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Comparative Antioxidant Properties of *Terminalia bellirica* (Gaertn.) Roxb. Fruit Extract: A Component of Ayurvedic Formulation 'Triphala'

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ABSTRACT

The medicinal plant *Terminalia bellirica (*Gaertn.*)* Roxb. is of great significance as its various parts have been used in the traditional system of medicine like Ayurveda , Unani and Siddha. The fruit of the plant is one of the components of the rejuvenating ayurvedic formulation-Triphala. The present study was carried out to investigate the phytoconstituents and also to evaluate the *in vitro* antioxidant potential of various extracts of *Terminalia bellirica* fruit. The fruits were extracted with different solvents. The qualitative phytochemical analysis confirmed the presence of a wide array of bioactive constituents such as alkaloids, flavonoids, sterols, glycosides, saponins, phenols and tannins. The extracts were evaluated for antioxidant potential *in-vitro* by total antioxidant assay, ferric reducing antioxidant potential (FRAP) assay and nitric oxide scavenging activity. Most of the polar extracts showed good antioxidant activity. The study revealed that aqueous acetone extract of *Terminalia bellirica* fruit is the most promising extract which exhibited maximum potential in a concentration-dependent manner.

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INTRODUCTION

Plant based traditional formulations constitute a rich and efficient antioxidant system to prevent oxidative stress and its associated pathogenesis [1]. As estimated by the World Health Organization (WHO), people in developing countries mostly rely on traditional medicine for their primary health needs [2]. Plant derived products are in focus than ever before to fight against current scenario of oxidative damage and related ailments. Plants, being the storehouse of a spectrum of antioxidant phytochemicals can be utilized for enhancing the efficient antioxidant system of the living species which fails once oxidative stress crosses certain threshold and leads to mild or chronic ailments [3]. The traditional system of medicines like Ayurveda, Siddha and Unani use herbal formulations to treat human sufferings since thousands of years and they contributed much towards the discovery of plant based therapeutics [4].

'Triphala', an age-old antioxidant rich ayurvedic formulation composed of equal parts of three fruits, *Terminalia chebula* (Retz), *Terminalia bellirica* (Gaertn.) Roxb. and *Phyllanthus emblica* (Linn.) has been prescribed in the traditional systems of medicine as an effective rejuvenator and remedy for various disorders like obesity, anemia, fatigue, constipation, tuberculosis in addition to its use against various infections [5, 6].

Terminalia bellirica (Gaertn.) Roxb. (Combretaceae), known as Bastard Myrobalan is a large deciduous tree found throughout India up to an elevation of 900 meters [7]. Apart from being an integral part in Triphala, the fruits are widely used in various traditional therapeutic formulations in the indigenous system of medicine either alone or in combination with other plant based drugs [7-8]. The fruits of *Terminalia bellirica* is a rich source of phytochemicals which include β-sitosterol, bellericanin, gallotannins, chebulagic acid, ellagitannins, gallic acid, ellagic acid and lignans [9,10]. The acetone and methanolic extracts of *Terminalia bellirica* fruits are reported to exhibit strong antioxidant activity [1, 10].

In spite of its medicinal importance, the fruits of *Terminalia bellirica* has not yet thoroughly been screened for antioxidant activity. The Present study is an attempt to assess antioxidant and free radical scavenging potential of different extracts of Terminalia bellirica fruits (TBF), and also to detect the presence of various bioactive components.

MATERIALS AND METHODS

Plant Material

Authenticated fruits of *Terminalia bellirica* (TBF) were purchased from Kerala Forest Research Institute, Peechi, India. After drying in shade for few days, the fruits were de-seeded, finely powdered and stored in an airtight container until used for extraction.

Preparation of extracts

The fruit powder (100 g) was mixed with 300 ml of each solvent in a conical flask, placed in an orbital shaker and allowed to percolate continuously for 72 hours. Prior to extraction, the fruit powder was defatted with n-hexane. The extracts were filtered through Whatman No.1 filter paper, concentrated to dryness in a rotary evaporator and stored in sterile vials at 4°C until further analysis.

Qualitative Phytochemical analysis

Qualitative phytochemical screening of all the extracts of TBF was carried out to detect the presence of various classes of phytochemicals using standard protocols [11,12].

In-vitro antioxidant screening

Total antioxidant activity

Total antioxidant capacity exhibited by various extracts of *Terminalia bellirica* fruit was evaluated by the method described by Prieto et al. [13] which is based on the reduction of Mo(VI) to Mo(V) by the antioxidant components in the extract and leads to the subsequent formation of green phosphate/Mo(V) complex with an absorption maximum of 695 nm.

The test tubes containing various extracts with a concentration of 100 µg/100 µL were mixed with 1 ml of the reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95ºC for 90 min. The absorbance of the mixtures, after cooling to room temperature, were measured at 695 nm using a spectrophotometer (Hitachi U-500) against methanol blank (0.3 ml). Ascorbic acid was used as the standard and total antioxidant capacities

of the extracts were expressed as the mean (mg of ascorbic acid equivalents per gram dry weight of extracts) of three replications.

Ferric reducing antioxidant potential (FRAP) assay

The ability of the antioxidants to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain [14]. The assay is based on single electron transfer and interprets the ferric reduction capacity of samples. The ferric ion Fe(III), used as an antioxidant probe is reduced to ferrous Fe(II) ion after extracting an electron from antioxidant samples. The ferrous ions, upon chelation with a chromogenic ligand (tripyridyl triazine, TPTZ) give a colored complex which is measured at 593 nm. The measured values in turn proportional to the ferric reducing antioxidant power of the sample [15].

An aliquot (200 µL) of an extract was added to 3 ml of FRAP reagent containing 10 parts of 300mM sodium acetate buffer at pH 3.6, 1 part of 10mM TPTZ solution and 1 part of 20mM $FeCl_3.6H_2O$ solution and the reaction mixture was incubated in a water bath at 37^0C for 30 minutes after which the increase in absorbance was measured at 593 nm. The FRAP results were expressed in µ mole Fe(II) per mg of dry weight of extract and calculated as mean value ± standard deviation (SD) for three replications.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was evaluated by the method previously reported by Sreejayan and Rao [16]. In this assay, Griess reagent is used to estimate the amount of nitrite ions formed through the reaction between nitric oxide generated from sodium nitroprusside and oxygen. Nitric oxide scavengers compete with oxygen and thereby lowering the production of nitrite ions.

The sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations of TBF extracts, incubated at room temperature for 150 minutes and 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The reaction mixture without the extracts served as the control [17].

Statistical analysis

All the experiments were carried out in triplicates and data reported are mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics 20 software by One-way Analysis of Variance (ANOVA), considering significant differences at $P < 0.05$. The IC_{50} values were calculated by regression analysis using Graph Pad Prism 5 (Graph Pad Software CA)

RESULTS

Qualitative phytochemical analysis

The phytochemical screening of the fruit extracts of the traditional medicinal tree, *Terminalia bellirica* revealed the presence of various constituents. The fruits contain a variety of bioactive phytochemicals such as alkaloids, flavonoids, sterols, glycosides, saponins, phenols and tannins. The results are summarized in Table: 1

Table 1: Qualitative phytochemical analysis of *Terminalia bellirica* fruit extracts

Extracts	Alkaloids	Flavonoids	Steroids	Glycosides	Saponins	Phenols	Tannins
TBF-Petroleum ether	_	_	+	_	_	_	_
TBF-Chloroform	_	_	+	+	_	_	_
TBF-Ethyl acetate	+	_	+	_	_	+	+
TBF-Acetone	_	+	+	_	_	+	+
TB-Methanol	+	+	_	+	+	+	+
TBF-Ethanol	_	+	_	_	+	+	+
TBF-Aqueous acetone	_	+	_	+	+	+	+
TBF-Aqueous methanol	_	+	_	+	+	+	+
TBF-Aqueous ethanol	_	+	_	_	+	+	+

(+) or (-) indicates the class of phytochemical absent or present

TBF - Terminalia bellirica fruit

The total antioxidant activity

The total antioxidant capacity of the *Terminalia bellirica* fruit extracts were calculated using the regression equation (Y= 0.134x+0.01) of ascorbic acid calibration curve (Figure:1) and the values are expressed in mg ascorbic acid equivalent/g plant extract and the values are presented in figure: 1 The highest antioxidant capacity was exhibited by the aqueous acetone extract (522.5 ±1.93 mg ascorbic acid equivalents/g extract) followed by aqueous ethanol, aqueous methanol, ethyl acetate, methanol and aqueous extracts with values 309.45 ± 2.85, 295 ±3.01, 283 ±2.21, 242.9 ± 3.65 and 211± 2.46 mg equivalents of ascorbic acid respectively. The petroleum ether, as well as chloroform extracts showed only minimal effect with 19.8 ± 2.87 and 35.35 ± 2.76 mg ascorbic acid equivalents/g extract.

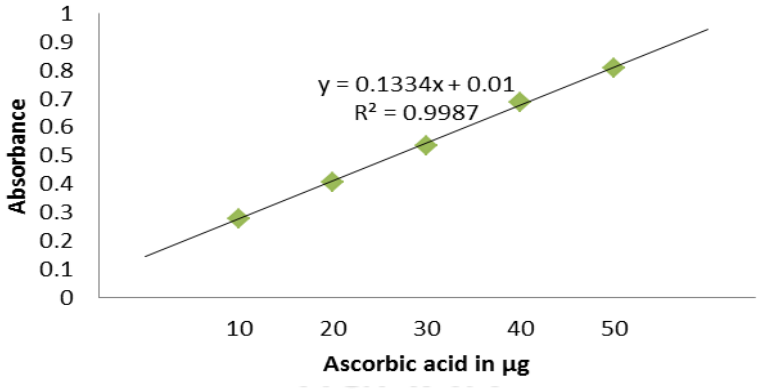

Figure 1: Calibration curve for standard Ascorbic acid

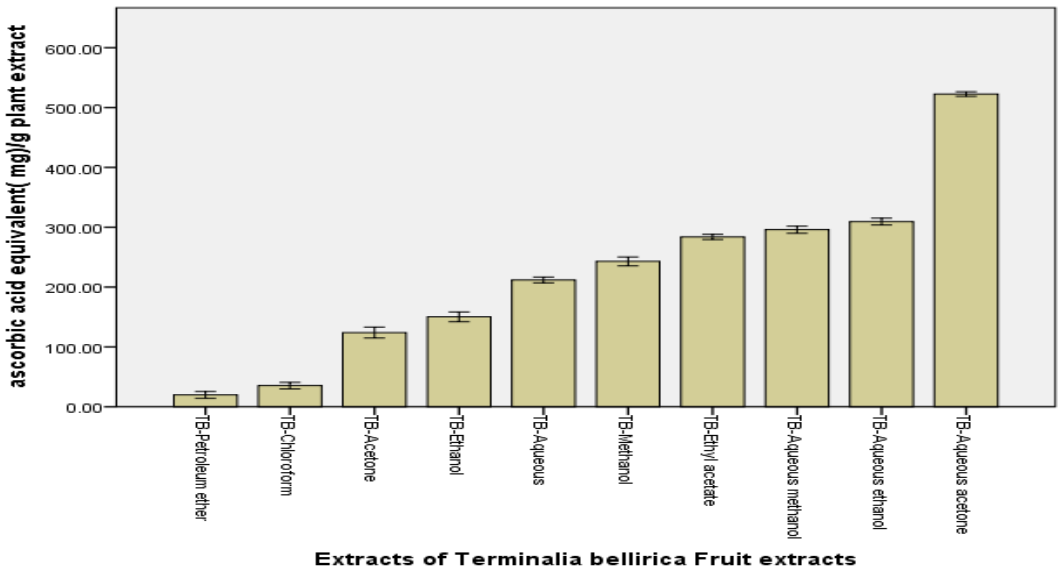

Figure 2: Total antioxidant activity of *Terminalia bellirica* fruit extracts

Ferric reducing antioxidant potential (FRAP) assay

As shown in figure: 3, the reducing power of aqueous acetone extract (14.89 ± 0.26 mol Fe(II)/mg plant extract) was markedly higher than the other extracts, while petroleum ether and chloroform extracts showed no significant reduction potential with values 0.26 ± 0.26 and 1.39 ± 0.005 mole Fe(II)/mg respectively.

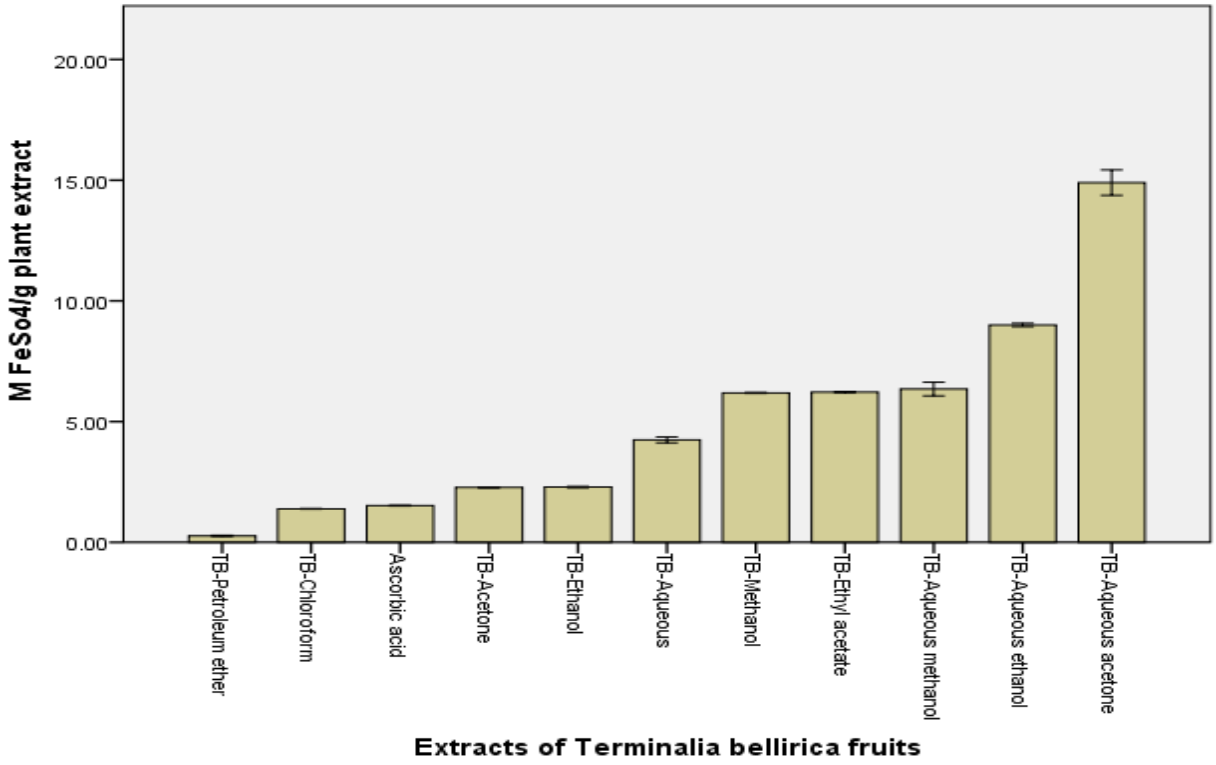

Figure 3: Ferric reducing antioxidant potential of *Terminalia bellirica* fruit extracts

Nitric oxide scavenging activity

It is evident from the results represented in Figure: 4 & 5 that aqueous acetone extract scavenged the NO_2 most efficiently with 79.7% (IC_{50} = 413.05± 13.48 µg/mL), when compared to the 92.8% (IC_{50}= 162.42± 8.98 µg/mL) inhibition by the standard ascorbic acid, while petroleum ether and chloroform extracts showed least activity in this regard with 7.2%(IC_{50}=6052±465.18µg /mL and 14.8%(IC_{50}= 4727± 463.6 µg/mL) of inhibition respectively.

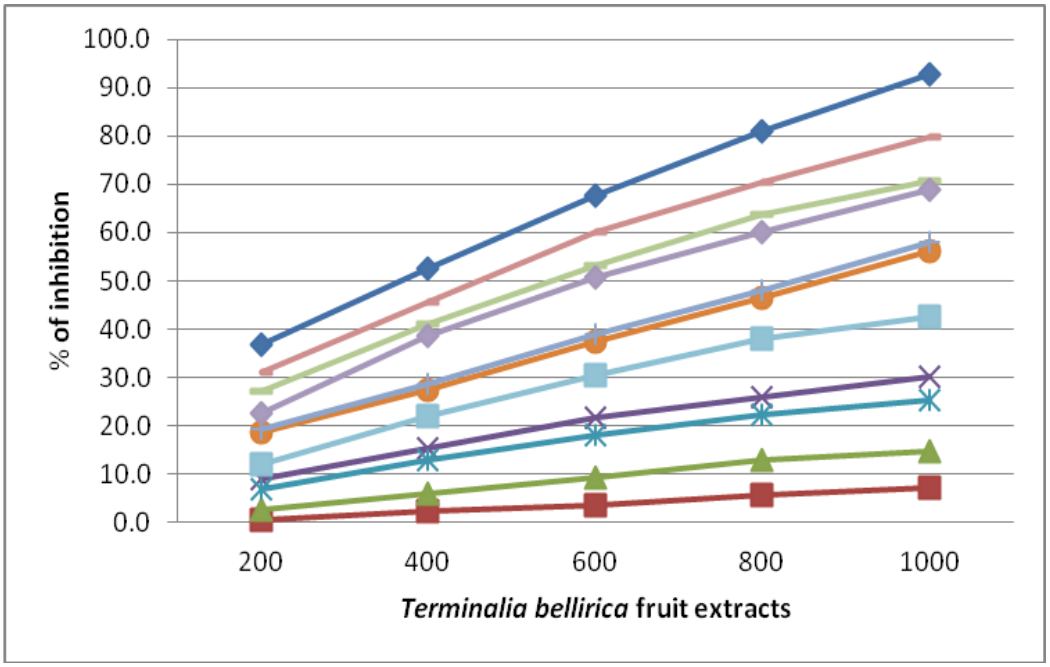

Figure 4: Nitric oxide scavenging activity of *Terminalia bellirica* fruit extracts

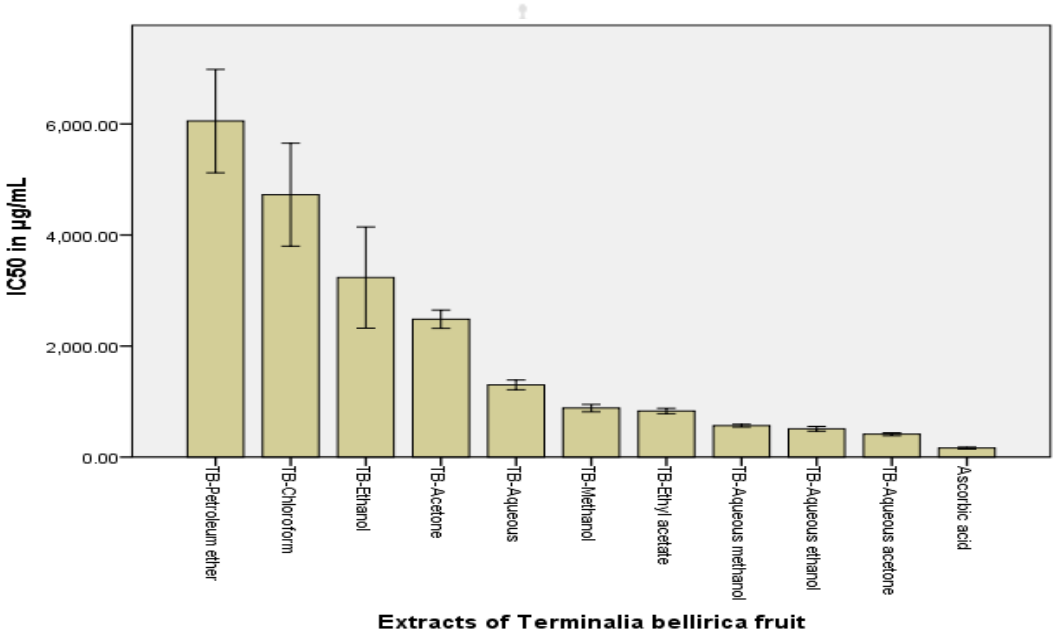

Figure 5: Nitric oxide scavenging activity- IC_{50} of *Terminalia bellirica* fruit extracts

DISCUSSION

Bioactive components such as alkaloids, tannins, carbohydrates, terpenoids, sterols, flavonoids and phenolics isolated from plants exert definite physiological action in human body [18] and play important role in the prevention of many diseases including cancer and other degenerative

disorders. Cellular damage by oxidation of macromolecules like DNA, proteins and membrane lipids is regarded as an important process during early carcinogenesis [19] and also very critical in the aging process as well as many degenerative and proliferative diseases. Gradual accumulation of free radicals can build up oxidative stress and induce cellular damage.

Antioxidant activities of phytochemicals that counteract ROS (reactive oxygen species) or free radicals are therefore highly desirable for potential anticancer compounds [20]. Triphala is extensively used as a rejuvenating agent in Indian system of alternative medicine and considered to possess significant antioxidant activity [21]. Preliminary phytochemical analysis of the extracts of *Terminalia bellirica* fruit revealed the presence of various constituents such as alkaloids, flavonoids, steroids, diterpenes, glycosides, saponins, phenols and tannins. Hence, the fruits of *Terminalia bellirica* are good reservoirs of bioactive phytochemicals that can counteract oxidative damage in cells.

Different extracts such as ethyl acetate, methanol: water, methanolic extract of various parts of *Terminalia bellirica* including aerial parts and fruits have been previously tested for antioxidant activities [3, 22, 23]. Hence the present study was focused on identifying the most antioxidant extract of *Terminalia bellirica* fruit using a wide range of solvent systems. The total antioxidant activity was determined on the basis of the reduction of Mo (VI) to Mo (V) followed by the subsequent formation of green phosphate/Mo (V) complex. The polar extracts showed good antioxidant capacity and among which aqueous acetone was found to be the one with highest antioxidant activity.

Ferric reducing antioxidant potential (FRAP) assay is based on single electron transfer that interprets the ferric reduction capacity of samples which is proportional to the ferric reducing antioxidant power of the samples. The maximum ferric reduction was observed in the aqueous acetone extract while aqueous ethanol also showed comparable reduction. The aqueous methanol, ethyl acetate and methanol extracts showed almost similar and good activity while the non-polar extracts have not shown significant reducing power. Nitric oxide scavengers lower the production of nitrite ions which in turn is a measure of the antioxidant capacity of the samples. The aqueous acetone extract, as similar to other antioxidant tests showed maximum activity.

Terminalia bellirica fruit is one of the major ingredient in many Ayurveda, Unani and Siddha polyherbal formulations which are used in India and United Kingdom [24]. *Terminalia bellirica* fruit was extracted with various solvents as well as with solvent combinations and investigated the antioxidant activity of each and found aqueous acetone as the most antioxidant one. It is the first report that aqueous acetone extract of *Terminalia bellirica* fruit possesses highest antioxidant activity among the other polar solvent systems used in the study.

CONCLUSION

The results of the current study showed that aqueous acetone extract of *Terminalia bellirica* fruit exhibited highest antioxidant potential. The fruits of *Terminalia bellirica* contain a wide range of phytoconstituents which in turn make the traditional medicinal fruit a promising natural antioxidant with good efficacy.

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CONFLICT OF INTEREST

Authors would like to disclose that there is no conflict of interest.

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