



The effect of regular intake of *Terminalia chebula* on oxidative stress in mice originated from *Salmonella typhimurium*

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Abstract

Typhoid is endemic and in most developing countries remains a public health problem. Due to an increasing resistance to antibiotics and the limited scope of the vaccine the requirement is to explore the efficacy of natural plant products in the treatment of this disease. In this study we have evaluated the aqueous extract of *Terminalia chebula* in connection with the oxidative stress generated in Swiss albino mice by *Salmonella typhimurium*. Mice pretreated through the oral route with the water extract of *T. chebula* at a dose of 500 mg/kg (T500) body wt for a period of 30 d exhibited a full protection against 1×10^5 Colony forming units (CFU) of *S. typhimurium* injected intraperitoneally. Mice pretreated with T500 for a period of 30 d followed with 50000 CFU of *S. typhimurium* showed a decrease in Xanthine oxidase activity by 31% and an increase in both glutathione peroxidase and glutathione reductase activity by 25% as compared to the infected saline treated control. The reduction in the oxidative stress indicated the effectiveness of the drug against *S. typhimurium* which can also be used against typhoid.

Keywords: Glutathione peroxidase, glutathione reductase, *Terminalia chebula*, typhoid, xanthine oxidase.

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INTRODUCTION

Typhoid fever remains an important cause of illness globally with the annual incidence at 21 million cases, of which 1-4% end fatally (Anonymous 2008). In Asia death due to typhoid was estimated at 90% by the WHO. Typhoid is caused by *Salmonella typhi*. Typhoid is characterized by a high fever, colic pain, inflammation, hepatic injury and diarrhea. *S. typhi* has also been reported to cause hepatic dysfunction and hepatic abscess (Soni et al. 1994). On the basis of a literature survey we also reported a number of symptoms (Khan et al. 2008). The treatment against this disease was antibiotics and vaccination. A major impediment to the effective chemotherapy of typhoid is the ever-increasing numbers of resistant strains of *S. typhi* (Goldstein et al. 1986, Eykyn and Williams 1987, Atoba and Charzt 2001).

The vaccines used against typhoid are Vi and Ty21a. 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 (Anonymous 2008). In view of the increasing resistant to antibiotics and limited scope of the vaccine the need of the hour is to evaluate the efficacy of natural plant products for the treatment of this infectious disease.

The majorities of the disease occur mainly due to the imbalance between the pro-oxidant and antioxidant homeostatic phenomenon in the body. The condition of prooxidant dominates either due to the enhanced generation of free radicals and/or their poor quenching/scavenging into the body. The ability of salmonellae to replicate within the macrophages allows this enteric pathogen to cause this disseminated disease. The bacterial entrance causes the production of superoxide

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and nitric oxide. Superoxide and nitric oxide react together to form peroxynitrite a strong biological oxidant. Consequently, pathological conditions characterized by oxidative stress can greatly elevate the production of peroxynitrite (Rastaldo et al. 2007). The exposure of isolated rat enterocytes to *Salmonella typhimurium* enterotoxin resulted in an increased Xanthine oxidase (XO) activity (Mehta et al. 1999).

Novel antimicrobial agents are being sought to combat this problem and also for use in cases of relapses and in the prevention of chronic intestinal carriage of the organism (DuPont 1993). Medicinal herbs represent a rich source from which novel antibacterial chemotherapeutic agents could be obtained. A large segment of the world population, especially in developing countries, depend on the traditional systems of medicine for therapeutic agents for a variety of diseases. We also reported a number of plants having the high medicinal value (Khan and Jain 2003).

Terminalia chebula Retz is reported to be antioxidant, hepatoprotective, antimicrobial (Malekzadeh et al. 2001, Aqil and Ahmad 2007), adaptogenic and anti-inflammatory (Pratibha et al. 2004). Comparing the symptoms of typhoid with the medicinal value of *T. chebula* we selected this plant as agent against salmonella. Moreover we reported the antimicrobial activity of the aqueous extract of *T. chebula* against *S. typhimurium* invitro and *invivo* (Khan and Jain 2009). In this article further investigations were done by using the same plant extract against *S. typhimurium* in Swiss albino mice. The effects of the drug is concerned with the enzymes of oxidative stress (XO), glutathione reductase (GR) and (GPX) was investigated using the above bacteria.

MATERIAL AND METHODS

Plant material

The fruit of *Terminalia chebula* Retz belongs to the family Combretaceae. It was purchased from the Okhla market in New Delhi and then was authenticated by the taxonomist of the University. Only authenticated fruits were used to prepare the extract.

Preparation of plant extract

Distilled water was used to wash the dried fruits to remove the impurities. After thorough washing the fruits were dried in the dark. Dried fruits were then converted into a powder form by continuously grinding and sieving. The powder obtained was soaked in distilled water overnight. It was then centrifuged at 3000 rpm for 15 min, filtered in a sterile condition and then lyophilized to obtain the sterile powder (T).

Microorganisms

In this experiment only *Salmonella typhimurium* (wild) was used. The standard strain of this pathogen was obtained from the National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India. This bacterial strain was further characterized and authenticated in the Department of Microbiology, Majeedia Hospital, New Delhi, India.

Animals

Animals used in this study were Swiss albino mice. Research was performed according to the internationally accepted principles for laboratory animal use. Mice weighing 20-25 g were used for the study. Animals were supplied by the Central Animal House, Hamdard University, New Delhi-62 and kept under standard laboratory condition for 12 h light dark cycle at $25 \pm 10^\circ\text{C}$. Mice were provided with a pellet diet (Lipton, India) and water ad libitum. The protocol was approved by Jamia Hamdard, Hamdard University, New Delhi-62, India. All the studies were conducted 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. Extra care was taken during the handling of animals.

Dose and dosage

The drug was prepared in saline. The dose of *S. typhimurium* was calculated and prepared according to Nasser, 2000. The drug was administered orally and *S. typhimurium* intraperitoneally. The animals were divided into five groups having 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 T100: Aqueous extract of *T. chebula* (100 mg/kg body wt)

Group T200: Aqueous extract of *T. chebula* (200 mg/kg body wt)

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

Group T500+B: Aqueous extract of *T. chebula* (500 mg/kg body wt) + 50000 CFU of *S. typhimurium* (wild)

Protective effect of T against *S. typhimurium* in Swiss albino mice

Seventy two Swiss albino mice were used in this experiment to test the efficacy of the drug (T) against *S. typhimurium*. The mice were divided into three sets. Each set has 24 mice. Four groups were in each set and each group contained six animals. In each set three groups of animals were pretreated orally with (T) at a dose of 100, 200 and 500 mg/kg body wt and the fourth group received saline 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 of 100000 colony forming units (CFU) of *S. typhimurium* (wild) intraperitoneally.

For the second set of animals the same experiment was repeated but the pretreatment of mice was for a period of twenty days. The third set of Swiss albino mice were pretreated in the same way for a period of thirty days and subjected to a challenge dose of 100000 CFU of *S. typhimurium*. Animals were observed for fourteen days and the death percentage was calculated.

Further, to know the protective effect of the drug (T), two groups of mice having six mice in each group were used. One group was pretreated orally with T500 for a period of thirty days and the other group was treated with saline which acted the control. Both the groups in this experiment were then subjected to a challenge dose of 2X100000 CFU of *S. typhimurium*. The death percentage in each group was then observed and recorded. From the death percentage the protective effect of above drug can be easily verified.

Biochemical estimations

Two groups of animals containing six mice in each group were used. The animals in one of the groups were pretreated orally with T500 and other with saline (S) for a period of

30 d. After 30 d of pretreatment, all the animals in both the groups were challenged with 50000 CFU of *S. typhimurium*. The animals in both groups (T500+B: Animals pretreated orally with T500 for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium*; S+B: Animals pretreated orally with saline for same period of time followed by challenge with the same doses of bacteria) were sacrificed at the 7th day of post infection (PI). Post mitochondrial supernatant was utilized for all biochemical estimations. All the biochemical estimations were completed within 24 h of animal sacrifice.

Post-mitochondrial supernatant (PMS) preparation

Livers were first aseptically removed and then homogenized in a chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17% w/v) using a Potter Elvehjem homogenizer. The homogenate was subjected to centrifugation at 800 Xg for 5 min at 4°C to separate the nuclear debris. The aliquot obtained was centrifuged at 10,500 Xg for 20 min to obtain PMS. Enzymes were then estimated by using the PMS.

Estimation of xanthine oxidase (XO)

Measurement of the activity of XO was based on the procedure described by Ali et al. (2000). The reaction mixture consisting of 0.1 mM xanthine and 0.5 M Tris-HCl buffer (pH 8.1) was incubated with the appropriate amount of enzyme source for 20 min at 37°C. The reaction was terminated by precipitating the enzyme using 10% perchloric acid. The mixture was then centrifuged at 4000 rpm for 10 min. The uric acid in the clear supernatant was determined to be at 290 nm. Results are expressed as mg of uric acid/mg protein.

Estimation of glutathione peroxidase (GPX)

The GPX activity was calculated according to the method of Mohandas et al. (1984). The mixture consisted of 1.44 mL PO₄ buffer (0.05 M, pH 7.0), 0.1 mL EDTA (1 mM), 0.10 mL sodium azide (1 mM), 0.05 mL GR (1 IU/mL), 0.1 mL GSH (1 mM), 0.10 mL NADPH (0.2 mM), 0.01 mL H₂O₂ (0.25 mM), and 0.1 mL PMS (10% w/v) with a total volume of 2.0 mL. Disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using a molar extinction coefficient of 6.22 x10³ M⁻¹cm⁻¹.

Estimation of glutathione reductase (GR)

The GR activity was calculated using the method of Mohandas et al. (1984). The mixture consisted of 1.60 mL of a sodium phosphate buffer (0.1 M, pH 7.4), 0.1 mL of EDTA (0.5 mM), 0.05 mL of oxidized glutathione (1 mM), 0.1 mL of NADPH (0.1 mM), and 0.15 mL of PMS (10% w/v) with a total volume of 2.0 mL. The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm at 30 sec intervals for 3.0 min. The activity was calculated using a molar extinction coefficient of $6:22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ and was expressed as nmol NADPH oxidized/min/mg protein.

Estimation of protein

The protein in all samples was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

The level of significance between different groups was analyzed by Dunnett's t-test after the application of analysis of variance (ANOVA).

RESULTS

The aqueous extract obtained from 100 grams of dried fruits of *T. chebula* on lyophilisation yielded 46 gms of the drug (T).

The Swiss albino mice pretreated with T100, T200 and T500 for a period of 10 d followed by a challenge with 100000 CFU of *S. typhimurium* showed 33.3%, 50%, and 33.3% death respectively. Animals pretreated orally with same doses of T for a period of 20 d followed by a challenge with the same dose of *S. typhimurium* exhibit 33.3%, 33.3% and 33.3% death respectively. Mice pretreated for a period of 30 days with the same doses of extract showed a 16.6%, 16.6% and 0% death respectively when exposed to a 100000 CFU dose of the above bacteria. In another experiment mice pretreated for 30 d and challenged with 2X100000 CFU of the above bacteria is shown in Table 1. Thus animals pretreated with T500 for a period of 30 d showed less death indicating that this is maximum protection. Thus 500 mg/kg body wt was considered as the best dose for further experiments as it showed the highest percentage of survival of the mice against *S. typhimurium*.

The XO level was assessed in the mice

Table 1. Protection study shown by different doses of the aqueous extract of *T. chebula* against *S. typhimurium* in Swiss albino mice. 100 = Aqueous extract of *T. chebula* (100 mg/kg body wt), T200 = Aqueous extract of *T. chebula* (200 mg/kg body wt), T 500 = Aqueous extract of *T. chebula* (500 mg/kg body wt). Pretreatment was done for a period of 10, 20 and 30 d.

Dose of Drugs (mg/kg body wt)	Number of Days for Which Dosing Was Done	Number of Animals Taken	Doses of <i>S. typhimurium</i>			
			1X10 ⁵ CFU	2X10 ⁵ CFU	1X10 ⁵ CFU	2X10 ⁵ CFU
			Number of Animals Died		% Death	
T 100	10	6	2	–	33.33	–
T 200	10	6	3	–	50	–
T 500	10	6	2	–	33.33	–
T 100	20	6	2	–	33.33	–
T 200	20	6	2	–	33.33	–
T 500	20	6	2	–	33.33	–
T 100	30	6	1	–	16.6	–
T 200	30	6	1	–	16.6	–
T 500	30	6	0	–	0.0	–
T 500	30	4	–	2	–	50

liver in those concerned with salmonellosis. The *S. typhimurium* infected saline group (SB) exhibited an increased in XO activity by 89% at day 7 of PI as compared to the saline treated control (group S). The group treated with T500 for a period of 30 d followed by a challenge with 50000 CFU of the same bacteria (T500 + B) showed a reduction in XO activity by 31% as compared to the SB group (Fig. 1).

The GPX activity was assessed in the mice liver in those concerned with salmonellosis. The saline treated animals infected with *S. typhimurium* (SB group) showed a decreased in the GPX activity by 13% at the 7th day of PI as compared to the saline treated control (group S). The group pretreated with T500 followed by a challenge with 50000 CFU of *S. typhimurium* (T500 + B) showed an increased level in the GPX activity by 25% as compared to the SB group (Fig. 2).

The GR activity was assessed in the mice liver in those concerned with the salmonella infection. Mice infected with *S. typhimurium* (50000 CFU) (SB group) exhibit a decreased in GR activity by 22% at the day 7th of PI as compared to the saline treated control (group S). The group treated with T500 for period of 30 d followed by a challenge with same dose of bacteria (T500 + B) showed an increased GR activity by 25% as compared to the SB group (Fig. 3).

The above study confirmed the protective role of the drug (T) against *S. typhimurium*. The drug exhibits this protective role by reducing oxidative stress.

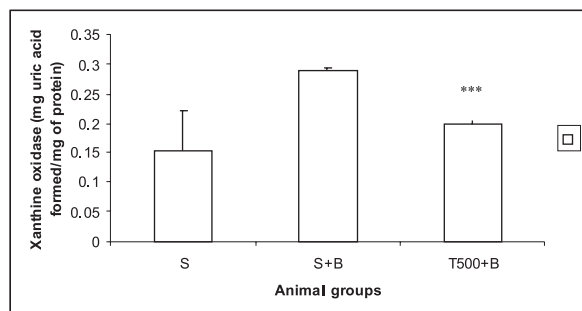


Fig. 1. Xanthine oxidase activity (mg uric acid formed/mg protein) induced in mice pretreated with drugs by *S. typhimurium*. S= Mice pretreated with saline for a period of 30 d. S+B= Mice pretreated with saline for a period of 30 d followed by a challenge with 50000CFU of *S. typhimurium*. T500+B= Mice pretreated with aqueous extract of *T. chebula* (500 mg/kg body wt) followed by challenge with 50000 CFU of same bacteria. Values are significantly different. ***P<0.001

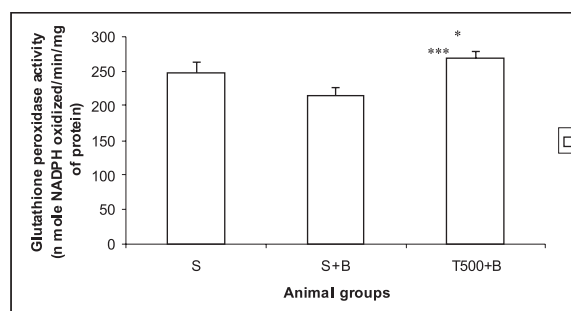


Fig. 2. Glutathione peroxidase (n mole NADPH oxidized/min/mg protein) induced in mice pretreated with drugs by *S. typhimurium*. Values are significantly different. ***P<0.05

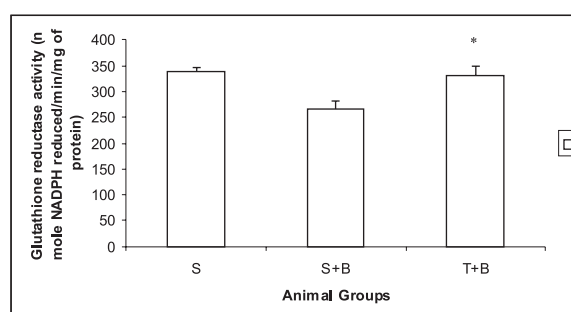


Fig. 3. Glutathione reductase (n mol NADPH reduced/min/mg protein) induced in mice pretreated with drugs by *S. typhimurium*. Values are significantly different. *P<0.05

DISCUSSION

Tannic acid represents the major constituent of the fruit of *T. chebula* (Naik et al. 2004). Some studies reported that tannic acid is bacteriostatic or bactericidal to some Gram positive and Gram negative pathogens (Kau 1980). *T. chebula* contains gallic acid as one of the chemical constituents. There have been reports of antimicrobial properties in gallic acid (Panizzi et al. 2002) and in some gallate esters. Moreover *T. chebula* has been also reported for its antimicrobial properties (Sato et al. 1997, Ahmad et al. 1998, Bonjar 2004).

T. chebula is also one of the Rasayana plants (Rege et al. 1999). 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. Chyawanprash is an ancient Ayurvedic preparation, which claimed to have health-promoting effects and has been advocated for degenerative diseases. It contains *T. chebula* as one of its components (Jose et al. 2001). Triphala, which is used in fever, cough, asthma, rheumatism, and inflammation of the lungs, contains *Emblica officinalis* Gaertn, *Terminalia chebula* and *Terminalia bellarica* Roxb in equal proportion. It was also reported to be hepatoprotective (Tasaduq et al. 2003, Tasduq et al. 2006) and immunomodulatory (Srikumar et al. 2005). The above properties

of T support our study regarding its protective effect against the above bacteria.

The XO activity increased in group SB, which in turn enhanced O_2^- production in the liver. The O_2^- radical has been of profound interest owing to its increased dominance *in vivo* in different disease conditions. Oxidation of hypoxanthine to uric acid with simultaneous generation of O_2^- and H_2O_2 has been observed to play a crucial role during an inflammatory condition and cancer. Compounds which possess both the superoxide scavenging activity as well as the XO inhibitory capacity are Quercetin, 7-neohe-speridosylluteolin, 4,7-dimethyl quercetin, and 3-rutinosylkaempferol. Quercetin is an important compound found in *T. chebula*. Thus it helps in reducing the production of uric acid by inhibiting XO. Decrease in uric acid production indicated the liver to be in good rather than in damaged condition. The infection of mice with *S. typhimurium* caused the damaged to liver as reported by (Khan et al. 2008, Khan and Jain 2009). Infection of the mice with *S. typhimurium* caused an

elevated level of uric acid thereby causing the increase of XO. In the present study we have shown a decrease in the level of uric acid in animals pretreated with T500 followed by a challenge with 50000 CFU of *S. typhimurium* as compared to the saline treated control group challenged with the same doses of the same bacteria. Thus our study supports the effectiveness of this drug against *S. typhimurium*.

GPX can catabolise peroxy nitrite *in vitro* as reported by Briviba et al. (1998) and also many other small biological molecules including glutathione, cysteine, methionine and tyrosine, and can react with peroxy nitrite or its toxic products. Peroxy nitrite is known to inactivate GPX by the oxidation of essential thiol or selenol (Asahi et al. 1997). Therefore, in the bacterial infected group the profound decrease in GPX activity could be the result of the inactivation of GPX by peroxy nitrite. An increase in the GPX activity by the herbs suggested that it catabolize peroxy nitrite and thus lowered salmonellosis.

The group treated with T500 followed by challenge with 50000 CFU of bacteria (T500+B) showed elevated levels of GR activity as compared to the saline treated group (SB) challenged with the same doses of the same bacteria. This again showed the protective effects of the drugs against *S. typhimurium*. The above said plant was also reported to have an antioxidant property (Lee et al. 2005, Lee et al. 2007, Tejesvi et al. 2008) which further confirmed our study on the antioxidant activity of the drugs against *S. typhimurium*.

In short the main phytochemicals present in the fruit of *T. chebula* are tanins, gallic acid and chebulinic acid (Naik et al. 2003). The other compounds present are chebulagic acid, ellagic acid, various other polyphenols and flavonoids. Reports have already been made regarding the hepatoprotective and

antimicrobial activities of chebulagic acid. Chebulagic acid showed a strong scavenging action for O₂⁻ and peroxy radicals and also inhibited reactive oxygen species (Kinoshita et al. 2007). Moreover ellagic acid is a polyphenol antioxidant (Vattem and Shetty 2005), while gallic acid was reported to be analgesic (Krough et al. 2000), antimicrobial, and antioxidant (Soong and Barlo 2006). Since typhoid is characterized by high fever, liver damage, and induction of oxidative stress so it can be predicted that the above phytochemicals can be a responsible way to reduce the risk of getting typhoid fever.

CONCLUSION

A crude aqueous extract of *T. chebula* was used against *S. typhimurium*. Animals pretreated with the drug followed by a challenge with *S. typhimurium* showed a resistance protection against the above bacteria. The drug exhibit protection by reducing oxidative stress. The need is now to explore the active principle of this extract against the bacteria. The route used for the administration of this drug was oral. It is the requirement at the present time to evaluate the potential of this drug using other route against the above bacteria. Further study is required to explore the drug at molecular level against the above pathogen.

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Duzenli *Terminalia chebula* Aliminin Farelerde, *Salmonella typhimurium* Kaynakli Oksidatif Stress Uzerine Etkisi

Özet

Tifo endemiktir ve gelismekte olan ülkelerin cogunda bir halk sagligi problemi olmaya devam etmektedir. Antibiyotiklere karsi artan direnc ve asinin sinirli kullanimi sebebiyle, bu hastaligin tedavisinde dogal bitki ürünlerinden faydalanma yollarini arastirmak bir gereklilik haline gelmistir. Bu calismada, Isvicre albino farelerinde *Salmonella typhimurium* tarafından olusturulan oksidatif stresin giderilmesiyle ilgili olarak, *Terminalia chebula*'nin sivi ozütleri incelenmistir. 30 gün boyunca, vücut agirligina gore 500 mg/kg (T500) dozda *T. chebula* su ozütü ile agiz yoluyla on muamele edilen fareler, enfekte tuzla muamele edilen kontrollere oranla, ksantin oksidaz aktivitesinde %31 oraninda azalma, glutanin peroksidaz ve glutanin redüktaz aktivitesinde %25'lik bir artis gosterdi. Oksidatif stresteki azalma, *S. typhimurium*'a karsi ilacin etkinligini gostermektedir. Bu ilac tifoya karsi da kullanilabilir.

Anahtar Sözcükler: Glutanin peroksidaz, glutanin redüktaz, ksantin oksidaz, *Terminalia chebula*, tifo.