS)	-
Positive control (Tap water)	+

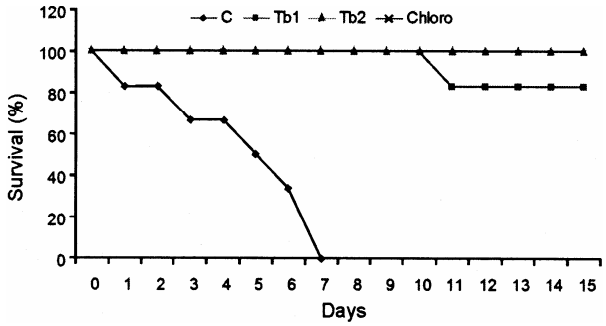

Fig. 2—Survival of mice pretreated with different dose of *T. belerica* for 30 days followed by a challenge with *S. typhimurium* ($2*LD_{50}$). Chloramphenicol (10 mg per kg body wt) was used as standard drug. Tb250=*T. belerica* (250 per kg body wt) and Tb500=*T. belerica* (500 mg per kg body wt).

no or relatively little harmful side effects and (c) due to development of resistance against antimicrobial drugs inducing antibiotics used against typhoid bacterium. As there are no specific herbal drug for typhoid, results of present study are highly encouraging and *T. belerica* appears to contain substances that have strong anti-*Salmonella* activity. Aqueous and alcoholic extracts exhibited inhibitory effect against *Salmonella* growth. MIC of both extracts was 12.5 mg/ml. At lower dose extract was

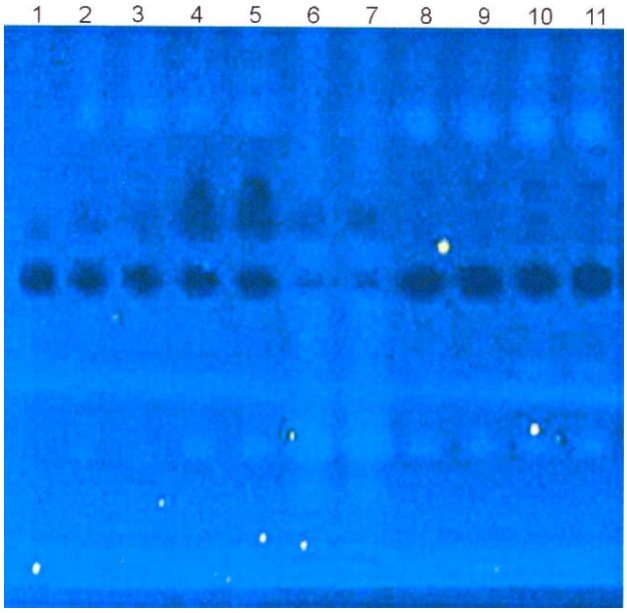

Fig. 3—HPTLC profile of fractionated methanol extracts of *T. belerica*. 01=Standard (Ellagic acid), (02-03=Methanol fraction, 04-05=Ethyl acetate fraction, 06-07=Butanol fraction, 08-09=aqueous fraction, 10-11=Crude extract fraction of *T. belerica*).

bacteriostatic while at high concentration it was bactericidal. Further, extract is highly protective and conferred 100% protection against experimental salmonellosis at a concentration of 500 mg per kg body weight. Furthermore, extract is non-cytotoxic and 100 mg/ml doses did not causes hemolysis of sheep erythrocytes. Partial purification of the crude extract was done using HPTLC, but further phytopharmaceutical studies are needed. Further research on local medicinal plants is expected to boost use of these plants in therapy against diseases caused by the test bacterial species.

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