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# Evaluation of *Terminalia bellerica* Roxb. Leaf Extracted in Different Solvents for Antioxidant Activities

L. SHERIN[1,*], M. MUSTAFA[1] and S. SHUJAAT[2,*]

[1]Department of Chemical Engineering, COMSATS Institute of Information Technology, Defence Road, Off Raiwind Road, Lahore, Pakistan
[2]Department of Chemistry, Lahore College for Women University, Lahore -54000, Pakistan

*Corresponding authors: Fax: +92 42 99203100; E-mail: lubnasherin@ciitlahore.edu.pk; shahidashujaat@yahoo.com

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*Terminalia bellerica*, an esteemed ayurvedic plant, is employed traditionally in the management of an array of diverse pathological conditions. The present work is aimed to assess antioxidant potential of plant leaf extracts. Crude methanol extract was fractioned with different solvents and antioxidant activities along with total phenolic and flavonoid content were investigated with different antioxidant testing models, including DPPH, ABTS, anti-lipid peroxidation and total antioxidant capacity assays. Ethyl acetate ($IC_{50}$ = 3.48 μg $mL^{-1}$) as well as chloroform ($IC_{50}$ = 4.55 μg $mL^{-1}$) extracts exhibited persuasive DPPH radical scavenging activity, much better than standard antioxidant Trolox ($IC_{50}$ = 6.17 μg $mL^{-1}$). In ABTS assay, ethyl acetate extract ($IC_{50}$ = 0.18 μg $mL^{-1}$) showed enhanced potential in comparison to other extracts and standard antioxidants Trolox and *n*-propyl gallate. Chloroform extract offered maximum protection against lipid peroxidation ($IC_{50}$ = 0.28 mg $mL^{-1}$) and highest total antioxidant activity (4.54 mM $g^{-1}$). All the extracts showed variable magnitude of phenolics and flavonoids content. In conclusion, ethylacetate and chloroform extracts of *Terminalia bellerica* leaf possess potent antioxidant potential which could be harnessed as economically viable source of natural antioxidants.

**Keywords: *Terminalia bellerica* leaf, Antioxidant activity, Anti-lipid peroxidation, DPPH assay, ABTS assay.**

## INTRODUCTION

In recent years, free radicals and antioxidants have been emerging field in consequence to modern lifestyle. Uncontrolled production and reactivity of free radicals, leading to oxidative stress, is implicated in the initiation and propagation of many diseases mostly due to damage of biologically significant sensitive targets[1]. Emerging data indicate that potential of antioxidants from natural sources could be exploited to manage various redox-dependent pathological conditions such as cancer, diabetes (type 2), artheriosclerosis and neurodegerative diseases[2]. Phyto-medicines or traditional medicinal plants have been protective against these diseases. Therapeutic effects of these plants are often considered to be propagated by oxidant mechanisms because they are rich in polyphenols[3]. Lipid peroxidation is a major cause of spoilage of food or lowers its nutritional value. Toxicity and spoilage of food especially oil industry due to its economical importance is a hot topic nowadays. Antioxidants are used in food to guard it from toxic effects of oxidation. Moreover, worldwide consumers are more conscious about synthetic food additives due to their strong side effects. Therefore, scientists and manufacturers are searching for natural antioxidants to replace the synthetic ones[4].

*Terminalia bellerica* (TB), commonly known as myrobalan or bhera is a large deciduous tree commonly found in plains of Southeast Asia[5]. It is an important medicinal plant in Ayurveda and is constituent of polyherbal formulation "Triphala" which is believed to revive and promote immunity and health[6]. Fruit of *Terminalia bellerica* is prescribed in ethnomedicine for liver and gastrointestinal tract disorders, cancer, dyspepsia, hypertension and rheumatism[7,8]. On the basis of phytochemical analysis, gallic acid, ethyl gallate, ellagic acid, β-sitosterol, chebulagic acid, galloyl glucose, galactose, glucose, mannitol and rhamnose has been reported in *Terminalia bellerica* fruit[9]. In present-day studies various biological activities of crude extracts of *Terminalia bellerica* fruit have shown antimutagenic, hepatoprotective, antimicrobial and anti-HIV and hypotensive potential[10-13].

As there is plenty of anecdotal evidence on the medicinal effects of *Terminalia bellerica* fruit, systematic studies on *Terminalia bellerica* leaf are also required to explore the therapeutic potential of this economical viable source. The present study is aimed to explore various *Terminalia bellerica* leaf extracts thoroughly for compounds with exceptional antioxidant potential.

## EXPERIMENTAL

1,1-Diphenyl-2-picryl hydrazyl radical (DPPH), 2,2´-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), Trolox (6-hydroy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (USA). All other chemicals and solvents used were of analytical grade available commercially.

**Plant material collection and extraction:** Leaf of *Terminalia bellerica* was collected from Bagh-e-Jinnah, Lahore Pakistan. Plant material was identified and confirmed with morphological techniques by plant taxonomist Prof. Dr Zaheer-ud-Din Khan (Department of Botany, GC University, Lahore Pakistan). A voucher specimen (GC. Herb. Bot. 256) was deposited in GCU herbarium.

Shade dried leaf powder (10.0 kg) was exhaustively extracted at room temperature with methanol. Crude methanol extract (TLM) was dried, redissolved in deionized water and partitioned with solvents of increasing polarity generating hexane fraction (TLH), chloroform fraction (TLC), ethyl acetate fraction (TLE) and *n*-butanol fraction (TLB) respectively. Solvent was excluded on rotary evaporator to yield respective fraction. Remainder was marked as aqueous extract (TLA) and freeze dried. Other all fractions were stored at 4 °C in refrigerator until further use.

**Anti-lipid peroxidation assay:** Anti-lipid peroxidation activity of samples and reference standards was assessed by slightly modified Halliwell *et al.*[14] protocol. KCl (1.15 %, 1.5 mL) and egg yolk (10.0 %, 1.0 mL) were added to appropriate series of sample's concentration. Lipid peroxidation was induced by adding up 0.2 mM ferric chloride (0.5 mL) in reaction mixture. After 1 h, incubation at 37 °C, reaction was stopped by adding ice-cold HCl (0.25 N, 2.0 mL) containing 0.38 % thiobarbituric acid (TBA), 15 % trichloroacetic acid (TCA) and 0.5 % butylated hydroxytoluene (BHT). It was heated for 1 h at 80 °C, followed by centrifugation at 3000 rpm. Pink adduct of malondialdehyde (MDA) and thiobarbituric acid was detected spectrophotometrically at 532 nm.

**DPPH scavenging assay:** Free radical-quenching ability of samples was evaluated by applying Blois protocol with certain modifications[15]. Reaction mixture comprised of sample solution (0.5 mL, in methanol/DMSO) and DPPH radical solution ($1 \times 10^4$ M, 2.5 mL). It was incubated at 37 °C for 0.5 h. Absorbance was recorded at 517 nm by UV-visible spectrophotometer. The percentage scavenging of DPPH was estimated as

$$\text{Scavenging (\%)} = \left(1 - \frac{(A_1 - A_2)}{A_0}\right) \times 100$$

where, $A_0$ = blank's absorption; $A_1$ = DPPH + sample solution absorption; $A_2$ = sample solution absorption.

**ABTS decolourization assay:** $ABTS^{\bullet+}$ decolourization potential was evaluated by applying Re *et al.*[16] protocol. Oxidation of ABTS stock solution (7.0 mM) was carried out by 2.45 mM $K_2S_2O_4$ to generate $ABTS^{\bullet+}$ radical cation. Absorption of this solution was adjusted at 0.70 (± 0.10) (734 nm). Reaction mixture contained 2.5 mL $ABTS^{\bullet+}$ and 0.5 mL sample solution and percentage quenching was estimated as:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}\right) \times 100$$

**Total antioxidant activity:** Total antioxidant activity of samples was calculated using Prieto *et al.*[17] protocol. Green phosphate molybdenum/Mo(V) complex was produced by Mo(VI) reduction at acidic pH. Sample solution (0.5 mL in methanol/DMSO) was pooled with 4 mL of reagent solution (0.6 M $H_2SO_4$, 4.0 mM ammonium molybdate, 28.0 mM sodium phosphate). It was incubated at 95 °C for 1.5 h. Absorbance was observed at 695 nm and total antioxidant activity was presented as mM equivalence of ascorbic acid per gram of tested sample.

**Total phenolic content[18]:** Total phenolic content was estimated using Folin-Ciocalteu reagent. 100 µL Folin-Ciocalteu reagent was added to 1.58 mL deionized water and 20 µL sample solution. Sodium carbonate solution (25 %, 300 µL) was added in it after 10 min, followed by incubation at 40 °C for 0.5 h. Absorbance was recorded at 765 nm and results were documented as gallic acid equivalent (GAE mg $g^{-1}$ dry wt.)

**Total flavonoid content[19]:** Sample solution (250 µL) was added in 1.5 mL deionized water and $NaNO_2$ solution (5 %, 90 µL), $AlCl_3$ solution (10 %, 180 µL) was added in the reaction mixture after 5 min stand followed by addition of 1 M NaOH solution (0.6 mL). Absorbance of final solution (3 mL, made up by deionized water) was recorded at 510 nm and results were documented as quercetin equivalent (QE mg $g^{-1}$ dry wt.).

**Statistical analysis:** Data were documented as mean value (± SD) of triplicates from two to three independent experiments. $IC_{50}$ values were calculated by linear regression analysis. Data were statistically examined using analysis of variance (ANOVA) and Duncan's test for significant values at $p < 0.05$ probability level.

## RESULTS AND DISCUSSION

In this study different *in vitro* antioxidant tests are used to analyze antioxidant activity of natural products comprehensively. As plant extracts exhibit their pharmacological properties through diverse mechanisms like free radical quenching, peroxides decomposition, transition metal ion binding and termination of chain reactions or acting as suppressing agents by blocking hydrogen abstraction, it is very unrealistic to use one *in vitro* assay for overall quantification of antioxidant potential of natural products[19]. Taking this fact into account, anti-lipid peroxidation, DPPH radical scavenging, ABTS decolourization and total antioxidant capacity assays were used as multidimensional antioxidant efficacy assessing tools for various fractions of *Terminalia bellerica* leaf. In all the assays they exhibited different level of antioxidant activity owing to various mechanisms of oxidative injury.

**Anti-lipid peroxidation assay:** In this assay, antioxidant potential is measured by evaluating the capability of test sample to impede the oxidation of polyunsaturated fatty acids into thiobarbituric acid reactive substances (TBARS). Peroxidation generates peroxyl radicals which decompose to malondialdehyde (MDA). It forms a stable product with thiobarbituric acid (TBA), which serves as a mean to quantify the level of

peroxidation[20]. This assay is very useful mean to assess lipid peroxidation *in vitro* due to its simplicity and reproducibility.

Initial screening of all fractions of *Terminalia bellerica* leaf at 5 mg $mL^{-1}$ concentration indicated that all of them were able to inhibit lipid peroxidation (Fig. 1). Analysis of $IC_{50}$ values indicated that TLC was most efficient in blocking peroxidation of lipids (Table-1). TLH and TLE fractions also offered good protection against lipid peroxidation with $IC_{50}$ values 0.356 mg $mL^{-1}$ and 0.527 mg $mL^{-1}$ respectively. These values were much better than reference antioxidant gallic acid ($IC_{50}$ = 2.22 mg $mL^{-1}$) and comparable to other standard PG ($IC_{50}$ = 0.42 mg $mL^{-1}$). TLM and TLB fractions showed moderate activity, while TLA was very poor in retarding lipid peroxidation.

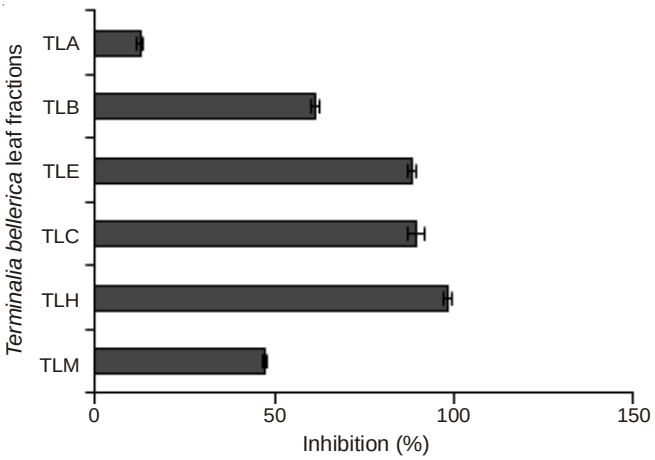


Fig. 1. Antilipid peroxidation potential of *Terminalia bellerica* leaf fractions (5 mg $mL^{-1}$); TLM:MeOH fr.; TLH:Hexane fr.; TLC: Chloroform fr.; TLE: EtOAc fr.; TLB: BuOH fr.; TLA: Aqueous fr.; Data are mean (n = 3) ± SD (n = 3, $p < 0.05$)

TABLE-1
*in vitro* RADICAL SCAVENGING AND ANTI-LIPID PEROXIDATION ACTIVITIES OF VARIOUS FRACTIONS OF *Terminalia bellerica* LEAF AND STANDARD ANTIOXIDANTS

| Samples/ standard antioxidants | Half efficiency concentration ($IC_{50}$)[a] | | |
|---|---|---|---|
| | DPPH scavenging activity (μg $mL^{-1}$) | $ABTS^{\bullet+}$ decolourization assay (μg $mL^{-1}$) | Antilipid peroxidation assay (mg $mL^{-1}$) |
| TLM | 5.51 ± 0.12[b] | 1.00 ± 0.16 | 1.30 ± 0.31 |
| TLH | 22.2 ± 0.24 | 2.82 ± 0.26 | 0.35 ± 0.09 |
| TLC | 4.55 ± 0.09 | 1.52 ± 0.18 | 0.28 ± 0.06 |
| TLE | 3.48 ± 0.22 | 0.18 ± 0.05 | 0.52 ± 0.12 |
| TLB | 19.8 ± 0.43 | 1.62 ± 0.27 | 3.00 ± 0.53 |
| TLA | 8.24 ± 0.33 | 8.11 ± 0.54 | 43.1 ± 0.33 |
| Gallic acid | 2.38 ± 0.04 | 0.60 ± 0.08 | 2.22 ± 0.02 |
| *n*-Propyl gallate | 2.31 ± 0.04 | 1.71 ± 0.03 | 0.42 ± 0.02 |
| Trolox | 6.17 ± 0.02 | 3.86 ± 0.03 | 0.08 ± 0.03 |

[a]$IC_{50}$ is the effective concentration of plant fraction that inhibits 50 % of initial DPPH, ABTS or TABRS concentration.
[b]Data are mean (n = 3) ± SD (n = 3, $p < 0.05$).

**DPPH radical scavenging assay:** The DPPH method is an easy and economical way to equate the antioxidant potential of plant extracts due to its sensitivity to active phytochemicals at very low concentration. It provides a rapid tool to analyze a large no of plant extracts in a very short time. DPPH is a stable free organic radical with an odd electron. Antioxidants reduce it through hydrogen atom donation to corresponding hydrazine. The whole reaction results in the change of colour of solution from purple to yellow which is measured calorimetrically[21]. DPPH quenching power of samples usually increases with phenolics concentration or their degree of hydroxylation.

Analysis of data indicated that all organic and aqueous fractions of *Terminalia bellerica* leaf possessed radical quenching activity. Dose-response curves of all the samples depicted that scavenging ability varied differently with concentration for different fractions (Fig. 2). At concentration range 1-30 μg $mL^{-1}$, TLM fraction exhibited quenching potential from 18 to 99 %, TLH quenched from 6 to 67 %, TLC quenched from 20 to 100 %, TLE quenched from 25 to 100 %, TLB quenched from 6 to 66 % and TLA quenched from 6 to 98 %. Among all the samples, TLE was strongest DPPH radical scavenger followed by TLC fraction. TLH and TLB fractions were very poor in scavenging the radical in comparison to other fractions. Analysis of Table-1 indicated that radical quenching potential of fractions decreased in the order of TLE ($IC_{50}$ = 3.48 μg $mL^{-1}$) > TLC ($IC_{50}$ = 4.55 μg $mL^{-1}$) > TLM ($IC_{50}$ = 5.51 μg $mL^{-1}$) > TLA ($IC_{50}$ = 8.24 μg $mL^{-1}$) > TLB ($IC_{50}$ = 19.85 μg $mL^{-1}$) > TLH ($IC_{50}$ = 22.12 μg $mL^{-1}$). Moreover, results indicated that TLE and TLC fractions were better radical quencher than reference antioxidant Trolox ($IC_{50}$ = 6.17 μg $mL^{-1}$).

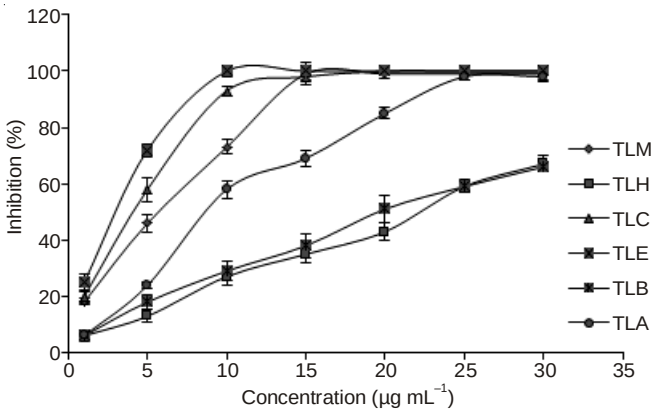


Fig. 2. Dose-response curves of *Terminalia bellerica* leaf fractions at multiple concentration against DPPH radical; TLM: MeOH fr.; TLH: Hexane fr.; TLC: Chloroform fr.; TLE: EtOAc fr.; TLB: BuOH fr.; TLA: Aqueous fr.; Data are mean (n = 3) ± SD (n = 3, $p < 0.05$)

**$ABTS^{\bullet+}$ decolourization assay:** This is an inhibition assay which measures the extend of antioxidant potential by electron donation of antioxidants to pre-formed $ABTS^{\bullet+}$ radical cation. The antioxidant components of plant extracts having a redox potential lower than that of $ABTS^{\bullet+}$ radical cation decolourize the colour of the radical, proportionate to its amount[21]. This method is rapid, sensitive and accurate and avoids unwanted reactions, especially interference by endogenous peroxidase in samples. Trolox equivalent antioxidant capacity (TEAC) values were obtained by calculating the percentage inhibition of each sample and comparing with Trolox, standard antioxidant.

Trolox equivalent antioxidant capacity values of various fractions of *Terminalia bellerica* leaf are depicted in Fig. 3. It is evident that highest value was scored by EtOAc fraction

(17.18 mM TE). TLM and TLC fractions also exhibited good values, which were better than standard antioxidants, GA and PG tested in the assay. Lowest TEAC values were achieved by TLA and TLH fractions. Comparison of $IC_{50}$ values (Table-1) indicated that TLE was very strong inhibitor of $ABTS^{\bullet+}$ radical and showed much lower half efficiency concentration ($IC_{50}$ = 0.181 μg $mL^{-1}$) than all the positive controls. TLM, TLC and TLB fractions exhibited good $IC_{50}$ values, which were comparable to standard antioxidants. According to results, aqueous fraction was not able to quench $ABTS^{\bullet+}$ radical efficiently and showed much higher $IC_{50}$ value.

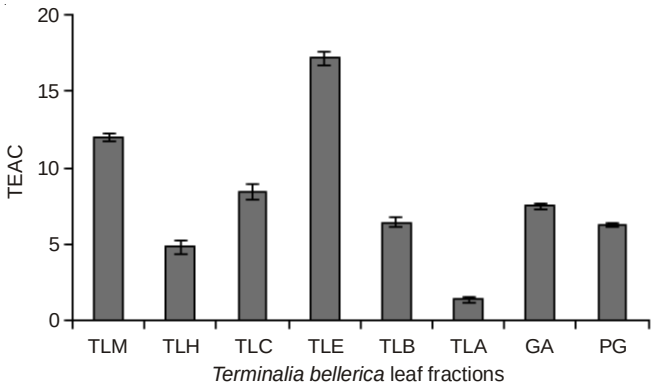


Fig. 3. TEAC values of *Terminalia bellerica* leaf fractions (mM Trolox eq. $g^{-1}$ dry wt.); TLM:MeOH fr.; TLH: Hexane fr.; TLC: Chloroform fr.; TLE: EtOAc fr.;TLB: BuOH fr.; TLA: Aqueous fr.; GA: Gallic acid; PG: Propyl Gallate; Data are mean (n = 3) ± SD (n = 3, $p < 0.05$)

The antioxidant activity displayed by various *Terminalia bellerica* fractions may be attributed to various tannins and polyphenols such as gallic acid, ellagic acid and ellagic tannins present in *Terminalia bellerica*. Literature reports suggest that these metabolites are natural antioxidants and most probably are responsible for the neutralization ability of radical cations of plant samples through their redox properties[22].

**Total antioxidant capacity assay:** Total antioxidant activity (TAA) of different fractions was assessed by the formation of phosphomolybdenum complex. The antioxidants in the sample reduce Mo(VI), producing a green Mo(V) complex which exhibits absorption at 695 nm[23].

Both organic and aqueous fractions of *Terminalia bellerica* leaf displayed various level of total antioxidant activity. Highest level was achieved by TLE, parallel to reference antioxidant PG. Among rest of the fractions, TLM and TLC fractions showed reasonable antioxidant activity.

**Total phenolic and flavonoid estimation:** Plant phenolics have become popular natural antioxidants owing to their strong radical neutralization potential. Phenolics may be simple one with solitary hydroxylated aromatic ring, or polyphenols with two phenolic subunits (flavonoids) or multiple phenol subuints (tannins)[24]. Flavonoids, constitute a special class of polyphenols with C6-C3-C6 flavone skeleton. It is reported that that they are strong antioxidant due to their ability to scavenge free radicals. Or they interact with other antioxidants to facilitate their localization and mobility in the microenvironment[25]. Their complex structure coupled with number and position of hydroxyl groups are key features for determining their antioxidant potential[26].

The total phenol assay by Folin-Ciocalteu reagent was used to quantify total phenolics in the test sample. Under basic conditions, phenolic compounds dissociate to form phenolate ion which reduces Folin-Ciocalteu reagent. The blue compound formed between phenolate ion and Folin-Ciocalteu reagent is measured spectrophotometrically. Total flavonoid content was also measured spectrophotometrically and expressed as quercetin eq. $g^{-1}$ dry wt. Among various *Terminalia bellerica* leaf fractions, highest phenolic content was displayed by TLE fraction (1.18 mM GAE). This fraction also showed highest flavonoid content among all the fractions. Good phenolic and flavonoid content was also detected in TLM, TLC and TLB fractions. A positive correlation existed between phenolic and flavonoid content and antioxidant activity of fractions (Tables 1 and 2). In almost all the assays performed in this study, either TLE or TLC fraction exhibited highest antioxidant activity, which might be attributed to highest phenolic and flavonoid content of these fractions.

TABLE-2
*in vitro* TOTAL ANTIOXIDANT ACTIVITY (TAA), TOTAL PHENOLICS AND FLAVONOIDS OF VARIOUS FRACTIONS OF *Terminalia bellerica* LEAF ALONG WITH STANDARD ANTIOXIDANTS

| Samples/ standard antioxidant | Total antioxidant activity (mM AAE $g^{-1}$)[a] | Total phenolics (mM GAE $g^{-1}$)[b] | Total flavonoids (mM QE $g^{-1}$)[c] |
|---|---|---|---|
| TLM | 3.97 ± 0.32[d] | 1.31 ± 0.06 | 1.18 ± 0.12 |
| TLH | 1.76 ± 0.08 | 0.68 ± 0.03 | 0.32 ± 0.06 |
| TLC | 2.83 ± 0.16 | 1.11 ± 0.11 | 1.06 ± 0.08 |
| TLE | 4.54 ± 0.24 | 1.81 ± 0.12 | 1.56 ± 0.16 |
| TLB | 0.22 ± 0.15 | 1.23 ± 0.14 | 0.96 ± 0.17 |
| TLA | 0.90 ± 0.08 | 0.82 ± 0.09 | 0.77 ± 0.02 |
| Gallic acid | 11.35 ± 0.80 | | |
| *n*-Propyl gallate | 4.54 ± 0.37 | | |
| Trolox | 3.97 ± 0.61 | | |

[a]Milli mole ascorbic acid equivalent.
[b]Milli mole gallic acid equivalent $g^{-1}$.
[c]Milli mole quercetin equivalent $g^{-1}$.
[d]Data are mean (n = 3) ± SD (n = 3, $p < 0.05$).

## Conclusion

Within the last few decades, strong evidence supporting the role of ethnomedicinal plants for drug discovery in various fields has been published. The aim of this research was to investigate *Terminalia bellerica* leaf for photochemical with potent antioxidant potential. For this purpose, different extracts of *Terminalia bellerica* leaf were subjected to through antioxidant screening. Results suggested that most of the fractions were active in all the *in vitro* testing systems employed however, ethylacetate and chloroform fractions emerged as profuse source of natural antioxidants with $IC_{50}$ values better than reference antioxidants, used as positive control. In most of the cases, a positive correlation existed between antioxidant power and total phenolic and flavonoid content of the fractions. This work reveals that *Terminalia bellerica* leaf can be an interesting source of natural antioxidant with potential use in different fields of food, cosmetics and pharmaceuticals. It could be a useful ingredient of dietary supplements or natural additives for food preservation. However, toxicity and antioxidant

mechanisms of the extracts should be further studied to gain more understanding of their antioxidant activity in food systems so that these could be used safely to increase shelf life.

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