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PHARMACOLOGY

PROTECTIVE EFFECT OF *EMBLICA OFFICINALIS* AGAINST *S.TYPHIMURIUM* THROUGH ITS ANTIOXIDANT ACTIVITY

K.H. Khan[1,2,*]

[1]*Centre for Biotechnology, Faculty of Science, Jamia Hamdard, Hamdard University, New Delhi-110062, India*
[2]*Assistant Professor, Medical Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India*

Abstract

Typhoid is a major public health problem caused by *Salmonella typhi*. *Salmonella typhimurium* (*S. typhimurium*) causes an invasive disease in mice that has similarity with human typhoid. The antibiotics used got resistant to the bacteria and has side effects while vaccine developed has little scope against this disease. In this study antioxidant activity of fruits of *Emblica officinalis* was explored against the oxidative stress generated by *S. typhimurium* in mice. Animals pretreated with EO500 for a period of 30 days followed by challenged with 50000 (CFU) of *S. typhimurium* exhibit a decrease in Xanthine oxidase activity by 36% and increase in glutathione peroxidase and glutathione reductase activity by 37% and 38% respectively as compared to mice infected with same doses of *S. typhimurium*. The study confirmed the lowering of oxidative stress in mice by this drug and can be used in typhoid.

Keywords: Antioxidant, *Emblica officinalis*, *Salmonella typhimurium*, Typhoid

Introduction

Salmonellosis are wide spectrum diseases for men and animals. *Salmonella typhi* (S. *typhi*) is the causative organism for human typhoid is a world wide problem as described by this author also[1]. It is characterized by high fever, diarrhea, inflammation, colic pain. *S. typhi* has been reported to cause hepatic dysfunction and hepatic abscess[2]. In our review article on typhoid, we also reported a number of symptoms of this disease[3]. The present treatment of typhoid is antibiotics and vaccination. A major draw back to the effective chemotherapy of typhoid is the ever-increasing numbers of resistant strains of *S. typhi*[4, 5, 6]. The vaccines used against this disease are Ty21a and Vi. However, the efficacy of Ty21a and Vi vaccines in children aged less than two years has not been explained, and neither of these vaccines are licensed for use in this age group as reported by WHO[7]. The resistant to antibiotic and limited scope of vaccine attracted the author to explore the efficacy of plant product against this disease.

A number of diseases develop in the body due to unbalance in the body between pro-oxidant and anti-oxidant homeostatic phenomenon. The increased generation of free radicals or their poor scavenging develops the pro-oxidant condition[8]. Bacterial entrance into the body causes the production of nitric oxide and superoxide. Nitric oxide and superoxide react together to form peroxynitrite which is a strong biological oxidant. The role of peroxynitrite molecule is to initiate lipid peroxidation, damages DNA, oxidizes thiol groups and modifies amino acetyl groups on protein. This oxidation and damage initiates a series of pathological conditions in the body leading to symptoms of diseases. The ability of Salmonella to replicate within the macrophages makes this enteric pathogen to cause disseminated disease. In pathological conditions there is an increase in the production of NO and superoxide anion which enhances the production of peroxynitrite. Consequently, pathological conditions characterized by oxidative stress can greatly elevate the production of peroxynitrite[9]. Further this author also reported the oxidative stress in mice caused by *S.typhimurium*[10, 1].

Herbal medicine has been improved in most of the developing countries not only as a way to rescue ancient tradition but also as an alternative solution to health problems. Medicinal herbs represent a rich source from which novel antibacterial chemotherapeutic agents could be obtained. We also reported a number of plants having high medicinal value in our review article on medicinal plants[11].

* Corresponding Author, *Email*: hamkishwar191@yahoo.co.in, kishwar@vit.ac.in, Mob: +91-9944327624, Phone: +91-416-2202536, Fax: +91-416-2243092/2240411

Triphala the combination of *Emblica officinalis* (ES), *Terminalia chebula* and *Terminalia bellarica* in equal ratio is used in fever, cough, asthma, rheumatism, and inflammation of the lungs. *Terminalia bellarica* was reported to be used against *Salmonella typhimurium* (*S.typhimurium*) from the laboratory of our University[12]. It was reported by the author that aqueous extract of *Terminalia chebula* exhibit antisalmonella typhimurium activities *invitro* and also *invivo*[13]. The author further reported that the animals pretreated with aqueous extract of *Terminalia chebula* at a dose of 500mg/kg body wt orally exhibited protection against 100000 CFU of *S. typhimurium* injected intraperitoneally[1, 13]. As the extract of the fruits of the above said plants showed its activity against *S. typhimurium*, enabled the author to explore the activity of lyophilized juice of ES, a component of triphala against the oxidative stress generated by *S. typhimurium*.

ES is also called as Amla or Indian gooseberry. It is used in jaundice, diarrhoea and has been reported to have antioxidant, antitumour, anti-inflammatory activity[14]. In a review article on ES this author has described it as antimicrobial, hepatoprotective, antoxidant, immunomodulatory, antipyretic, analgesic, cytoprotective and also reported for having a number of other medicinal value[15]. The author studied the sign and symptoms of this disease and compared it with the biological activities of this plant which further enabled him to select the lyophilized juice of ES against *S. typhimurium*. Despite many therapeutic effects of Indian gooseberry, relatively little data is available on the antioxidant activity of Amla against oxidative stress induced by *S. typhimurium*. Therefore an *invivo* study was performed to determine the antioxidant effect of lyophilized fruit juice against *S. typhimurium* in mice.

This author reported the protective effect of this drug (EO500) against the enzymes of oxidative stress induced in swiss albino mice against *salmonella typhimurium*[16]. The present study is the extension of the previous work. In this study the effect of lyophilized juice of ES were mainly studied on other enzymes of oxidative stress namely xanthine oxidase (XO), glutathione peroxidase (GPX) and glutathione reductase (GR) induced experimentally by *S. typhimurium* in mice.

S. typhimurium causes an invasive disease in mice that has similarity with human typhoid[17]. Consequently, the murine model of salmonellosis has been used extensively to explain potentially clinical relevant mechanisms of antisalmonella host-defense[18, 19, 20, 21]. The aim of this study is therefore to evaluate the efficacy of the lyophilized juice of ES against *S. typhimurium* in mice so to develop herbal drugs with no side effect against typhoid.

Material and Methods

Plant material

Emblica officinalis (ES) belongs to the family *Euphorbiaceae*. Fruits were purchased from Okhla market of New Delhi, India. Further it was authenticated by University taxonomist. Before processing, it was confirmed that the materials were freed from contamination, and had no microbial growth.

Preparation of plant extract

Fruits of (ES) were washed thoroughly with double distilled water to remove dust particle and other impurities. Fruits were then deseeded and crushed to obtain juice. The juice collected were centrifuged at 3000 rpm for 10 minutes and filtered in sterile condition and finally lyophilized to obtain dry powder (EO). Since EO is hygroscopic care was taken to handle this drug.

Microorganisms

The microorganism used in the experiment was *S. typhimurium* (wild). The standard strain of this pathogen was obtained from National Salmonella Phage Typing Centre, Lady Harding Medical College, New Delhi, India. This strain was further characterized and authenticated in the Department of Microbiology, Majeedia Hospital, New Delhi, India. Further it was confirmed by growing on Triple Sugar Iron (TSI) agar as it produces black colony against pinkish background. The confirmed colonies were then selected for the experimental purposes.

Animals

Swiss albino mice weighing 20-25 gms were used as an animal model for this study. These 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± 10 oC. Mice were provided with pellet diet (Lipton, India) and water ad labitum. Protocol was approved by Jamia Hamdard, Hamdard University, New Delhi-62, India where this work was done. All the studies were conducted according to ethical guidelines of the Jamia Hamdard Animal Ethics committee and "Commiittee for the Purpose of Control and Supervision of Experiments on Animals" (CPCSEA). Much care was taken during the handling and maintenance of the mice in an ethical manner.

Dose and Dosage

The drug was prepared in saline. The dose of *S. typhimurium* was calculated and prepared according to Nasser[22]. Drug was administered orally and *S. typhimurium* intraperitoneally into

animals. The animals were divided into groups having six animals in each group. The study comprised of following treatment schedule.

Group S: Normal saline.

Group S+B: Normal Saline+50000CFU of *S. typhimurium* (wild).

Group EO500: Lyophilised juice of *Emblica officinalis* (500mg/kg body wt).

Group EO500+B: Lyophilised juice of *Emblica officinalis* (500mg/kg body wt)+ 50000CFU of *S. typhimurium* (wild).

Biochemical estimations

For this study eighteen swiss albino mice were taken. They were then carefully distributed into three groups. There were six animals in each group. First and second groups were pretreated with saline and third group with EO500 orally for a period of 30 days. After pretreatment for 30 days the mice in second and third groups were subjected to a challenge dose of 50000 CFU of *S. typhimurium* intraperitoneally. The animals of all groups (S: Animals pretreated with saline only for 30 days, S+B: Animals pretreated with saline for a period of 30 days followed by challenge with 50000 CFU of *S. typhimurium*. EO500+B: Animals pretreated orally with EO500 followed by challenge with same doses of bacteria) were sacrificed at 7th day of post infection (PI). For all biochemical estimation, post mitochondrial supernatant was used. Precaution was taken to complete all biochemical estimations within 24 hrs of animal sacrifice.

Preparation of post-mitochondrial supernatant (PMS)

Livers of the experimental animals were aseptically removed and then homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17% w/v) using potter elvehjem homogenizer. The homogenate obtained was subjected for centrifugation at 800g for 5 min at 4 ^{o}C to separate the nuclear debris. The aliquot obtained was again centrifuged at 10,500g for 20 min to obtain PMS. Enzymes were estimated by using PMS.

Xanthine oxidase (XO) estimation

The activity of XO was measured by the procedure adopted by Ali et al[23]. Reaction mixture consisting of 0.1 mM xanthine and 0.5 M Tris-HCl buffer (pH 8.1) was incubated with appropriate amount of enzyme source for 20 min at 37°C. The reaction was stopped by precipitating the enzyme using 10% perchloric acid. The mixture was then centrifuged at 4000 rpm for 10 min. Uric acid in the clear supernatant was determined at 290 nm. Results are expressed as mg of uric acid/mg protein.

Estimation of glutathione peroxidase (GPX)

GPX activity was assayed according to the method of Mohandas et al[24]. 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) in 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 x10^3 $M^{-1}cm^{-1}$.

Estimation of glutathione reductase (GR)

GR activity was assayed by the method of Mohandas et al[24]. The assay system consisted of 1.60 ml of 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) in a total volume of 2.0 ml. The enzyme activity was quantified by measuring the disappearance of NADPH at 340nm at 30-s intervals for 3.0 min. The activity was calculated using a molar extinction coefficient of 6:22 x10^3 $M^{-1}cm^{-1}$ and is expressed as nmol NADPH oxidized/ min/mg protein.

Protein estimation

Protein in all samples was determined by the method of Lowry et al[25] using bovine serum albumin as a standard.

Statistical analysis

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

Results

Fresh fruits (250 gms) produced 250 ml juice. The juice on lyophilization yields 32.8 gm of drugs (EO).

XO level was significantly assessed in the mice liver in concerned with *S. typhimurium* infection. Animals pretreated with saline for a period of 30 days followed by challenge with *S. typhimurium* (S+B) showed increased XO activity by 89% at 7th day of PI as compared to saline treated control (S). Mice pretreated with EO500 for same period of time and then subjected to same doses of *S. typhimurium* (EO500+B) showed a reduced in XO activity by 36% as compared to S+B group (Fig 1).

GPX activity was significantly assessed in the mice liver in concerned with salmonellosis. Mice pretreated with saline for a period of 30 days followed by challenge with S. typhimurium (S+B) showed a decrease in GPX activity by 13 % at 7th day of PI as compared to saline treated control (S). Animals pretreated with EO500 for 30 days followed by challenge with same doses of bacteria

(EO500+B) showed an increase in GPX activity by 37% as compared to SB group (Fig 2).

Fig 1: Xanthine oxidase activity (mg uric acid formed/mg protein) induced by *S. typhimurium* in mice pretreated with EO for a period of 30 days. S = Saline, S+B = Saline+ 50000 CFU of *S.typhimurium*, EO500+B = Lyophilized juice of *Emblica officinalis* (500mg/kg body wt) + 50000 CFU of *S. typhimurium*. Values are significantly different. ***P< 0.001

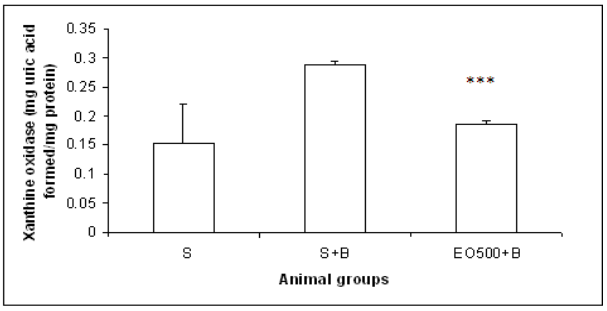
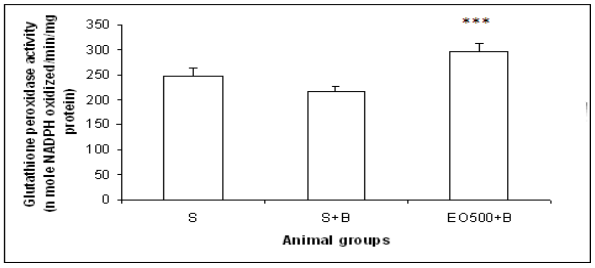
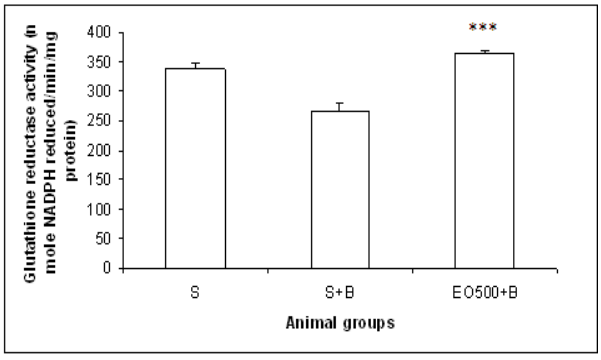


Fig 2: Glutathione peroxidase activity (n mole NADPH oxidized/min/mg protein) induced by *S typhimyrium* in mice pretreated with drug for a period of 30 days. S = Saline, S+B = Saline+ 50000 CFU of *S.typhimurium*, EO500+B = Lyophilized juice of *Emblica officinalis* (500mg/kg body wt) + 50000 CFU of *S.typhimurium*. Values are significantly different. ***P< 0.001



The activity of GR was significantly determined in the mice liver in concerned with S.typhimurium infection. S. typhimurium infected saline group (S+B) showed a decrease in GR activity by 22 % at 7th day of PI as compared to saline treated control (S). The group pretreated with EO500 for a period of 30 days and challenged with same doses of bacteria showed an increase in GR activity by 38% as compared to S+B group (Fig3).

Fig 3: Glutathione reductase activity (n mol NADPH reduced/min/mg protein) induced by *S typhimurium* in mice pretreated with drug for 30 days. S = Saline, S+B = Saline+50000 CFU of *S.typhimurium*, EO500+B = Lyophilized juice of *Emblica officinalis* (500mg/kg body wt) + 50000 CFU of *S.typhimurium*. Values are significantly different. ***P< 0.001



Discussion

Crude EO was deliberately used in this experiment in order to be faithful to the traditionally prescribed formulation. ES belongs to one of the Rasayana plant[26]. The main feature of Rasayana plants are to prevent ageing, strengthen life, reestablish youth, and brain power and prevent diseases[27]. They increase the resistance of the body against various drastic conditions. Chyawanprash is one of the ancient Ayurvedic preparation, which has been claimed to have health-promoting effects and also used for various degenerative diseases. It contains ES as one of its major constituents[28]. Triphala, which is used in asthama, fever, cough, rheumatism, and inflammation of the lungs, contains ES, *Terminalia chebula* and *Terminalia bellarica* in equal proportion. ES was also reported to have antisalmonellae activity invitro by using well diffusion method[29].

S. typhimurium enterotoxin when exposed to isolated rat enterocytes resulted in an elevated XO activity[30]. This enzyme (XO) is capable of generating $O_2^{\cdot-}$ by converting hypoxanthine or xanthine to uric acid. XO is normally found in the liver. During severe liver damage, XO is released into the blood, so a blood assay for XO is a way to determine if liver damage has happened.

In this experiment XO activity got increased in group SB, which in turn enhanced O_2- production in liver. O_2-radical has been of profound interest owing to its increased dominance *invivo* 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 inflammatory condition and cancer. Compounds which possess both the superoxide scavenging activity as well as XO inhibitory capacity are Quercitin,7-neohespiridosylluteolin, 4,7-dimethyl quercitin, 3-rutinosylkaempferol. Quercitin is an important compound found in ES31. Thus it helps in reducing the production of uric acid by inhibiting XO. Decrease in uric acid production indicated liver to be in good rather than in damaged condition. The infection of mice with *S. typhimurium* caused damaged to liver as reported by us13. Infection of mice with *S. typhimurium* caused elevated level of uric acid thereby causing the increase of XO. In the present study it was shown that there is decrease in the level of uric acid in animals pretreated with EO500 followed by challenge with 50000 CFU of *S. typhimurium* as compared to saline treated control challenged with same doses of same bacteria. Thus our study supports the effectiveness of this drug against *S. typhimurium*. Triphala has been reported to decrease the level of Xanthine oxidase, lipid peroxidation

and also restore the level of glutathione[32] which further supports our study.

The main function of glutathione peroxidase (GPX) is to protect the body from oxidative damage. It reduces lipid hydroperoxides to their corresponding alcohols and also reduces free hydrogen peroxide to water. They are the major enzymes that remove hydrogen peroxide generated by SOD in cytosol and mitochondria by oxidizing the tripeptide glutathione (GSH) into its oxidized form (GSSG).

GPX can catabolise peroxynitrite *invitro*[33] and many other small biological molecules including glutathione, cysteine, methionine and tyrosine, can react with peroxynitrite or its toxic products. Peroxynitrite is known to inactivate GPX by the oxidation of essential thiol or selenol[34]. Therefore, in bacteria infected group the profound decrease in GPX activity could be the result of inactivation of GPX by peroxynitrite. Increased in the GPX activity by the herbs suggested to catabolize peroxynitrite and thus lowers salmonellosis. ES have been further reported to normalize the level of GPX in stress condition[35, 36] which also supports our study.

GR reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant. The group treated with EO500 followed by challenge with 50000CFU of *S. typhimurium* (EO500+B) showed elevation in levels of GR activity as compared to saline treated group (S+B) challenged with same doses of same bacteria. This again showed the protective effects of drugs against *S. typhimurium*. Further, ES have been also reported further to increase GR activity[36] which finally supports our study.

Further in support of our study the literature described ES as an antioxidant also[37, 38, 39, 40, 41]. It provided protection against radiation[42]. It is reported to be used against oxidative stress during ischemic-reperfusion injury[43] and also ameliorates hyperthyroidism and hepatic lipid peroxidation[44].

As far as phytochemical is concerned, ES is a good source of polyphenol, flavones, tannins and other bioactive substances that are strong antioxidant and might contribute to the healthy effect[14]. Flavonoids are plants polyphenols which are available in the fruits45. ES has been reported to contain, ascorbic acid, carotenoids, ellagic acid[46]. The fruit also contains phyllembic acid, gallic acid[14], emblicanin A and emblicanin B47, geraniin, quercetin, quercetin 3-β-D-glucopyranoside, kaempferol 3-β-D-glucopyranoside, isocorilagin, kaempferol[31] and vitamin C[48].

Some studies reported that tannic acid is bacteriostatic or bactericidal to some Gram (+)ve and Gram (-) ve pathogens[49]. There have been reports of antimicrobial properties in gallic acid50 and in some gallate esters. Polyphenolic compounds of ES were reported for its anti oxidant acitivities by scavenging free radical. Gallic acid, ellagic acid, ascorbic acid, emblicanin A, and emblicanin B have been reported as free radical scavenger[39]. Gallic acid and tannic acid were also reported to have antioxidant activity[51]. Moreover gallic acid was also reported to be a major compound that showed highest NO scavenging activity[52]. Ellagic acid scavenges peroxynitrite derived radicals and consequently inhibits peroxynitrite induced oxidation and nitration reactions[53].

Geraniin, quercetin 3-β-D-glucopyranoside, kaempferol 3-β-D-glucopyranoside, isocorilagin, quercetin, and kaempferol were reported for their antioxidant activity[31]. 5-hydroxymethylfurfural, gallic acid, β-daucosterol and ellagic acid were reported to exhibit high antioxidant activity[14]. Vitamin C in ES accounts for approximately 45-70% of the antioxidant activity[48]. A number of reports regarding the antioxidant activity of ES were also made[54, 55, 39, 56, 57,58].

Preliminary phytochemical screening of the plant extract gave positive test for alkaloids, tannins, phenolic compounds, carbohydrates and amino acids, which might be in part responsible for antipyretic and analgesic activities[59]. Thus the protective effect of this drug is considered due to the combined effect of the active principle present in the fruit through its antioxidant activity.

Conclusion

The author mainly studied the effect of EO on the enzymes of oxidative stress namely xanthine oxidase (XO), glutathione peroxidase (GPX) and glutathione reductase (GR) induced experimentally by *S. typhimurium* in mice. *S. typhimurium* induces the oxidative stress in mice by increasing the level of XO and decreasing the level of GPX and GR. The drugs further reduced the activity of XO and increased the level of GPX and GR. Thus the drug provided protection to infected mice by acting as an antioxidant and lowering the oxidative stress generated by *S. typhimurium*. This reduction in the oxidative stress confirmed the effectiveness of the drug against *S. typhimurium* and so can also be used against typhoid. The route used for the administration of this drug was oral. It is the requirement of the present time to evaluate the potential of this drug using other route against the above bacteria. Since crude extract has been used in this experiment so effort is required to purify the

active principle and study this protection at molecular level. Further the drug can be put forward for clinical trial in man against typhoid.

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