Terminalia Chebula Reduces the Oxidative Stress Induced by *Salmonella typhimurium* in Mice and May Reduce the Risk of Getting Typhoid

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**Abstract:** Typhoid fever is a serious systemic infection caused by the enteric pathogen *Salmonella enterica* serovar typhi. WHO conservatively estimates the annual global incidence of typhoid fever at 21 million cases, of whom 1-4% end fatally. Keeping in consideration of increasing resistant of this disease to antibiotics and the limited available vaccine against *salmonella* infection, the need of the hour is to evaluate the efficacy of the natural plant products which can be useful for this disease. *Salmonella typhimurium (S. typhimurium)* causes an invasive disease in mice that has similarity with human typhoid. Mice pretreated orally with aqueous extract of *Terminalia chebula* at a dose of 500 mg/kg (T500) body wt for a period of 30 days exhibit a full protection against 100000 CFU of *S. typhimurium* injected intraperitonially. Mice pretreated with T500 for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium* also showed a reduction of 5.6% catalase activity and 97.69% in the level of lipid peroxidation (LPO) as compared to saline control mice subjected to same dose of bacteria. The same infected mice showed an increased level of reduced glutathione (GSH) by 62% as compared to the same saline control infected mice. The data indicated that regular intake of *Terminalia chebula* reduced the development of oxidative stress in mice and so can also reduce the risk of getting oxidative stress in *S. typhi* infection in man which finally reduces the possibility of getting typhoid fever.

**Key words:** Typhoid · Oxidative stress · *Terminalia chebula* · LPO · Catalase

**INTRODUCTION**

Typhoid fever is caused by *Salmonella enterica* serovar typhi (*S. typhi*) a highly virulent and invasive enteric bacterium. World Health Organization (WHO) estimated that 21 million cases of typhoid fever occur per year globally, of which 1-4% cases end fatally. In Asia 90% of death due to typhoid was estimated by WHO [1]. *S. typhi* usually enters into the body via ingestion of food or water contaminated with excreta from typhoid fever cases or asymptomatic carriers of the bacterium. Although typhoid fever is largely considered an endemic disease, epidemics do occur, frequently as a result of breakdowns in water supplies and sanitation systems. Typhoid is characterized by high fever, colic pain, inflammation, hepatic injury and diarrhea. A number of other symptoms have been also reported [2].

Increasing multidrug resistance of *S. typhi* reduces the effective treatment options, increases treatment costs and results in higher rates of serious complications and deaths. In recent years, the accessibility and affordability of the Vi and Ty21a vaccines have greatly improved. In Asia, Vi vaccination of school-age and preschool-age children (aged 2-4 years) in the high incidence urban slums of major cities was estimated to be “very cost effective” under WHO definitions. However, the efficacy of the Vi and Ty21a vaccines in children aged <2 years has not been demonstrated and neither of the vaccines is licensed for use in this age group [1]. In view of the increasing resistant to the antibiotics and limited scope of vaccine the need of the hour is to evaluate the efficacy of the natural plant products for the treatment of this infectious disease.
The origin of disease of multifactorial nature is being understood now due to the vitiation in basic heamostatis balance phenomenon in the body [3]. It is increasingly being realized now that majorities of the diseases are mainly due to the imbalance between pro-oxidant and anti-oxidant homeostatic phenomenon in the body. Prooxidant condition dominates either due to increased generation of free radicals and/or their poor quenching/scavenging into the body [4].

Peroxynitrite is a strong biological oxidant that can be formed in vivo by the nearly diffusion-controlled reaction of nitric oxide and superoxide. Peroxynitrite initiates lipidperoxidation, damages DNA, oxidizes thiol groups and modifies amino acetyl groups on protein. The oxidation and damage induce various pathological conditions [5]. Catalase removes hydrogen peroxide. It catalyses conversion of hydrogen peroxide, to water and molecular oxygen. The specific activity of catalase in Salmonella typhimurium and other enteric bacteria increased at the onset and during the stationary phase. Hydrogen peroxide was produced by S. typhimurium cultures during the exponential and stationary growth phases [6].

Salmonellae are bacterial pathogens that have evolved sophisticated strategies to evade host immune defenses. They are widely distributed in nature and cause a wide spectrum of diseases in man and animals [7]. S. typhi and S. typhimurium, the causative organisms for human and rodents/murines respectively, have been extensively used to understand the pathophysiology of disease [8]. S. typhimurium causes an invasive disease in mice that has similarity with human typhoid [9]. Consequently, the murine model of salmonellosis has been used extensively to explain potentially clinical relevant mechanisms of antialmonella host-defence [10-13]. Mice were thus used as animal model for in vivo study and salmonellosis was experimentally induced by S. typhimurium.

Terminalia chebula (Haritaki) fruits are a very important part of ayurveda. According to Charak, the most eminent ayurvedic physician, Haritaki is the best fruit for rejuvenation and disease cure. Its uses have been described in the first chapter of his text "Charak Samhita"; the best yet known text for Ayurvedic medicines and formulations. With the advent of tools and technology of biotechnology and molecular biology researchers are exploring this plant against a number of diseases. Terminalia chebula is used in India to treat many diseases such as parasitic infections, digestive diseases, irregular fevers, urinary diseases, flatulence, constipation, ulcers, vomiting and colic pain. It is reported to be antimicrobial [14-18], hepatoprotective [19, 20], anti-inflammatory [21], immunomudulatory [22], antioxidant [23-28] and adaptogenic [29]. Seeing the above properties, the fruit was selected for the experimental work against salmonella.

MATERIALS AND METHODS

Plant Material: Terminalia Chebula comes under the family Combretaceae. It was purchased in the local markets of Okhla, New Delhi. The fruit was then identified and authenticated by Dr. M.P. Sharma, Department of Botany, Jamia Hamdard, New Delhi-110062, India.

Preparation of Extract: Fruits of Terminalia chebula were first washed with water to remove the dust and impurities and then dried in shade. Powdered forms of the fruits were then made by continuously grinding and sieving. The powered form was soaked in distilled water for overnight. It was then centrifuged at 3000 rpm for 15 minutes, filtered in sterile condition and further lyophilized to get the sterile powder (T).

Microorganism Used: S. typhimurium (wild) was used as microorganism in this experiment. The above standard strain was obtained from National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India. This bacterium was further identified, characterized and authenticated in the Department of Microbiology, Majeedia Hospital, New Delhi, India. Only authenticated strain was used.

Animals and Maintenance: Animal model used in this study was laboratory bred Swiss albino mice. About 5-7 weeks of age and weighing 20-25 gm of mice were used in this study. Animals were supplied by Central Animal House, Hamdard University, New Delhi-62. Mice were kept under standard laboratory condition for 12 hrs light dark cycle at 25± 10°C and were provided with pellet diet (Lipton, India) and water ad libitum. Research was conducted according to the internationally accepted principles for laboratory animal use.

Protective Effect of T Against S. typhimurium in Swiss Albino Mice: Swiss albino mice were taken to test the efficacy of drug (T) against S. typhimurium. The mice were divided into three sets. Four groups were there in each set. Each group contains six animals. Three groups of animals were pretreated orally with (T) at a dose of 100,
200 and 500 mg/kg body wt and fourth group which received saline act as a control. The doses of 100, 200 and 500 mg/kg body wt were represented as T100, T200 and T500 respectively in this study. After 10 days of pretreatment with drugs, the animals were exposed to a challenge dose (10^5 CFU) of *S. typhimurium* (wild) intraperitoneally.

Same experiment was repeated for the second set of Swiss albino mice but the pretreatment was done for a period of twenty days. The third set of animals were pretreated in the same way for a period of thirty days and subjected to a challenge dose (10^5 CFU) of above bacteria. Mice were observed for fourteen days and the percent survival rate was calculated.

Again two groups of mice having six mice in each group were taken. One group was pretreated orally for a period of thirty days with T500 and other group with saline which act as a control. Both the groups were then subjected to a challenge dose of 2×10^5 CFU of *S. typhimurium*. The percent survival rate was again determined.

**Biochemical Estimations:** The animals were divided into two groups having six animals in each group. One of the groups was pretreated orally with T500 and other with saline (S) for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium*. The animals in both groups (T500+B: Animals treated orally with T500 followed by challenge with 50000 CFU; S+B: Animals treated orally with saline for a period of 30 days followed by challenge with same doses of same bacteria) were sacrificed at 7th day of post infection (PI). For all biochemical estimations the post mitochondrial supernatant was utilized. All the biochemical estimations were completed within 24 hrs of animal sacrifice.

**Post-mitochondrial Supernatant (PMS) Preparation:** Livers were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17% w/v) using Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The aliquot obtained was centrifuged at 10, 500 g for 20 min to obtain PMS. Enzymes like catalase and glutathione were estimated from PMS. A portion of the PMS was centrifuged at 105,000g for 60 min at 4°C. The pellet was washed with PO4 buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%). This pellet was considered to be the microsomal fraction and was suspended in PO4 buffer (0.1 M, pH 7.4) containing KCl (1.17%) and was then used for the estimation of LPO.

**Catalase Activity (CAT):** PMS was used to assess the activity of catalase. The assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml PMS (10% w/v) in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmole H2O2 consumed/min/mg protein by using molar extinction coefficient of 0.081×10^3 M^-1 cm^-1. Catalase activity was assayed by the method of Claiborne [30].

**Lipid Peroxidation (LPO):** LPO was estimated by using hepatic microsomes by the method of Wright *et al.* [31]. The reaction mixture, in a total volume of 1.0 ml, contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml of hepatic microsome (10% w/v), 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM) was incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 ml trichloroacetic acid (TCA) (10% w/v). Following which, 1.0 ml thiobarbituric acid (TBA) (0.67% w/v) was added and all the tubes were placed in a boiling water bath for 20 min. The amount of malonaldehyde (MDA) formed in each of the samples was assessed by measuring the optical density of supernatant at 535 nm using a spectrophotometer against a reagent blank and then calculated using a molar extinction coefficient of 1.56×10^5 M^-1/cm^-1.

**Estimation of Reduced Glutathione (GSH):** Reduced glutathione in the liver was determined by the method of Jollow *et al.* [32]. 1.0 ml of PMS (10% w/v) was precipitated with 1.0 ml of sulfosalicylic acid (4%). The samples were kept at 4°C for at least one hour and then subjected to centrifugation at 1200 xg for 20 minutes at 4°C. The assay mixture contained 0.1 ml of PMS (10% w/v), 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (100 mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm.

**Statistical Analysis:** The level of significance between the different groups is based on Student’s *t*-test by Fisher, followed by the ANOVA test.

**RESULTS**

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

Swiss albino mice pretreated with T orally at a dose of 100, 200 and 500 mg/kg (T100, T200 and T500) body wt for a period of 10 days showed 66.7, 50 and 66.7%
Table 1: Protection study shown by different doses of water extract of *Terminalia chebula* against *S. typhimurium* in Swiss albino mice

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*T100 = Aqueous extract of Terminalia chebula* (100 mg/kg body wt), *T200 = Aqueous extract of Terminalia chebula* (200 mg/kg body wt), *T500 = Aqueous extract of Terminalia chebula* (500 mg/kg body wt), Pretreatment was done for a period of 10, 20 and 30 days

Fig. 1: Catalase activity (n mol H$_2$O$_2$ decomposed/ min/protein) induced by *S. typhimurium* in Swiss albino mice pretreated with drugs for a period of 30 days. S = Mice treated orally with saline for a period of 30 days, S+B = Mice treated with saline orally for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium*, T 500+B = Mice pretreated with aqueous extract of *Terminalia chebula* (500 mg/kg body wt) followed by challenge with 50000 CFU of *S. typhimurium*

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Catalase activity was significantly assessed in mice infected experimentally with *S. typhimurium*. Saline group (SB) infected with *S. typhimurium* (50000 CFU) showed increased CAT activity by 15% at 7th days of PI as compared to saline treated control (Group S without infection). The group treated with T500 for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium* (T500+B) exhibit reduced CAT activity by 5.6% as compared to SB group (Fig. 1).

The malonaldehyde (MDA) level in liver was significantly (p<0.01) assessed at day 7th of PI. Bacterial infection in saline group (SB) showed an increased LPO (nmol MDA formed/h/g of tissue) level by 68% at day 7th of PI. The SB group showed highly significant (p<0.001) increased LPO level as compared to saline treated control group. Mice pretreated orally with T500 followed by challenge with same doses of bacteria significantly (p<0.001) inhibited formation of MDA contents at day 7th of PI as compared to SB group. There was a decrease of 97.69% in the level of LPO as compared to SB significantly (Fig. 2).

GSH activity was significantly assessed in mice liver infected with *S. typhimurium* experimentally. Saline group infected with *S. typhimurium* at a dose of 50000 CFU (SB) showed a decreased level of GSH by 24 % at 7th day of PI as compared to saline treated control (Group S without infection). The group treated with T500 followed...
DISCUSSION

We deliberately used a crude aqueous extract in order to be faithful to the traditionally prescribed formulation. Mice pretreated with drug and challenged with *S. typhimurium* exhibit significant protection against *S. typhimurium* as assessed by survival experiment and estimation of enzymes of oxidative stress.

Rasayana plants prevent ageing, reestablish youth, strengthen life and brain power and prevent diseases [33]. They increase the resistance of the body against any onslaught. *Terminalia chebula* is also one of the Rasayana plant [29]. Chyawanprash is an ancient Ayurvedic preparation, which has been claimed to have health-promoting effects and have been advocated for degenerative diseases. It contains *Terminalia chebula* as one of its constituents. Triphala, which is used in fever, cough, asthma, rheumatism and inflammation of the lungs, contains *Emblica officinalis*, *Terminalia chebula* and *Terminalia bellarica* in equal proportion. This plant also contains tannic acid and gallic acid as their chemical constituent. There have been reports of antimicrobial properties in gallic acid [34]. The above reasons supports our work regarding the protective effects of above drugs against *S. typhimurium* also.

The specific activity of catalase in *S. typhimurium* and other enteric bacteria increased at the onset and during the stationary phase. Addition of hydrogen peroxide to *S. typhimurium* cultures during the exponential growth phase stimulated CAT synthesis. Hydrogen peroxide was produced by *S. typhimurium* cultures during the exponential and stationary growth phases [6]. Increased CAT activity in infected animals with *S. typhimurium* and decreased in activity of CAT in animals treated with drugs followed by challenge with *S. typhimurium* indicated the effectiveness of above drugs against the above bacteria.

The main function of the immune system is the defense of the host organism against infectious agents and malignant tumors. Macrophages, neutrophils and other phagocytic cells are key components of the antimicrobial and tumoricidal immune responses, which is due to the fact that these cells are capable of generating large amounts of highly toxic molecules, reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). The ability of *Salmonella* to replicate within the macrophages makes this enteric pathogen to cause disseminated disease. Professional phagocytic cells like macrophages generate nitric oxide (NO) that acts as a potent agent to limit the growth of many intracellular pathogens including *Salmonella*.

The most direct interaction between nitric oxide (NO) and superoxide anion is their rapid iso-stoichiometric reaction to form peroxynitrite [35, 36]. Under physiological conditions, the generation of peroxynitrite is low and

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**Fig. 2**: Percentage lipid peroxidation (Percentage of nmol MDA formed/h/g of tissue) induced by *S. typhimurium* in mice pretreated with drugs for a period of 30 days. S = Mice treated orally with saline for a period of 30 days, S+B = Mice treated with saline orally for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium*, T 500+B = Mice pretreated with aqueous extract of *Terminalia chebula* (500 mg/kg body wt) followed by challenge with 50000 CFU of *S. typhimurium*. Values are significantly different. ***P<0.001

**Fig. 3**: GSH content (m mol conjugate Glutathione/gm tissue) in Swiss albino mice pretreated with drugs for a period of 30 days followed by challenge with *S. typhimurium*. S = Mice treated orally with saline for a period of 30 days, S+B = Mice treated with saline orally for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium*, T 500+B = Mice pretreated with aqueous extract of *Terminalia chebula* (500 mg/kg body wt) followed by challenge with 50000 CFU of *S. typhimurium*. Values are significantly different. ***P<0.001

by challenge with 50000 CFU of *S. typhimurium* showed an increased level of GSH by 62% as compared to SB group (Fig. 3).
potential oxidative damage is prevented by endogenous antioxidant defenses. At physiological PH, peroxynitrite damages protein directly and decomposes into toxic products that include nitrogen dioxide gas, hydroxyl radical and nitronium ion. However, in pathological conditions, modest elevations in the simultaneous production of NO and superoxide anion can greatly stimulate the formation of peroxynitrite. Consequently, pathological conditions characterized by oxidative stress can greatly elevate the production of peroxynitrite [37]. Homolytic cleavage of peroxynitrous acid (protonated peroxynitrite) yields a hydroxyl radical and a nitrogen dioxide radical. Peroxynitrite is considered to be a potent pathophysiologically relevant cytotoxin. It initiates lipid peroxidation [38], damages DNA [39], oxidizes thiol groups [40] and modifies amino acetyl groups on protein [41]. The oxidation and damage induce various pathological conditions.

It was postulated that ellagic acid scavenges peroxynitrite-derived radicals and consequently inhibits peroxynitrite-induced oxidation and nitration reactions [42]. Ellagic acid is one of the constituent of Terminalia chebula [43, 44] and so can scavenge peroxynitrite derived radical. Terminalia chebula was also reported to reduce LPO [45]. The elevated activity of LPO in saline treated control mice challenge with S. typhimurium and decreased in activity of LPO in mice treated with drugs followed by challenge with same dose of S. typhimurium indicated the effectiveness of the drug against S. typhimurium.

Glutathione is considered to be the most powerful, most versatile and most important of the body's self-generated antioxidants. The liver, spleen, kidneys, pancreas, lens and cornea, have the highest concentrations in the body. It is used by the liver to detoxify many toxins. There is an increase in the level of Glutathione in mice pretreated with drugs followed by challenge with bacteria as compared to the saline treated control infected with same doses of S. typhimurium. Thus the above study confirmed the reduction of oxidative stress in mice induced experimentally by S. typhimurium.

CONCLUSION

Overall, we have shown that water extracts of Terminalia chebula, used extensively in the Indian traditional system of medicine, reduced the oxidative stress in mice which was experimentally induced by S. typhimurium. Pretreatment of mice with drugs at a dose of 500 mg/kg body weight for a period of 30 days protected the animals against 100000 CFU of bacteria. The drugs showed a reduced level of CAT, LPO and increased level of GSH in infected mice as compared to infected saline control. It is therefore concluded that regular intake of the drug can reduce the infection in mice with salmonella. It is already known that S. typhimurium causes an invasive disease in mice that has similarity with human typhoid. So it can be predicted that the regular intake of this drug may reduce the risk of getting typhoid fever. The administration of drug to the mice in this study was done orally. It is now required to test the efficacy of this drug through other route against S. typhimurium. Further study is required to explore the drug at molecular level against the above pathogens.

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