Global Journal of Pharmacology 5 (1): 07-17, 2011
ISSN 1992-0075

Thank You, a Good Research Antioxidant, Antimicrobial and Toxicity Studies of the Different Fractions of Fruits of *Terminalia belerica* Roxb

[1]*M. Badrul Alam,* [1]*Ronok Zahan,* [1]*Mahedi Hasan,*
[1]*M. Maruf Khan,* [1]*M. Saifur Rahman, Nargis* [2]*S. Chowdhury and* [3] *M. Ekramul Haque*

[1]Department of Pharmacy, Atish Dipankar University of Science and Technology, Banani, Dhaka, Bangladesh
[2]Department of Pharmacy, Manarat International University, Mirpur, Dhaka, Bangladesh
[3]Department of Pharmacy, BRAC University, Mohakhali, Dhaka, Bangladesh

Abstract: The crude methanolic extract of the fruits of *Terminalia belerica* Roxb. along with its various organic fractions were tested to evaluate the toxicity, antioxidant (*in vitro* and *in vivo*) and antibacterial activity. The different assay methods, including total antioxidant activity, scavenging free radical, authentic peroxynitrite and reducing power assessment were used to evaluate the antioxidant potential of the crude extract and its organic fractions. While antimicrobial activities were evaluated using agar disc diffusion method and measuring their MIC value. The EtOAc fraction, showed strong activity in all the model systems tested and in peroxynitrite model this fraction (IC_{50} = 1.29±0.18 µg/ml) exerted four-fold stronger activity than standard penicillamine (IC_{50} value of 5.20±0.32 µg/ml). The reducing power of the extract was found to be concentration dependent. The administration of the extract/fractions at a dose of 200 and 400 mg/kg body weight to the male Wistar rats increased the percentage inhibition of reduced glutathione, superoxide dismutase and catalase significantly. Whereas, lipid peroxidation level in hepatotoxic rats markedly decreased at a dose of 400 mg/kg body weight after 7 days. EtOAc fractions showed also the highest antibacterial effect against *Staphylococcus aureus* with zone of inhibition 30.00±0.25 mm. The Brine shrimp lethality bioassay was used to determine the toxicity of the extracts where dichloromethane (CH_2Cl_2) fraction showed highest activity (LC_{50} = 39.02±1.16 µg/ml). In conclusion, the study clearly indicated that the extract/fractions of *Terminalia belerica* Roxb possesses good antioxidant and antimicrobial activity along with moderate toxicity.

Key words: Antioxidant · *Terminalia belerica* · Free radical · Antibacteiral · Toxicity

INTRODUCTION

Free radicals cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [1, 2]. Undoubtedly, *in vivo* suppression of free radicals is important for the human body to eliminate the toxicity induced by free radicals. For several years, many researchers have been investigated powerful and nontoxic antioxidants from natural sources, especially edible or medicinal plants to prevent the free radical related disorders in human, which replaces the synthetic compounds because of their probable carcinogenic activity and harmful to the lungs and liver [3].

Plants produce wide array of bioactive principles and constitute a rich source of medicines. In many developing countries, traditional medicine is one of the primary health care systems [4, 5]. Large scale evaluation of the local flora exploited in traditional medicine for various biological activities is therefore necessary. Isolation and characterization of the bioactive principles ultimately leading to new drug development. In view of this our attention has been focused particularly *Terminalia belerica* Roxb. belongs to the family "Combretaceae", commonly known as Bohera. It is routinely used as traditional medicine by some tribal people of various districts of Bangladesh, to get remedies from several

Corresponding Author: M. Ekramul Haque, PhD Professor and Chairman, Department of Pharmacy, BRAC University, 66-Mohakhali, Dhka-1212, Bangladesh.
Tel: +8801711952286, E-mail: haque_md.ekramul@yahoo.com.

ailments such as fever, cough, diarrhea, skin diseases and oral thrush [6]. Chemical substances of β-sitosterol, gallic acid, ethyle gallate, galloyl glucose, a new triterpene, the belleric acid and chebulagic acid have been isolated from fruits of *T. belerica* [7]. Fruit extracts of *T. bellerica* possess hepatoprotective, antimicrobial and antihypertensive activity [8-10]. Based on these reports our studies have been designed to examine whether the methanolic extract and organic soluble fractions of *T. belerica* fruit exerts *in vitro* and *in vivo* antioxidant activity. In addition, we investigated whether these extract/fractions have any antimicrobial and toxic effect.

MATERIALS AND METHODS

Plant Materials: The fruits of *Terminalia belerica* were collected from the village Kachuria under Mollahat thana of Khulna district, Bangladesh during the month of October 2009. The sample was identified at Bangladesh National Herbarium, Dhaka, whereas the voucher specimen has been deposited. Its DACB Accession Number is AD/2908.

Chemicals: Ammonium molybdate, Folin-chiocaltu phenol reagent, were purchased from E. Merck (Germany). 1,1-diphenyl- 2-picryl-hydrazyl (DPPH), ascorbic acid, quercetin and potassium ferric cyanide and DL-penicillamine (DL- 2- amino- 3- mercapto- 3-methylbutanoic acid) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The high quality DCFH-DA and DHR 123 (dihydrorhodamine 123) and ONOO- were purchased from Molecular Probes (Eugene, Oregon, USA) and Cayman (Ann Arbor, MI, USA), respectively. All other chemicals and reagents were of analytical grade.

Preparation of Plant Extract: Fruits of *T. belerica* were dried in an oven at 37°C and then powdered with a mechanical grinder, passing through sieve #40 and stored in a tight container. The dried powdered material (1.5 kg) was refluxed with MeOH for three hours. The total filtrate was concentrated to dryness, *in vacuo* at 40°C to render the MeOH extract (490 g). This extract was suspended in H_2O and then successively partitioned with dicholoromethane (CH_2Cl_2), ethylacetate (EtOAc) and normal butanol (n-BuOH) to afford the CH_2Cl_2 (200 g), EtOAC (60 g) and n-BuOH (110 g) fractions along with the residue (120 g) present in aqueous phase.

Test Microorganisms: Both Gram positive and Gram negative bacterial strains were obtained from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6059, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonus aeruginosa* ATCC 27853, *Salmonella paratyphi* ATCC 9150, *Salmonella typhi* ATCC 13311, *Shigella dysenteriae* ATCC 9361, were used as test microorganism. The strains are maintained and tested on Nutrient Agar (NA) for bacteria.

In vitro Antioxidant Activity

The Amount of Phenolic Compounds and Flavonoids: The total phenolic content of methanolic extract and several organic fractions were determined using Folin–Ciocalteu reagent [11]. *T. belerica* fruit extracts (100 µl) were mixed with the Folin–Ciocalteu reagent (500 µl) and 20% sodium carbonate (1.5 ml). The mixture was shaken thoroughly and made up to 10 ml with distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was measured with Shimadzu UV-160A spectrophotometer (Kyoto, Japan). These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid.

The flavonoids content was determined by aluminium chloride colorimetric method [12]. Quercetin was used to make the calibration curve. The different concentration of plant extract (0.5 ml) were separately mixed with 95% ethanol (1.5 ml), 10% aluminum chloride (0.1 ml), 1M potassium acetate (0.1 ml) and distilled water (2.8 ml). After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. All the determinations were carried out in duplicates. These data were used to estimate the flavonoid contents using a standard curve obtained from various concentration of quercetin.

Determination of Total Antioxidant Activity: The antioxidant activity of the MeOH extract and other organic fractions were evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al*. [13]. The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green

phosphate/Mo(V) complex at acid pH. Extract (0.3 ml) was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling to room temperature. Methanol (0.3 ml) is used as the blank experiment. The antioxidant activity is expressed as the number equivalents to ascorbic acid using the following formula:

$$C = (c \times V)/m$$

Whereas: C—total antioxidant activity, mg/g plant extract, in Ascorbic acid; c—the concentration of ascorbic acid established from the calibration curve, mg/ml; V— the volume of extract, ml; m—the weight of pure plant extract, g.

Free Radical Scavenging Activity Measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH): The free radical scavenging activity of MeOH extract and other organic fractions, based on the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical and was determined by the method described by Braca *et al*. [14]. Plant extract (0.1 ml) was added to 3ml of a 0.004% MeOH solution of DPPH. Absorbance at 517nm was measured after 30 min and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0]$ x 100, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/ standard. IC_{50} value was calculated from the equation of the line obtained by plotting a graph of concentration (µg/ml) versus% inhibition.

Measurement of the ONOO-Scavenging Activity: The ONOO- scavenging activity was measured by monitoring the oxidation of DHR 123, by modifying the method of Kooy *et al*. [15]. The DHR 123 (5 mM), in dimethylformamide, was purged with nitrogen, stored at -80°C and used as a stock solution. This solution was then placed on ice and kept from exposure to light, prior to the study. The buffer used was consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride, at pH 7.4 and 100 mM diethylenetriaminepentaacetic acid (DTPA), each of which were prepared with high quality deionized water and purged with nitrogen. The final concentration of the DHR 123 was 5 µM. The background and final fluorescent intensities were measured after 5 minutes treatment, both with and without the addition of authentic ONOO-. The DHR 123 was oxidized rapidly by authentic ONOO- and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc.), with excitation and emission wavelengths of 480 and 530 nm, respectively. The results were expressed as the mean±standard error (n=3) of the final fluorescence intensity minus the background fluorescence. The effects were expressed as the percentage inhibition of the DHR 123 oxidation. IC_{50} was calculated from the equation of line obtained by plotting a graph of concentration (µg/ml) versus% inhibition.

Reducing Power Activity: The reducing power of *T. belerica* fruits were determined according to the method previously described [16]. Extracts at different concentrations in 1 ml of 10% DMSO were mixed with 2.5 ml of phosphatebuffer (0.2M, pH 6.6) and 2.5 ml potassium ferricyanide $[K_3Fe(CN)_6]$ (1%) and then the mixture was incubated at 50°C for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

***In vivo* Antioxidant Activity**

Animals: Male Wistar rats with a mean weight of 175 g ±5.2 were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR,B). Animals were maintained under standard environmental conditions (temperature: (24.0±1.0°), relative humidity: 55-65% and 12 h light/12 h dark cycle) and had free access to feed and water ad libitum. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Animal Grouping and Extract Administration: Twenty male rats were randomized into four groups consisting of five each group. Group 1 served as normal control and was given distilled water alone (0.5 ml) per day for seven days with the aid of oropharyngeal cannula.

Groups 2 animals served as hepatotoxic control, treated with CCl_4 in a single dose of 0.5 ml administered orally for seven days. Group 3 and 4 were treated like the normal control except that they received 0.5 ml of the extract corresponding to 200 and 400 mg/kg body weight, respectively. Again group 3 - 4 was given 0.5 ml of CCl_4 on the seventh day after 6 h of extract administration. All the animals from each group were sacrificed by ether anesthesia 24 h after their respective 21 daily doses of the extract and distilled water. The liver from each animal was excised, rinsed in ice cold 0.25 M sucrose solution and 10%w/v homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 5000 rpm for 60 min at 4°C. The supernatant obtained was used for the estimation of catalase, superoxide dismutase, lipid peroxidation (TBARS) and reduced glutathione.

Determination of Catalase Activity: The activity of catalase was assayed according the method described by Pari and Latha [17]. The liver was homogenized in 0.01 M phosphate buffer (pH 7.0) and centrifuge at 3000 rpm. The reaction mixture consisted of 0.2M hydrogen peroxide (0.4 ml), 1 ml of 0.01M phosphate buffer (pH 7.0) and 10% w/v liver homogenate (0.1 ml). The reaction of the mixture was stopped by adding 5% dichromate-acetic acid ($K_2Cr_2O_7$ in glacial acetic acid) reagent (2 ml). The changes in the absorbance was measured at 620 nm and percentage inhibition was calculated using the equation:

$$\% \text{ catalase inhibition} = [(Ao - A1) / A0] \times 100$$

Where; A_o is the absorbance of the control and A_1 is the absorbance of the sample extract.

Determination of Superoxide Dismutase Activity: Superoxide dismutase was assayed as described by Naskar *et al.* [18]. The liver homogenates were prepared in Tris (ethylenediamine tetraacetic acid) buffer centrifuged for 40 min at 10000 r.p.m at 4°C, the supernatant was used for the enzyme assay. 2.8 ml Tris-EDTA (composed of 49.78 mM Tris 0.0012 mM EDTA; pH 8.5) 100µl Pyrogallol (2mM) were taken in the cuvette scanned for 3 min at 420 nm wavelength. Then 2.8 ml Tris-EDTA (pH -8.5), 100µl Pyrogallol, 50µl tissue homogenate were taken and scanned for 3 min at the same wavelength. Percentage inhibition was calculated using this equation:

$$\text{Rate (R)} = (\text{final OD-initial OD})/3$$
$$\% \text{ of inhibition} = [(\text{blank OD-R})/\text{blank OD}] \times 100$$

Determination of Reduced Glutathione Activity: Reduced glutathione was determined using the modified method of Ellman [19]. An aliquot of 1.0 ml of supernatant of liver homogenate was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was measured at 412 nm. The percentage inhibition of GSH was calculated using the following equation:

$$\% \text{ reduced glutathione inhibition} = (A_o - A_1)/Ao \times 100$$

Where; A_o is the absorbance of the control and A_1 is the absorbance of the sample extract.

Estimation of Lipid Peroxidation: Lipid peroxidation in the liver was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) using the modification method of Niehius and Samuelsson [20]. In brief, 0.1 ml of liver homogenate (10% w/v) was treated with 2 ml of (1:1:1 ratio) TBATCA-

Hcl reagent (thiobarbituric acid 0.37%, 15% trichloroacetic acid and 0.25 N HCl). All the tubes were placed in a boiling water bath for 30 min and cooled. The amount of malondialdehyde formed in each of the samples was assessed by measuring the absorbance of clear supernatant at 535 nm against reference blank. Percentage inhibition was calculated using the equation:

$$\% \text{ lipids Inhibition} = \{A_o - A_1\}/Ao \times 100$$

Whereas; A_o is the absorbance of the control and A_1 is theabsorbance of the sample extract.

Antimicrobial Activity: The dried fruit extract/fractions (MeOH, CH_2Cl_2, EtOAc, n-BuOH and aqueous) of *T. belerica* fruits was dissolved in 10% DMSO to get a concentration of 500 µg/ml and sterilized by filtration by 0.45 µm Millipore filters. Standard antibacterial agents Amoxicillin (10µg/disc) and Kanamycin (30 µg/disc) were prepared. Antimicrobial tests were then carried out by agar diffusion method [21] and a method modified by Olurinola [22] using 100 µl of suspension containing 108 CFU/ml of bacteria spread on nutrient agar (NA). Bacteria were cultured overnight at 37°C for 72 hour used as inoculums. Nutrient agar (20 ml) was dispensed into sterile universal bottles. These were then inoculated, mixed gently and poured into sterile petri dishes. After setting

a number 3-cup borer (6 mm) diameter was properly sterilized by flaming and used to make three to five uniform cups/wells in each Petri dish. A drop of molten nutrient agar was used to seal the base of each cup. The cups/wells were filled with 50µl of the extract concentration of 500 µg/ml and allow for diffuse (45 minutes). The plates were incubated at 37°c for 24 hours for bacteria. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicate. The extract/fractions that showed antimicrobial activity were later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial sample using suitable method.

Determination of Minimum Inhibitory Concentration (MIC): MIC values were also determined for microorganisms, which were determined as sensitive to the extract in disc diffusion assay. In order to determine the MIC values, extract or fractions were dissolved in 10% DMSO to make a concentration of 50 mg/ml. The extract or fractions were diluted in a simple dilution manner to make concentrations in the range of 10, 5, 2.5, 1.25, 0.625, 0.312 mg/ml. 0.1 ml of the extract or fractions were then added to each hole. The MIC was taken as the lowest concentration of extracts or fractions that caused a clear to semi clear inhibition zone around the hole. All the tests were repeated in triplicates.

Brine Shrimp Lethality Bioassay: The toxic potentiality of the different fractions of the plant extracts was evaluated using Brine Shrimp lethality bioassay method [23], where 6 graded doses (viz,. 5µg/ml, 10µg/ml, 20µg/ml, 50µg/ml, 100µg/ml, 200µg/ml) were used. Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine solution with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment. DMSO was used as solvent and also as a negative control. The median lethal concentration LC_{50} of the test sample after 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. Vincristine sulfate was used as standard.

Statistical Analysis: All values were expressed as the mean±standard error of three replicate experiments. The analysis was performed by using student's t test. $p<0.001$ and <0.005 were considered to be statistically significant.

RESULTS

In vitro Antioxidant Activity

Total Phenolic and Flavonoid Contents: The content of total phenols in the extract and fractions of *T. belerica.* was determined using the Folin–Ciocalteu assay, calculated from regression equation of calibration curve ($y = 0.013x + 0.127$, $r^2 = 0.988$) and is expressed as gallic acid equivalents (GAE). The content of the total phenols in the fractions decreased in the order of EtOAc > n-BuOH > MeOH > CH_2Cl_2 > aqueous fractions and the flavonoid contents of the whole plant extract and fractions in terms of quercetin equivalent (the standard curve eqation: $y = 0.009x – 0.036$). The flavonoid content in the fractions decreased in the order of EtOAc > n-BuOH > MeOH > aqueous fractions > CH_2Cl_2. (Table 1).

Total Antioxidant Activity: Percentage yield of methanolic extract and different organic fractions of *T. belerica* fruits and their total antioxidant capacity are given in table 1. Total antioxidant capacity of *T. belerica* fruits is expressed as the number equivalents to ascorbic acid. Total antioxidant capacity of EtOAc fractions showed the highest and was found to be 274.5±0.45 mg/gm equivalent of ascorbic acid, followed by, MeOH, n-BuOH, Aqueous fraction and CH_2Cl_2 145.4±0.21, 148.9±0.69, 30.4±0.12 and 24.0±1.01 mg/gm equivalent of ascorbic acid, respectively.

DPPH Radical Scavenging Activity: All the fractions of *T. belerica* fruits demonstrated H-donor activity. EtOAc fractions showed the highest DPPH scavenging activity with the IC_{50} value of 5.04±0.08 µg/ml, followed by n-BuOH and MeOH extract/fractions with the IC_{50} value of 12.32±0.16 and 25.11±0.22 µg/ml, respectively. CH_2Cl_2 and aqueous fractions had no activity within the experimental concentration range. EtOAc fractions showed the two fold higher activity than the standard ascorbic acid (IC_{50} 10.30±0.11µg/ml) (Table 2).

Peroxynitrite ($ONOO^-$) Scavenging Activity: The $ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123. The MeOH extract and its organic soluble fractions exhibited significant $ONOO^-$ scavenging effects in a dose-dependent manner, with IC_{50} values of 1.29±0.18 µg/ml for EtOAc fraction and exerted activity four-fold stronger than a well known $ONOO^-$ scavenger, penicillamine, with an IC_{50} value of 5.20±0.32 µg/ml.

Table 1: Yield, total amount of plant phenolic compounds, flavonoids and total antioxidant capacity of methanolic extract and soluble organic fraction of *Terminalia belerica*

Sample	Yield (%)	Total phenols mg/g plant extract (in GAE)[a]	Total flavonoids mg/g plant extract (in QA)[b]	Total antioxidant capacity mg/g extract (in ASC)[c]
MeOH	32.66%	260.62±0.12	69.98±0.32	148.9±0.69
CH_2Cl_2	13.33%	43.85±0.19	1.96±0.41	24.0±1.01
EtOAc	4.00%	440.00±0.02	112.73±0.23	274.5±0.45
n-BuOH	7.33%	317.38±0.21	87.24±0.12	145.4±0.21
H_2O	8.00%	22.77±0.15	10.36±0.02	30.4±0.12

[a]Gallic acid equivalents (GAE, mg/g of each extract) for the total phenolic content.

[b]Quercetin equivalents (mg/g of each extract) for the total flavonoid content.

[c] Ascorbic acid equivalents (mg/g of each extract) for the total antioxidant capacity.

The GAE, QA and ASC values are expressed as means±SEM of triplicate experiments.

Table 2: Antioxidant activities of the *Terminalia belerica* fruits extract on DPPH and $ONOO^-$

Sample	[a]DPPH IC_{50}(µg/ml)	[b]$ONOO^-$ IC_{50}(µg/ml)
MeOH	25.11±0.22*	5.16±0.61*
CH_2Cl_2	> 80	> 80
EtOAc	5.04±0.08*	1.29±0.18**
n-BuOH	12.32±0.16#	10.85±0.28**
H_2O	> 80	73.08±2.41*
Ascorbic acid	12.30±0.11	
L-penicillamine		5.20±0.32

[a]DPPH is the free radical scavenging activity (IC_{50}: µg/ml), [b]$ONOO^-$ is the inhibitory activity of authentic peroxynitrite (IC_{50}: µg/ml), *$p < 0.001$ by student's test for values between the sample and the control. **$p < 0.005$ by student's test for values between the sample and the control. # not significant.

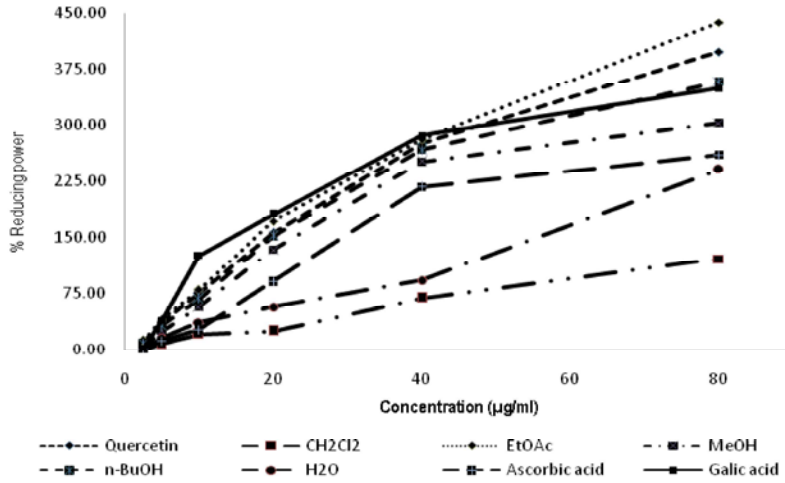

Fig. 1: Reducing power of MeOH extract and fractions of *T. belerica*, quercetin, ascorbic acid and galic acid by spectrophotometric detection of Fe^{3+} to Fe^{2+} transformation. Results are mean±SEM of three parallel measurements.

MeOH extract came in second with respect to IC_{50} values, 5.16±0.61 and possess similar activity with standard. However, n-BuOH and aqueous extract/fractions have moderate activity with IC_{50} values of 10.85±0.28 and 73.08±2.41 µg/ml, respectively (Table 2).

Reducing Power Ability: For the measurement of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of extract and organic fractions. Like the antioxidant activity, the reducing power of *T. belerica* increased with increasing concentration of the sample. Figure 1 shows the reductive capabilities of the *T. belerica* compared with quercetin, ascorbic acid and galic acid. All *T. belerica* extract and fractions concentrations tested showed higher activities and these differences were statistically significant ($p<0.001$).

Table 3: Effect of MeOH extract and its organic soluble fractions of *T. belerica* on LPO, antioxidant enzymes and GSH in CCl_4 induced liver damage in male Wistar rats

Sample	CAT	SOD	GSH	TBARS
Normal Control	91.27±0.19	88.22±0.11	89.55±0.13	95.67±0.17
CCl_4 control	32.12±0.39	42.92±0.21	33.85±0.23	45.17±0.57
M1	32.12±0.25*	38.92±0.21*	35.95±1.03*	51.17±0.77*
M2	38.12±0.11*	47.22±0.22*	42.15±0.34*	58.17±0.37*
C1	ND	ND	ND	ND
C2	ND	ND	ND	ND
E1	42.12±0.29*	48.92±0.21*	45.95±0.03*	61.17±0.57*
E2	67.12±0.11*	73.22±0.22*	68.45±0.53*	87.17±0.17*
B1	35.12±0.19*	42.92±0.22*	40.95±0.13*	54.17±0.17*
B2	53.12±0.51*	60.22±0.12*	57.45±0.13*	71.17±0.77*
A1	ND	ND	ND	ND
A2	ND	ND	ND	ND

Values are Mean±SEM, (n = 5), $^{**}p < 0.001$ by student's test for values between the sample and the CCl_4 control. M = Methanolic extract, C = Dicholoromethane (CH_2Cl_2) fraction, E = Ethylacetate (EtOAc) fraction, B = n-Butanol (BuOH) fraction and A = Aqueous (H_2O) fraction. 1 = 200 mg/kg body weight and 2 = 400 mg/kg body weight. ND = Note done.

Table 4: Antimicrobial activity of MeOH extract and different organic fractions of the *T. belerica* fruits on various bacterial strains by agar diffusion method

	[a]Zone of inhibition in diameter (mm) (n=3)						
Fraction	MeOH	CH_2Cl_2	EtOAc	n-BuOH	Aqueous	Kanamycin[b]	Amoxicilline[b]
Gram Positive							
Bacillus cereus	10.12±0.75	NA	12.77±2.05	8.80±0.15	5.80±0.15	30.16±0.95	22.89±0.65
Bacillus subtilis	10.24±0.25	NA	11.46±1.15	9.8±0.85	NA	23.19±0.45	21.59±0.25
Staphylococcus aureus	24.14±1.67	NA	30.00±0.25	12.8±1.25	7.80±0.15	26.61±0.55	28.71±0.55
Gram Negative							
Escherichia coli	25.36±0.75	NA	28.67±0.15	19.52±0.25	7.16±1.70	22.43±0.45	11.45±0.75
Pseudomonus aeruginosa	16.19±0.55	NA	27.50±0.65	10.53±0.73	6.15±0.75	25.34±0.25	12.81±0.65
Salmonella paratyphi	8.39±1.75	NA	15.83±0.95	9.83±0.45	NA	25.56±1.05	14.45±0.65
Salmonella typhi	17.41±2.75	NA	20.27±0.75	8.87±2.75	5.20±0.85	25.89±0.75	16.75±0.25
Shigella dysenteriae	9.19±0.95	NA	14.83±1.75	6.51±0.75	NA	25.83±0.95	20.49±1.65

[a] Values of the observed diameter zone of inhibition (mm) excluding cap diameter. Incubation conditions for bacteria - 24 hours at 37°C. Assay was performed in triplicate and results are the mean of three values±Standard Deviation.

[b] Reference standard

NA- Zone of inhibition < 5 mm consider as no activity.

In vivo Antioxidant Activity

Estimation of lipid peroxidation (LPO), Enzymic (CAT, SOD) and non Enzymic (GSH) Antioxidant System: Reduced activities of enzymic (CAT, SOD), non enzymic (GSH) antioxidant system and lipid peroxidation (LPO) level of liver homogenate were summarized in table 3. There was a significant decrease in the percentage inhibition of CAT, SOD and GSH in CCl_4 treated rats than the normal control group. However, the percentage inhibition of SOD, CAT and GSH were significantly increased after oral administration of extract/fractions at 200 and 400 mg/kg body weight in a dose dependent manner. EtOAc fraction at a dose of 400 mg/kg body weight showed the highest percentage inhibition activity in both enzymic (67.12±0.11% for CAT and 73.22±0.22% for SOD) and non enzymic (68.45±0.53% for GSH) antioxidant system. Since aqueous and CH_2Cl_2 and aqueous fractions showed lower activity in *in vitro* model, test for *in vivo* model were not done.

In vivo lipid peroxidation study of rats treated with CCl_4 showed a significant increase ($p < 0.001$) in TBARS when compared with normal control group. Treatment with *T. belerica* fruits extract/fractions for 7 days were able to lower the rise in TBARS level dose dependently as shown in table 3. EtOAc showed the highest lowering effect in TBARS level (87.17±0.17) at dose of 400 mg/kg body weight.

Antibacterial Effect: Tablet 4 expressed the antibacterial activity (zone of inhibitions) of extract and fractions of the *T. belerica* fruits. MeOH, EtOAc and n-BuOH extract/fractions have inhibitory function for all the test bacteria. EtOAc fractions showed high activity against *Staphylococcus aureus, Escherichia coli and*

Table 5: Minimum Inhibitory Concentration of MeOH extract and different organic fractions of the *T. belerica* fruits on various bacterial strains by agar diffusion method

	Minimum Inhibitory Concentration (MIC) (mg/ml)				
Sample	MeOH	CH_2Cl_2	EtOAc	n-BuOH	Aqueous
Gram Positive					
Bacillus cereus	2.5	ND	2.5	10	ND
Bacillus subtilis	5	ND	1.25	2.5	ND
Staphylococcus aureus	0.625	ND	0.312	2.5	ND
Gram negative					
Escherichia coli	0.625	ND	0.312	5	ND
Pseudomonus aeruginosa	2.5	ND	0.625	2.5	ND
Salmonella paratyphi	5	ND	5	10	ND
Salmonella typhi	2.5	ND	1.25	10	ND
Shigella dysenteriae	5	ND	5	>10	ND

ND: Not done due to least zone of inhibition or no zone of inhibition

Table 6: LC_{50} data of test samples of *T. belerica* and Vincristine sulphate

Sample	LC_{50} (µg/ml)
Mean±SE[a]	
MeOH	95.32±1.63
CH_2Cl_2	39.02±1.16
EtOAc	64.92±1.56
n-BuOH	64.65±3.13
H_2O	> 200
Vincristine sulphate	1.225±0.11

aValues of toxicity (LC_{50}) were expressed as the mean±standard error of three experiments.

Pseudomonus aeruginosa (zone of inhibition 30.00±0.25, 28.67±0.15 and 27.50±0.65 mm respectively). MeOH extract was found inhibitory against all the tested bacteria (zone of inhibition range 8.39±1.75 to 24.14±1.67 mm). n-BuOH fraction showed moderate activity against all the tested microorganism (zone of inhibition range 6.51±0.75 to 19.52±0.25 mm). CH_2CH_2 fraction was found completely inactive against all the test organisms.

Minimum inhibitory concentration (MIC) values of crude extract and various fractions of the *T. belerica* fruit against susceptible bacteria were represented in table 5. *Staphylococcus aureus* and *Escherichia coli* the most sensitive bacteria showed the variable MIC ranges of (0.312-2.5 mg/ml) and (0.625-5.0 mg/ml), respectively. All the extract/fractions showed moderated activity against *Pseudomonus aeruginosa.*

Toxicity Assay: As summarized in table 6, the toxicity exhibited by the crude MeOH extract as well as the organic soluble fractions of the *T. belerica* fruits showed potent activity against the positive control (vincristine sulphate). The toxicity of the MeOH extract and its fractions on the BSLA increased in the order of CH_2Cl_2 > MeOH > EtOAc > n-BuOH > H_2O and LC_{50} values were 39.02±1.16, 55.32±1.63, 74.92±1.56, 84.32±1.13 and > 200 µg/ml, respectively.

DISCUSSION

Phenolic constituents are very important in plants because of their scavenging ability due to the presence of hydroxyl groups [24]. A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical scavengers [25]. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the compounds having antioxidant property and the formation of a green phosphate/ Mo(V) complex with a maximal absorption at 695 nm. This assay is successfully used to quantify vitamin E in seeds. This method is simple and independent on other antioxidant measurements and is commonly employed for plant extracts [13].

The stable DPPH radical model is widely used and was found relatively quick method for the evaluation of free radical scavenging activity. DPPH• is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [26]. Based on the data obtained from this study, DPPH radical scavenging activity of EtOAc fractions of *T. belerica* fruits was significantly lower than standard. It was revealed that organic soluble fraction of *T. belerica* fruits did show the proton donating ability and could serve as free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in human body.

Formation of reactive peroxynitrite ($ONOO^-$) form the combination of NO• and $O^{\bullet-}_2$ leads to serious toxic reactions with biomolecules such as protein, lipids and nucleic acids [27]. High concentration of nitric oxide (NO) has deleterious effects, so it is necessary to regulate the production of NO strictly [28]. When NO is produced by macrophages, the nitric oxide radical can be converted into peroxynitrites, which will cause diverse chemical reactions in a biological system including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport and oxidation of biological thiol compound [29].

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [30]. The reducing properties are generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [31]. Our data on the reducing power of the tested extracts suggested that it is likely to contribute significantly towards the observed antioxidant effect.

Ccl_4 Is one of the most commonly used hepatotoxin in the experimental study of liver damage [32]. The hepatotoxic effects of this chemical is mostly based on membrane lipid peroxidation. Consequently, trichloromethyl radical leads to severe cell damage [33]. In the present study, a single dose of CCl_4 developed significant hepatic damage and oxidative stress, leads to increase lipid peroxidantion. The treatment with different fractions of *T. belerica* fruits was able to reduce the level of lipid peroxides in a dose dependent manner as compared with the hepatotoxic group.

Superoxide dismutase has been reported as one of the most important enzymes in the enzymatic antioxidant defense system [33]. It removes superoxide anion by converting it to hydrogen peroxide and prevents the toxic effect caused by this radical. CCl_4 Induced hepatic damage lead to decrease in percentage inhibition of SOD and after administration of plant extract/fractions increased the percent inhibition of SOD, revealed the efficient protective mechanism of this plant.

Catalase, another antioxidant enzyme, is widely distributed in the animal tissues and decomposes H_2O_2 and protects the cells from highly reactive hydroxyl radicals [34]. Yeh and Yen [35], reported that four different phenolic acids induced antioxidant enzymes SOD, catalase and glutathione peroxidase. Thus increased the percentage inhibition of catalase after administration of extract/fractions probably due to the presence of the phenolic compounds in the extract/fractions.

Reduced Glutathione (GSH) is a tripeptide, non enzymatic biological antioxidant present in the liver. It protects cellular proteins against reactive oxygen species generated from exposure to CCl_4. [36]. The ability of plant extracts to reactivate the hepatic glutathione reductase was reflected by decreasing the level of lipid peroxidation. This result agrees with the earlier report of Bhandarkar and Khan [37].

Phytoconstituents such as saponins, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial and fungal infections. The results of our study are in agreement with the study of Elizabeth KM [38] who reported that the crude and methanolic extracts of fruits of *T. belerica* fruits were effective against several human pathogenic bacteria with zone sizes ranging from 15.5-28.0 mm and 14.0-30.0 mm, respectively. The extract/fractions were found most inhibitory against *Staphylococcus aureus*. Higher susceptibilities of Gram positive bacteria may be attributed probably due to the differences in chemical composition and structure of cell wall of both types of microorganisms.

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the crude extracts to assess the toxicity towards brine shrimp, which could also provide an indication of possible toxicity of the test materials. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay [23]. The variation in BSLA results (Table 4) may be due to the difference in the amount and kind of toxic substances (e.g. tannins, flavonoids, triterpenoids, or coumarins) present in the crude extracts. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 100 ppm or µg/ml) to brine shrimp is indicative of the presence of potent toxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which crude plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds.

In conclusion, the results of the present study indicate that the MeOH extract and its various fractions exhibit interesting antioxidant properties via various *in vitro* and *in vivo* model and also show potent antibacterial activity with moderate toxicity. These results of the investigation do not reveal that which chemical compound is responsible for aforementioned activity. To explore the lead compounds liable for aforementioned activity from this plant are in progress.

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