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Antioxidant, Cytotoxic and Analgesic activities of the methanolic fruit extract of *Terminalia belerica* Roxb.

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Abstract *Terminalia belerica* Roxb. has a long ethnomedicinal tradition of being used as a remedial agent in various diseases. The present study aimed to investigate antioxidant and cytotoxic activity of the methanolic extract of *T. belerica* fruits. Antioxidant potential of the extract was evaluated by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, nitric oxide scavenging assay, reducing power and total antioxidant capacity and the hydrogen peroxide (H_2O_2) scavenging assay. The extract showed significant activities in all antioxidant assays compared to ascorbic acid in a dose dependent mode. In the DPPH, the NO and the H_2O_2 scavenging assays, the extract of *T. belerica* displayed significant antioxidant activities with the IC_{50} values of 179.16, 96.553 and 134.29 µg/mL, respectively. In addition to strong reducing power, total antioxidant activity of the extract was also found to increase in a dose dependent manner. The extract also showed moderate cytotoxic activity in brine shrimp lethality bioassay and the LC_{50} value was found to be 7.612 µg/mL. The analgesic activity was evaluated using acetic acid-induced writhing test in mice. The extract, at a dose of 500 mg/kg, showed a maximum of 19.71 % inhibition ($p < 0.05$) of writhing reaction compared to the reference drug diclofenac-sodium (66.96 %).

Keywords *Terminalia belerica*, antioxidant, DPPH, cytotoxicity and analgesic

Introduction

Terminalia belerica belongs to the Family 'Combretaceae' known as Belleric myrobalan (Bengali name-'Bohera') has been used for thousands of years in traditional medicine for the treatment of several diseases such as hepatitis, dysenteric diarrhea, eye disease, cough and breathing problems. Earlier phytochemical works on 'bohera' fruit revealed the presence of gallo- and ellagitannic acids phyllemblin, mannitol, sugars, hydrocarbons, tetratriacontane, ditriacotanol, resin, tritriacontanone [1]. Fruit extract of *T. belerica* produced fall in blood pressure of rats at a concentration of 70mg/kg body weight [2]. Literature reviews pointed out that no studies combining the antioxidant and cytotoxic activities of the fruit have so far been undertaken. Coupled with our continuous interest of pharmacological screening of Bangladeshi medicinal plants, in this study we aimed to investigate antioxidant and cytotoxic activities of the methalonic extract of *T. belerica*.

Materials and Methods

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), ammonium molybdate, hydrogen peroxide (H_2O_2), sodium nitroprusside, Methanol, DMSO (dimethyl sulfooxide) and Ammonium molybdate was purchased from Merck, Germany and ethylene diamine tetra acetic acid (EDTA), sodium phosphate, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride from BDH, England. Ferric

chloride was obtained from Thomas Baker and Potassium ferricyanide was from Guandong Chemical Reagent, China. Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. Diclofenac-Na was collected from Square Pharmaceuticals Ltd., Bangladesh. All other reagents were of analytical grade.

Plant Material

The plant *Terminalia belerica* was collected from Dhaka in the month of May 2007. A voucher specimen for this collection has been maintained in Bangladesh National Herbarium, Dhaka, Bangladesh.

Extraction

The powdered plant sample (500 g) was soaked in 1.5 L of methanol for 16 days and then filtered through a cotton plug followed by Whattman filter paper number 1. The extract was concentrated with a rotary evaporator and it afforded 15 g of the methanol extract.

Animal

For the experiment Swiss albino mice of either sex, 3-4 weeks of age, weighing between 20-25 g, 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 ºC), relative humidity: 55-65 % and 12 hrs light / 12 hrs dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to experimentation [3]. All protocols for animal experiment were approved by the institutional animal research ethical committee.

Phytochemical Screening

The freshly prepared crude extract was qualitatively tested for the presence of various phytochemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorff's reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann- Burchard reagent. Gum was tested using Molisch reagent and concentrated sulfuric acid; reducing sugars with Benedict's reagent. These were identified by characteristic color changes following standard procedures described by Ghani, 2003 [4].

Antioxidant Assays

DPPH Radical Scavenging Activity

Qualitative Analysis

Qualitative assay was performed by the method of Sadhu *et al.*, [5]. Test samples were developed on a TLC plate (solvent system, $CHCl_3$: MeOH = 4:1) and sprayed with 0.004% w/v DPPH solution in MeOH using an atomizer. The positive activity was detected by the discolored (pale yellow) spots on a reddish purple background.

Quantitative Analysis

A methanolic solution of DPPH solution (0.15%) was mixed with serial dilutions (1 µg to 500 µg) of *T. belerica* extracts and after 30 min, the absorbance was recorded at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a positive control. The inhibition curve was plotted and the IC_{50} values were determined.

Assay of Nitric Oxide Scavenging Activity

The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) was mixed with different concentrations of methanolic extract of *T. belerica* dissolved in methanol and incubated at room temperature for 150 min. The same reaction mixture without the methanol extract but the equivalent amount of methanol served as the control. After the

incubation period, 0.5 ml of Greiss reagent [1 % sulfanilamide, 2 % H_3PO_4 and 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride] was added. The absorbance of the chromophore formed was read at 546 nm [6] using a Hach, DR-4000U spectrophotometer.

Determination of Total Antioxidant Capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al. (1999) [7]. 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. 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM 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 (Hach, DR-4000U) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Reducing Power

The reducing power of *Terminalia belerica* was determined according to the method previously described by Oyaizu, 1986 [8]. Different concentrations of *Terminalia belerica* extract (25–500 μg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1 %). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference antioxidant.

Scavenging of Hydrogen Peroxide

The ability of the extracts to scavenge hydrogen peroxide (H_2O_2) was determined by the method based on that of Ruch *et al*. [9] Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Positive control ascorbic acid and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of standard or extract solutions (3.4 mL) were added to 0.6 mL of H_2O_2 solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated as follows [10]:

$$\% H_2O_2 \text{ Scavenging} = 100 \times (\text{Absorbance of Control} - \text{Absorbance of Sample})/\text{Absorbance of Control}$$

Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay was used for probable cytotoxic action [11-13]. The eggs of Brine Shrimp (*Artemia salina* Leach) was collected from local pet shops and hatched in a tank at a temperature around 37 ◦C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution the sample was prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). With the help of a pasteur pipette living nauplii were exposed to different concentrations of the extracts.

Preparation of Test Groups

20 mg of sample was dissolved in 2 ml of DMSO to obtain a solution having concentration of 10 μg/ml. From that test solution different volumes were added to premarked glass vials or test tubes containing 5 ml of seawater and 10 shrimp nauplii, so as to make the final concentration of samples in the vials or test tubes 200 μg/ml, 100 μg/ml, 90 μg/ml, 80 μg/ml, 70 μg/ml, 60 μg/ml, 50 μg/ml, 40 μg/ml, 30μg/ml, 20 μg/ml and 10 μg/ml.

Counting of Nauplii

After 24 hrs, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

Analgesic Screening

Acetic Acid-Induced Writhing Test

The analgesic activity of the samples was also studied using acetic acid-induced writhing model in mice. Test samples and vehicle were administered orally 30 min before intraperitoneal administration of 0.7 % acetic acid but Diclofenac-Na was administered intraperitonially 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as 'writhing' for the next 10 min [14].

Statistical Analysis

The results are expressed as MEAN ± SEM. The statistical analysis of the results was shown using one way analysis of variance (ANOVA) followed by Dunnett's test as appropriate using SPSS 11.5 software. Differences between groups were considered significant at a level of $p < 0.05$.

Results and Discussion

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases [15-18]. Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute to shift the balance toward an adequate antioxidant status. Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years [19]. In this study, antioxidant potential of the methanol extract of the fruits of *T. belerica* was evaluated by using the reducing power and total antioxidant capacity, DPPH, the NO and the H_2O_2 scavenging assays,.

The DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants [20]. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The TLC-based qualitative DPPH spray revealed the presence of significant antioxidant activity in the alcoholic extract of *T. belerica* indicated by the presence of a yellowish spot on the reddish purple back ground of the TLC plate. In the quantitative assay the extract exhibited a notable dose dependent inhibition of the DPPH activity, with a 50% inhibition (IC_{50}) at a concentration of 179.16 µg/mL while the IC_{50} value of the positive control, ascorbic acid, was found 55.91 µg/mL (Figure 1).

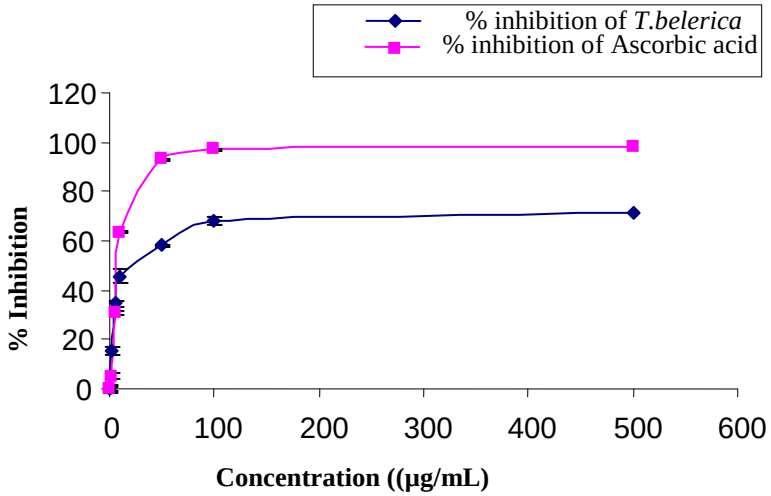

Figure 1: The DPPH scavenging activity of T. belerica (values are the average of triplicate experiments and represented as mean± standard deviation)

The result of NO scavenging activity of the *T. belerica* extract is shown in Figure 2. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_{3-} and NO_2 are very reactive. These compounds are responsible for altering the structural and functional behavior of many cellular components. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25 °C for 2 hrs resulted in linear time-dependent nitrite production, which is reduced by the extract of *T. belerica*. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. The IC_{50} value of the extract is 96.553 µg/mL while ascorbic acid showed IC_{50} value of 47.684 µg/mL. Preliminary phytochemical screening of the extract showed the presence of flavonoids and tannins (Table 1)**.** Flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [20-21].

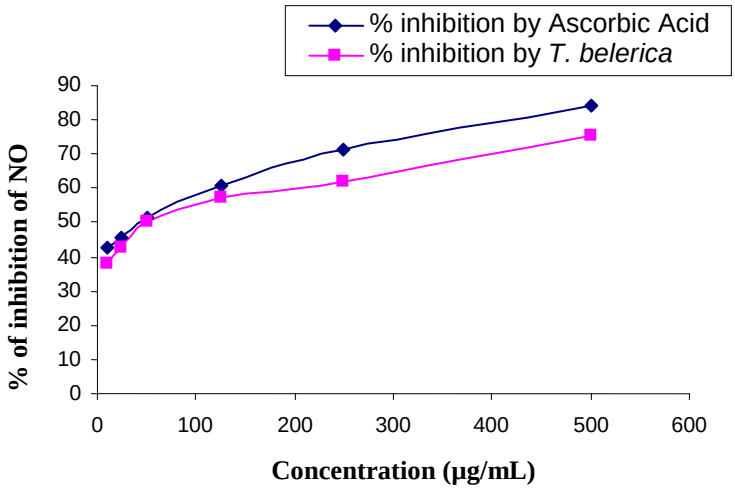

Figure 2: Nitric oxide scavenging activity of T. belerica vs. Ascorbic acid

Table 1: Result of chemical group tests of the methanol extract of *T. belerica*

Extract	Carbohydrate	Tannin	Flavonoid	Saponin	Gum	Steroid	Alkaloid
Methanolic extract of *T. T. belerica*	+	+	+	-	+	-	+

(+) =Presence; (–) =Absence

Total antioxidant capacity of the extract, expressed as the number of gram equivalents of ascorbic acid, is shown in Figure 3. The extract showed good total antioxidant activity, which was also found to increase in a dose dependent manner. Figure 4 represents the reductive capabilities of the plant extract compared to ascorbic acid.

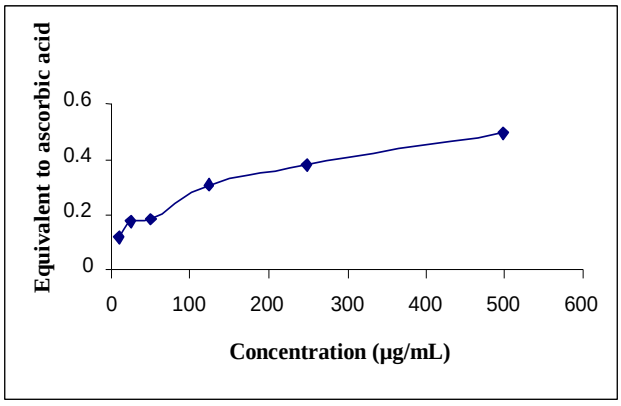

Figure 3: Total antioxidant capacity of T. belerica vs. Ascorbic acid

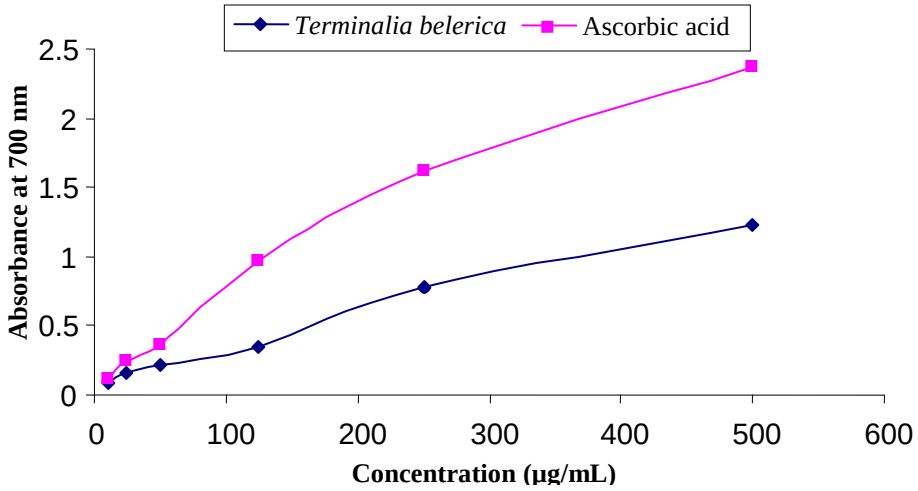

Figure 4: Reducing power of T. belerica vs. Ascorbic acid

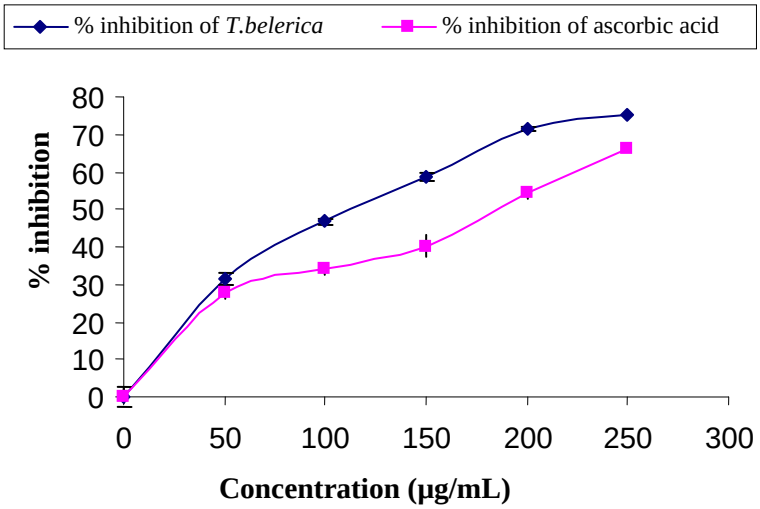

Figure 5: H_2O_2 scavenging activity of T. belerica (values are the average of triplicate experiments and represented as mean± standard deviation)

The reducing power of extract of *T. belerica* was found remarkable and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased. Earlier authors [22] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones [23], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [24].

The ability of the methanol extract of *T. belerica* to scavenge hydrogen peroxide is determined according to the method of Ruch et al. (1989) [9]. The scavenging ability of various extracts with hydrogen peroxide is shown in Figure 5 and compared with the standard ascorbic acid. It is noticed that the extract is capable of scavenging hydrogen peroxide in an amount-dependent manner. The percentage of scavenging hydrogen peroxide is determined with 50 mg/ml of the methanol extracts of the *T. belerica* is 29.13 %. Scavenging activities on hydrogen peroxide for the standards ascorbic acid at the same dose are 35.32 %, respectively. The results reveal that the extract has the hydrogen peroxide scavenging effect. Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells [25]. Therefore, removing of H_2O_2 is very important for antioxidant defense in cell or food systems. Dietary polyphenols have also been shown to protect mammalian and

bacterial cells from cytotoxicity induced by hydrogen peroxide, especially compounds with the [26]. Therefore, the phenolic compounds of the Ichnocarpus extract may probably be involved in removing the H_2O_2.

The cytotoxic results of the *T. belerica* fruit extract, as determined by brine shrimp lethality bioassay is given in Table 2. The brine shrimp assay is an excellent assay to investigate bioactivity of plant extracts. *T. belerica* fruit extract showed moderate cytotoxic activity with LC_{50} of 7.612 µg/mL. Moderate cytotoxic effects of crude extracts indicate that it can be selected for further cell line assay; because many scientists have shown a direct correlation between cytotoxicity and activity against the brine shrimp nauplii using plant extracts [27].

Table 2: Brine shrimp lethality bioassay of methalnolic extract of *T. belerica*

Sample	LC_{50} (µg/ml)	Regression Equation	R^2
Vincristine Sulfate (Positive control)	0.35	y = 32.104x + 64.66	0.9809
T. belerica extract	7.612	y = 19.126x + 33.141	0.973

Table 3: Effect of *T. belerica* extract on acetic acid induced writhing in mice

Group	Treatment & Dose	No of writhing[†]	% of protection
Group I	0.7 % acetic acid (10 mL/Kg)	34.5 ± 0.866[*]	0.00
Group II	Diclofenac sodium (25 mg/kg)	11.4 ± 0.430 [*]	66.96
Group III	Extract of *T. belerica* (250 mg/kg)[‡]	31.3 ± 1.067 [*]	9.28
Group IV	Extract of *T. belerica* (500 mg/kg)[‡]	27.7 ± 0.768 [*]	19.71

Each value is presented as the mean ± SEM (n=5). *p < 0.05 compared with the control group (Dunnett's test)

Aforementioned antioxidant results of the *T. belerica* fruit extract promoted us to investigate the analgesic potential of the plant using acetic acid induced writhing test in animal models. The results of acetic acid induced writhing test are given in Table 3. The extract significantly and dose dependently inhibited the acetic acid induced writhing in mice (19.71%, $p < 0.05$ and, 9.28 %, $p < 0.05$ for 500 and 250 mg/kg body weight respectively). Acetic acid-induced writhing model represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipids [28]. The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics The response is thought to be mediated by peritoneal mast cells [29], acid sensing ion channels [30] and the prostaglandin pathways [31].

Conclusion

In conclusion, it can be said that the antioxidant, analgesic and cytotoxic activities shown by the *T. belerica* fruit extract lend credence in favor of the various uses of *T. belerica* in folk medicine. However, extensive pharmacological studies in molecular level are required to understand underlying mechanism of these actions and eventually to isolate active compounds responsible for such activities in *T. belerica* fruit extract.

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Conflict of Interest

The authors declare that there is no conflict of interest to reveal.

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