

Phytochemical analysis, phenolic content and antioxidant properties of different parts of *Terminalia bellirica* (Gaertn.) Roxb.- A comparative study

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Terminalia bellirica (Gaertn.) Roxb., commonly known as *Baheda*, is one of the three ingredients of the well known Ayurvedic formulation *Triphala* which is very effective in gastrointestinal tract and eye and brain related problems. In the present study the fruits (TBF), leaves (TBL) and bark (TBB) of *T. bellirica* were analyzed and phytochemical analysis for triterpenoids and phenolics was done by HPTLC. The Total phenolic content and Total flavonoid content of the samples were in the order TBL>TBF>TBB with TBL showing the highest TPC and TFC with 15.8 mg GAE/g and 33.3 mg QE/g, respectively. Ellagic acid was the most predominant constituent and was found to be present in all the samples of *T. bellirica*, its percentage being maximum in case of leaves (4.863 %). Their antioxidant potential was also determined. It was observed that the leaves of *T. bellirica*, which are rich in phenolics and flavonoids, also exhibit the highest antioxidant potential as evidenced by the better DPPH radical scavenging ability as well as the total antioxidant capacity.

Keywords: *Terminalia bellirica*, Phenolics, HPTLC, Antioxidant activity.

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Medicinal plants, being an important aspect of various traditional medicine systems, have been used therapeutically all around the world. Although the various systems of traditional medicine, eg. *Ayurveda*, Chinese Traditional Medicine, *Unani*, Tibetan Medicine, Amazonian or African Medicine, may be based on different theoretical and cultural models, they all integrate phytotherapy into their doctrine. According to World Health Organization (WHO) estimates, more than 80 % of the people in developing countries depend on the traditional medicine for their primary health needs. It is generally estimated that over 6000 traditional plants in India are used in folk and herbal medicine, representing about 75 % of the medicinal needs of the 3rd world countries.

Terminalia bellirica (Gaertn.) Roxb. syn. *Myrobalanus bellirica* (Gaertn.) Roxb. commonly known as *Baheda* in Hindi and referred to as Beleric Myrobalan in English, belongs to family Combretaceae. It is a large, deciduous tree, about 10 to 20 m high, buttressed at base. The bark is bluish grey with many fine vertical cracks. The wood is yellowish grey, hard, no heartwood, annual rings

indistinct; pores very scanty, large, frequently subdivided, joined by wavy, concentric bands of soft loose cellular tissue. Leaves alternate, crowded at the end of the branchelets, broadly elliptic or elliptic-ovate, 10 - 20 cm long and 7 - 16 cm broad, rounded or rarely acuminate at apex, narrowed at base, entire along margins, both surfaces glabrous in old, coriaceous. Mature leaves are glabrous and usually punctuate on the upper side. Flowers sessile, in axillary spikes, green to yellow, with an offensive odour; upper part of spike contains male flowers and lower one hermaphrodite; bracts are linear, caducous. Calyx pubescent outside, wooly inside with long brown hairs; teeth rhomboid, acute at apex. Fruits drupe, sub globose, grey, velvety, narrowed into a short stalk, 5-angled or ridged when dried¹. It is found throughout India, Bangladesh, Bhutan, Cambodia, China, Indonesia, Laos, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka, Thailand and Vietnam². The generic name '*Terminalia*' comes from Latin word 'terminus' or 'terminalis' (ending), and refers to the habit of the leaves being crowded or borne on the tips of the shoots³.

T. bellirica possesses an esteemed status in medicinal plants with diverse biological potentials.

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It has been shown to possess a large spectrum of medicinal properties such as analgesic, anticancer, antidepressant, antidiabetic, antidiarrhoeal, anti-ulcer, immunomodulatory, anti-spasmodic and bronchodilatory, antifertility, antihypertensive, antifungal, antimicrobial, anti-inflammatory and antioxidant activities^{4,5}. The bark is a mild diuretic and is useful in anaemia and leucoderma. The fruit is bitter, pungent, acrid, digestible, antipyretic, laxative, astringent, brain tonic, intoxicating, anthelmintic, useful in thirst, vomiting, dyspepsia, bilious headache bronchitis, sore throat, inflammations, strangury, asthma, corneal ulcers and in diseases of the eye, nose, heart and the bladder. Half ripe fruit is considered purgative. The seed kernels are reported to be narcotic. The pulp of the kernels is used in dropsy, piles, diarrhea, leprosy and in fever. The oil is considered a good application for the hair. The gum is believed to be demulcent and purgative¹.

The mature dried fruits are effective in the treatment of diarrhoea, dysentery and parasites. In traditional medical system, the fruits are used for anemia, asthma, cancer, colic, constipation, dysuria, headache, hypertension, inflammations and rheumatism. A decoction of fruits may be taken internally and can be used externally as eye wash in the treatment of ophthalmological disorders. It also promotes eyesight and hair growth. Fresh fruits are diuretic, digestive, expectorant, antipyretic and antiemetic; pulp of fresh fruits is lithotriptic and anthelmintic. *T. bellirica* is known to lower the lipid level in hypercholesterolemic animals. Its well known *Ayurvedic* formulation *Triphala* is very effective in gastrointestinal tract and eye and brain related problems⁶.

In our present study, we have carried out the comparative analysis of different parts of *T. bellirica*, viz. fruits, stem bark and leaves, for physicochemical analysis, phenolic and flavonoid contents, qualitative as well as quantitative HPTLC analysis and antioxidant activity.

Methodology

Chemicals and reagents

Ellagic acid, gallic acid, ferulic acid, β -sitosterol, lupeol, stigmaterol and ursolic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Whatman (Florham Park, NJ) No. 1 filter paper was used for filtration of the samples. Other chemicals and solvents were purchased from Merck Chemicals, Mumbai, India.

Collection of plant material, extraction and sample preparation

Different parts of *T. bellirica*, viz. fruits, stem bark and leaves were collected in the months of April from Lucknow, the region topographically known as Upper-Gangetic plain of India. They were identified, authenticated and deposited (specimen numbers NBR/PH/227356 to NBR/PH/227358) in the departmental herbarium of the Pharmacognosy Division, National Botanical Research Institute, Lucknow, India for future reference.

Air-dried (40–50 °C), powdered plant materials, viz. fruits, stem bark and leaves (10 g each) were extracted with 95 % ethanol (3x100 mL each) by stirring with a magnetic stirrer for 30 min each time. The extracts were filtered through Whatman No. 1 filter paper, concentrated under vacuum at reduced temperature (below 50 °C) by rotary evaporation (Büchi, USA), and lyophilized (Freezone 4.5; Labconco, USA) under high vacuum (133 × 10⁴ mbar) at -40±2 °C to yield the respective samples. These extracts obtained from fruits, stem bark and leaves were coded as TBF, TBB and TBL respectively and stored at 5 °C. These were used for examination of antioxidant activity. For HPTLC analysis standard solutions of β -sitosterol, lupeol, stigmaterol and ursolic acid (0.5 mg/mL) were prepared in chloroform while those of ellagic acid, gallic acid, ferulic acid (0.5 mg/mL) were prepared in methanol. Sample stock solutions of 10 mg/mL concentration for HPTLC analysis were prepared by dissolving the extracts in methanol. These were further diluted for obtaining samples of final concentrations of 1 mg/mL for estimation of total phenolic and total flavonoids content.

Determination of physicochemical parameters

Quantitative analysis for total ash, water soluble and acid insoluble ash, ethanol and water soluble extractives, total sugar and starch content and tannin were carried out according to the standard methods as per WHO guidelines⁷.

Estimation of total phenolic and flavonoid contents

The total phenolic content (TPC) of the methanolic extracts of the different samples was determined using a modified colorimetric Folin-Ciocalteu method⁸. A calibration curve was prepared using different concentrations of standard gallic acid solutions, each time an analysis was run. Total phenolic content in the samples was calculated from the standard curve,

and the results were expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of extract. Total flavonoid content (TFC) of the different samples was also determined using the aluminum chloride colorimetric assay⁹ and expressed as milligrams of quercetin equivalents per gram (mg QE/g) of extract. All determinations were done in triplicates and averaged.

HPTLC Analysis

A Camag (Muttentz, Switzerland) HPTLC system, comprising of a Linomat 5 automatic applicator, a twin trough plate development chamber (20 x 10 x 4 cm), Camag TLC scanner 3 and win-CATs 4 software, was used for analysis. Pre-coated silica gel 60 F254 plates (20 x 10 cm, 0.2 mm thickness Merck, Darmstadt, Germany) on aluminium sheets were used as adsorbent layers. Solutions of the standard and samples of known concentration were applied to the plates as bands 6 mm wide, 10 mm from the bottom of the plate. Plates were developed to a distance of 9 cm in a twin trough glass chamber previously saturated with mobile phase vapor for 15 min. Toluene: ethyl acetate : formic acid (7:3:1) was used as mobile phase for the triterpenoids, viz. lupeol, β -sitosterol, stigmasterol and ursolic acid. After development the plates were dried, sprayed with anisaldehyde sulphuric acid reagent and heated at 105 °C for 10 min. The peak areas were recorded by scanning densitometry at 400 nm. Toluene: ethyl acetate: formic acid (5:5:1) was used as mobile phase for the analysis of phenolics, viz. ellagic acid, gallic acid, ferulic acid. After development the plates were dried and observed under UV light. The peak areas were recorded by scanning densitometry at 254 nm. The slit width used was 5 mm x 0.45 mm for all the analyses. The presence of the triterpenoids and the phenolics mentioned above was confirmed by comparing the R_f values and the spectra of the standards with corresponding spots in the extracts.

In vitro antioxidant activity

DPPH radical scavenging - The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay was carried out on the basis of the Blois method with slight modification¹⁰. Antioxidant activities of the methanolic extracts of the different samples were expressed as IC₅₀, defined as the concentration of the test material required to cause a 50 % decrease in initial DPPH concentration. Ascorbic acid was used as the positive control.

Total antioxidant capacity (TAC) by phosphomolybdate antioxidant assay- The total antioxidant capacity of the methanolic extracts of the different samples was measured spectrophotometrically using the phosphomolybdenum method¹¹, based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of specific green phosphate/Mo (V) compounds. Sample solution (0.3 mL, 40–300 μ g) was combined in an eppendorf tube with 3.0 mL of reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate, and 4 mmol/L ammonium molybdate). The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of each aqueous solution was measured at 695 nm against a blank. A typical blank solution containing 3.0 mL of reagent solution and 0.3 mL of methanol was incubated under the same conditions as the rest of the samples. AA was used as the standard, and the total antioxidant capacity was expressed as ascorbic acid equivalents (AAEs).

Ferric reducing antioxidant power (FRAP) assay- The ferric ions (Fe³⁺) reducing antioxidant power assay (FRAP) of the methanolic extracts of the different samples was determined by the method of Oyaizu with slight modifications^{12,13}. Substances that have a reduction potential react with potassium ferricyanide (Fe³⁺) to form potassium ferrocyanide (Fe²⁺), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of Fe³⁺ ions. Different concentrations of methanol extract in 1 mL of distilled water were mixed with sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10 %) were added to the mixture. The 2.5 mL of this solution was mixed with distilled water (2.5 mL) and FeCl₃ (0.5 mL, 0.1 %), and the absorbance was measured at 700 nm against the blank and absorbance vs. concentration graph was plotted. Increased absorbance of the reaction mixture indicates an increase of reduction capability.

Results and discussion

Physicochemical analysis

Various physicochemical parameters like the ash content and extractive values were checked for the

three plant parts and their results are summarized in Table 1. The stem bark exhibited considerably high ash values as compared to the leaves and the fruit. The water soluble extractives were higher as compared to the ethanol soluble extractives with fruits showing the highest water soluble contents.

Total phenolic and flavonoid contents

The total phenolic and the total flavonoid contents of the methanolic extracts of different parts of *T. bellirica* were determined and the results expressed as milligram of gallic acid equivalents per gram (mg GAE/g) of extract for TPC and as milligrams of quercetin equivalents per gram (mg QE/g) of extract for TFC, respectively (Table 2). The TPC of the samples were in the order TBL>TBF>TBB with TBL showing the highest TPC with 15.8 mg GAE/g while TBF and TBB contained only 8.3 and 1.8 mg GAE/g, respectively.

Table 1 — Physicochemical analysis of different parts of *T. bellirica*

Parameter(s)	Plant parts		
	Stem bark	Leaves	Fruits
Ash values (%)			
Total ash	21.38	9.91	4.2
Acid insol. ash	0.20	2.52	0.2
Water sol. ash	19.60	7.87	1.12
Extractive values (%)			
Ethanol soluble	2.66	13.83	12.80
Water soluble	18.16	27.15	43.33
Sugar (%)	0.22	0.21	0.20
Starch (%)	0.31	0.25	0.37
Tannin (%)	0.084	0.36	0.30

*Values are mean of three determinations each

The TFC of the samples were also in the order TBL>TBF>TBB with TBL showing the highest TFC with 33.3 mg QE/g. TBF and TBB contained only 9.6 and 3.4 mg QE/g, respectively.

HPTLC analysis

The ethanolic extracts obtained from fruits, stem bark and leaves of *T. bellirica* were subjected to qualitative as well as quantitative HPTLC analyses for the presence of different triterpenoids and phenolics under the conditions described earlier. Table 3 summarizes the different compounds found to be present in them. It was observed that TBF contained stigmaterol, ellagic acid and gallic acid while TBL contained β-sitosterol, ursolic acid, ellagic acid and gallic acid. TBB was found to contain lupeol, stigmaterol, ellagic acid and ferulic acid. Ellagic acid was the only phytoconstituent present in all the three parts of *T. bellirica*.

Results obtained from quantitative HPTLC analysis (Table 4) show that the phenolics are present in larger quantity as compared to the triterpenoids. Ellagic acid was the most predominant constituent and was found to be present in all the samples of *T. bellirica*, its percentage being maximum in case of TBL (4.863 %) followed by TBF (2.892 %) and lowest in TBB (0.196 %). The ethanolic extract of the fruits (TBF) contain a significantly high percentage of gallic acid (1.695 %) as compared to leaves (TBL) (0.213 %). Lupeol and ferulic acid although found in the TBB are present in very low amounts. Leaves were found to be richer in triterpenoids as compared to the fruits and bark.

Table 2 — TPC, TFC and antioxidant activities of methanolic extracts of different parts of *T. bellirica*

Plant parts	Total phenolic content* (mg GAE/g)	Total flavonoid content* (mg QE/g)	DPPH Radical Scavenging Ability IC ₅₀ (µg/mL)	Total Antioxidant Capacity by
				Phosphomolybdate Antioxidant Assay (µmol AA/g extract)
Fruit	8.3	9.6	64.67	256
Leaves	15.8	33.3	53.81	294
Bark	1.8	3.4	100.7	162
AA	-	-	4.6	-

*Values are mean of three determinations each

Table 3 — Qualitative HPTLC analysis of terpenoids and phenolics present in ethanolic extract of different parts of *T. bellirica*

Plant parts	Terpenoids				Phenolics		
	β-Sitosterol	Lupeol	Stigmaterol	Ursolic acid	Ellagic acid	Gallic acid	Ferulic acid
Fruit	-	-	+	-	+	+	-
Leaves	+	-	-	+	+	+	-
Bark	-	+	+	-	+	-	+

Table 4 — Quantitative HPTLC analysis of terpenoids and phenolics present in ethanolic extract of different parts of *T. bellirica*

Plant parts	Terpenoids (%)			Phenolics (%)			
	β -Sitosterol	Lupeol	Stigmasterol	Ursolic acid	Ellagic acid	Gallic acid	Ferulic acid
Fruit	-	-	0.031	-	2.892	1.695	-
Leaves	0.419	-	-	0.342	4.863	0.2133	-
Bark	-	0.018	0.066	-	0.196	-	0.030

In vitro antioxidant activity

DPPH radical scavenging ability - DPPH radicals react with suitable reducing agents losing color stoichiometrically with the number of electrons consumed which is measured spectrophotometrically at 517 nm. The IC₅₀ values for the DPPH radical scavenging activities of the extracts of different parts of *T. bellirica* are given in Table 2. The order of DPPH activity was TBL>TBF>TBB showing a direct correlation between their antioxidative capability and their TPC as well as TFC (Fig. 1).

Total antioxidant capacity by phosphomolybdate antioxidant assay – The total antioxidant capacity of the extracts was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695 nm. A direct correlation was found to exist between the concentration of the extract used and the spectrophotometrically measured phosphomolybdenum complex. The total antioxidant capacity of the extracts of different parts of *T. bellirica* are given in Table 2. The order of total antioxidant capacity was TBL>TBF>TBB showing a direct correlation between their antioxidative capability and their TPC as well as TFC (Fig. 1).

Ferric reducing anti-oxidant power (FRAP) assay- Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species. The reducing power of the extracts was measured using Fe³⁺ to Fe²⁺ reduction assay. The product was visualized by forming the intense Prussian blue color complex and then measured spectrophotometrically at λ 700 nm. As shown in Fig. 2, a higher absorbance value indicates a stronger reducing power of the samples. All the samples showed reducing capacity in a concentration dependant manner. However, its reducing power was weaker than that of ascorbic acid, which exhibited the strongest reducing power.

The present studies thus show that the content of both the phenolics and flavonoids is maximum in the leaves of *T. bellirica* and they also exhibit good antioxidant potential, as evidenced by the better

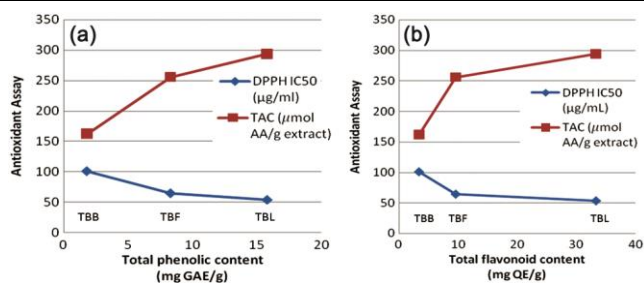


Fig. 1 — Correlation between total phenolic content and total flavonoid content with antioxidant activities, DPPH (IC₅₀) and Total antioxidant capacity (TAC; μ mol AA/g extract), of methanolic extracts of different parts of *T. bellirica*. (TBB: *T. bellirica* bark; TBF: *T. bellirica* fruits; TBL: *T. bellirica* leaves)

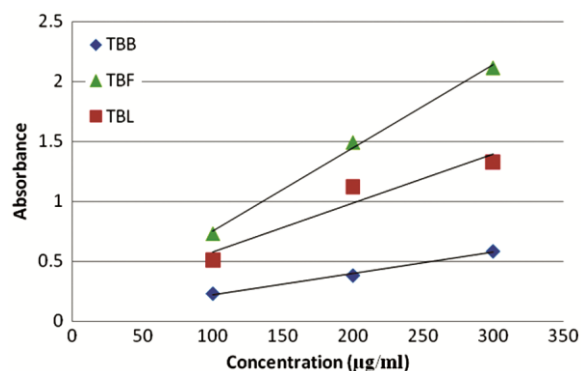


Fig. 2 — Ferric reducing anti-oxidant power of methanolic extracts of different parts of *T. bellirica* (TBB: *T. bellirica* bark; TBF: *T. bellirica* fruits; TBL: *T. bellirica* leaves)

DPPH radical scavenging ability as well as the total antioxidant capacity (Fig. 3).

Traditional significance of study to farmers/researchers/society

Traditional medicines, including *T. bellirica*, have been used in *Ayurveda* and other traditional systems of medicine for the benefit of humans through ancient times. The present studies have shown that *T. bellirica* is rich in flavonoids and phenolics. Polyphenols and flavonoids, which are naturally occurring antioxidants and have been reported to exhibit anti-carcinogenic and anti-inflammatory actions and contribute to the prevention of cardiovascular disease, may therefore be responsible for various activities exhibited by *T. bellirica*.

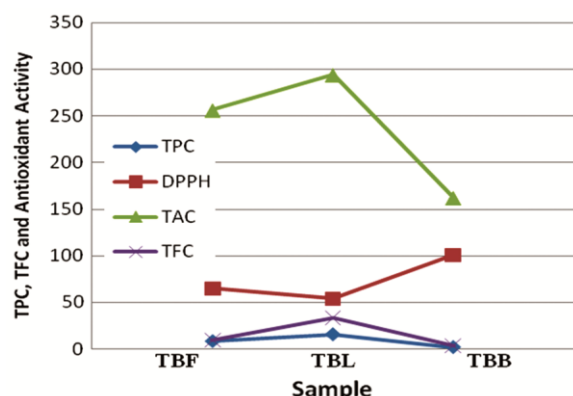


Fig. 3 — TPC, TFC, DPPH (IC₅₀) and TAC (μmol AA/g extract) of methanolic extracts of different parts of *T. bellirica* (TBF: *T. bellirica* fruits; TBL: *T. bellirica* leaves; TBB: *T. bellirica* bark)

Conclusion

T. bellirica is a well known medicinal plant that possesses a wide spectrum of activities, such as anticancer, antidepressant, antidiabetic, immunomodulatory and antihypertensive activities. Although different parts of the plant have been used for medicinal purposes, major work has been focused on the fruits. The present studies have shown that the different parts of *T. bellirica* contain significant amounts of phenolics and flavonoids and also exhibit good antioxidant potential, although the leaves exhibit the highest values as evidenced by the better DPPH radical scavenging ability as well as the total antioxidant capacity. Flavonoids and phenolics are known to be helpful against human cancers, arteriosclerosis, ischemia and inflammatory diseases, which are partially caused by exposure to oxidative stress. Also, there has been considerable interest in the use of naturally occurring antioxidants for treatment or prophylaxis of various oxidative stress-related diseases. Thus, *T. bellirica* leaves have the potential to be used against oxidative stress related diseases although further detailed studies are required.

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