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## Antioxidant, cytotoxic and analgesic activities of the methanolic fruit extract of *Terminalia chebula* Retz.

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### ABSTRACT

The present study was aimed to investigate antioxidant, analgesic and cytotoxic activity of the methanolic extract of *Terminalia chebula* Retz. fruits. Antioxidant potential of the extract was evaluated by using nitric oxide scavenging assay, reducing power and total antioxidant capacity. The extract showed significant activities in all antioxidant assays compared to ascorbic acid in a dose dependent mode. In nitric oxide scavenging assay, the IC<sub>50</sub> value of the extract was found to be 51.3 µg/mL while the IC<sub>50</sub> value of ascorbic acid was 77.4 µg/mL. In addition to strong reducing power, total antioxidant activity of the extract was also found to increase in a dose dependent manner. 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 44.17 % inhibition ( $p < 0.05$ ) of writhing reaction compared to the reference drug diclofenac-sodium (66.96 %). The extract also showed moderate cytotoxic activity in brine shrimp lethality bioassay and the LC<sub>50</sub> value was found to be 97.36 µg/mL.

**Key Words:** *Terminalia chebula* Retz., antioxidant, analgesic, cytotoxicity.

### INTRODUCTION

*Terminalia Chebula* has been extensively used in ayurveda, unani & homoeopathic medicine and has become cynosure of modern medicine. The Sanskrit name 'Haritaki' is rich with meaning, referring to the yellowish dye (harita) that contains the god Siva (Hari, i.e. the Himalayas and that it cures (harayet) all the diseases (Das, 1991). Its other commonly used Sanskrit name, Abhaya, refers to the 'fearlessness' it provides in the face of the disease. According to Indian mythology, this plant originated from the drops of ambrosia (Amrita) which fell on the earth when Indra was drinking it (Srikanthmurthy et al., 2000). *T. Chebula* possesses a wide variety of activities like antimicrobial (Sato et al., 1997), antioxidant (Cheng et al., 2003), antiviral (Jeong et al., 2002), anticarcinogenic (Hushum et al., 2002), hypocholesterolemic (Thakur et al., 1988), radioprotective (Gandhi et al., 2005), antispasmodic & antipurgative (Miglani et al., 1971).

Literature reviews pointed out that no studies combining the antioxidant, analgesic 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, analgesic and cytotoxic activities of the methanolic extract of *T. chebula*.

### MATERIALS AND METHODS

#### Chemicals and drugs

Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. Ferricchloride was obtained from Sigma Chemical Co and Potassium ferricyanide was from May and Backer, Dagenham, UK. Methanol, DMSO (dimethyl sulfoxide) and Ammonium molybdate was purchased

from Merck, Germany. Diclofenac-Na was collected from Square Pharmaceuticals Ltd., Bangladesh.

#### Plant material

The plant *Terminalia chebula* Retz. was collected from Dhaka in the month of May 2013. 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 Whatman 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 20-25gm, 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:  $24.0 \pm 1.0^\circ\text{C}$ ), 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 (Chatterjee, 1993). 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

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and concentrated sulfuric acid; reducing sugars with Benedict's reagent. These were identified by characteristic color changes following standard procedures described by Ghani (2005).

#### **Antioxidant Assays**

##### *Assay of nitric oxide scavenging activity*

The procedure was 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 (10mM) in phosphate buffered saline (PBS) was mixed with different concentrations of methanolic extract of *T. chebula* dissolved in methanol and incubated at room temperature for 150 minutes. 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<sub>3</sub>PO<sub>4</sub> and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride] was added. The absorbance of the chromophore formed was read at 546 nm (Sreejayan and Rao, 1997) 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). 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 3ml 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 minutes. 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 chebula* was determined according to the method previously described by Oyaizu, 1986. Different concentrations of *Terminalia chebula* 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<sub>3</sub>Fe(CN)<sub>6</sub>] (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<sub>3</sub> (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.

##### *Brine Shrimp Lethality Bioassay*

Brine shrimp lethality bioassay was used for probable cytotoxic action (McLaughlin, 1998; Meyer *et al.*, 1982; Persoone, 1980). 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 hours, 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<sub>50</sub>) 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 intraperitoneally 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 (Ahmed *et al.*, 2004).

##### *Statistical Analysis*

Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the vehicle control group. *p* values < 0.05 were considered to be statistically significant when compared to the control.

## **RESULTS AND DISCUSSION**

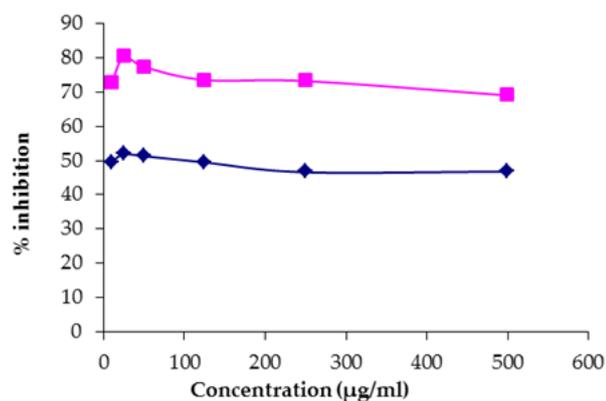
Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases (Halliwell and Gutteridge 1999; Finkel and Holbrook, 2000). Antioxidants act as a major 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 (Jayaprakash and Rao, 2000). In this study, antioxidant potential of the methanol extract of the fruits of *Terminalia chebula* Retz. was evaluated by using nitric oxide scavenging assay, reducing power and total antioxidant capacity.

The result of NO scavenging activity of the *Terminalia chebula* Retz. extract is shown in figure 1. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO<sub>2</sub>, N<sub>2</sub>O<sub>4</sub>, N<sub>3</sub>O<sub>4</sub>, NO<sub>3</sub>, and NO<sub>2</sub> are very reactive. These compounds

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

Extract	Carbohydrate	Tannin	Flavonoid	Saponin	Gum	Steroid	Alkaloid
Methanolic extract of <i>P. P. emblica</i>	+	+	+	-	+	-	+

(+) =Presence; (-) =Absence



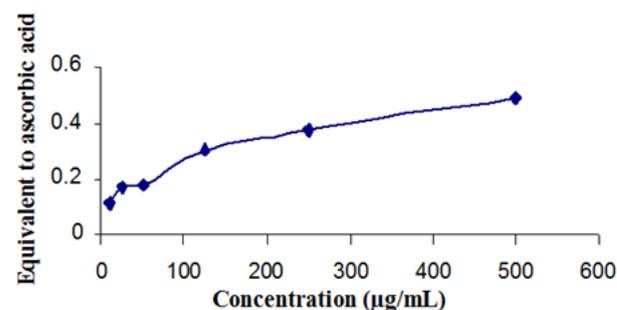
**Figure 1: Nitric oxide scavenging activity of *Terminalia chebula* Retz. vs. ascorbic acid.**

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 hours resulted in linear time-dependent nitrite production, which is reduced by the extract of *T. chebula*. 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<sub>50</sub> value of the extract is 51.3 µg/mL while ascorbic acid showed and IC<sub>50</sub> value of 77.4 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 (Rice-Evans *et al.*, 1997; Jorgensen *et al.*, 1999).

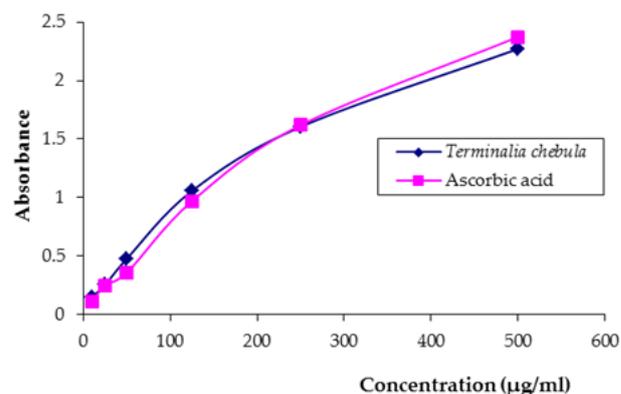
Total antioxidant capacity of the extract, expressed as the number of gram equivalents of ascorbic acid, is shown in figure 2. The extract showed good total antioxidant activity, which was also found to increase in a dose dependent manner. Figure 3 represents the reductive capabilities of the plant extract compared to ascorbic acid. The reducing power of extract of *Terminalia chebula* Retz. was found remarkable and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased. Earlier authors (Tanaka *et al.*, 1988) 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 (Duh *et al.*, 1999), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990).

**Table 2: Brine shrimp lethality bioassay of methanolic extract of *T. chebula*.**

Sample	LC <sub>50</sub> (µg/ml)	Regression Equation	R <sup>2</sup>
Vincristine Sulfate (Positive control)	0.35	y = 32.104x + 64.66	0.9809
<i>T. chebula</i> extract	97.36	y = 46.476x + 42.412	0.9404



**Figure 2: Total antioxidant capacity of *Terminalia chebula* Retz. vs. ascorbic acid.**



**Figure 3: Reducing power of *Terminalia chebula* vs. ascorbic acid.**

The cytotoxic results of the *Terminalia chebula* Retz. 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. *Terminalia chebula* Retz. fruit extract showed moderate cytotoxic activity with an LC<sub>50</sub> of 97.36 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 (Martin *et al.*, 1995)

Aforementioned antioxidant results of the *Terminalia chebula* Retz. 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 (44.17%, p < 0.05 and, 15.94%, p < 0.05 for 500 and 250 mg/kg body weight respectively). Acetic acid-induced writhing model

**Table 3: Effect of *Terminalia chebula* Retz. extract on acetic acid induced writhing in mice.**

Group	Treatment and Dose	Writhing <sup>†</sup>	No. of writhing	% of protection
Group I	0.7% acetic acid (10mL/Kg)	34.5 ± 0.866*	100	0.00
Group II	Diclofenac sodium (25mg/kg)	11.4 ± 0.430*	33.04	66.96
Group III	Extract of <i>Terminalia chebula</i> Retz. (250mg/kg) <sup>‡</sup>	29.0 ± 1.00*	84.06	15.94
Group IV	Extract of <i>Terminalia chebula</i> Retz. (500mg/kg) <sup>‡</sup>	19.26 ± 1.016*	55.83	44.17

<sup>‡</sup>Administered 45 min before 0.7% acetic acid administration (10 ml/kg)

<sup>†</sup>Counted for 15 min, starting 5 min after acetic acid administration;

<sup>\*</sup>p<0.05 vs. control, Dunnett's t-test; values are mean ± S.E.M (n = 3)

represents pain sensation by triggering localized inflammatory response. Such pain stimulus leads to the release of free arachidonic acid from tissue phospholipids (Ahmed *et al.*, 2006). 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 (Ronaldo *et al.*, 2000), acid sensing ion channels (Voilley, 2004) and the prostaglandin pathways (Vogel and Vogel, 1997).

## CONCLUSION

In conclusion, it can be said that the antioxidant, analgesic and cytotoxic activities shown by the *Terminalia chebula* Retz. fruit extract lend credence in favor of the various uses of *Terminalia chebula* Retz. 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. chebula* fruit extract.

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