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Antioxidant Studies on Aqueous and Organic Extracts of *Terminalia bellerica* Roxb. Bark

L. SHERIN*, S. SHUJAAT and R. NAZIR

Department of Chemistry, Lahore College for Women University, Lahore 54000, Pakistan

*Corresponding author: Tel: +92 300 4330910; E-mail: nedo_e@yahoo.com

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Traditional Ayurvedic plant, *Terminalia bellerica* Roxb. (TB), has been widely used for the management of a number of clinical disorders in South East Asia. In view of better understanding of its pharmacological action, *in vitro* antioxidant activities of aqueous and organic extracts (hexane, ethyl acetate, chloroform and butanol) of *Terminalia bellerica* Roxb. bark were evaluated with different antioxidant testing models, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), antilipid peroxidation and total antioxidant capacity assays. Total phenolic and flavonoids content was also analyzed. Results showed that both chloroform (IC_{50} = 1.00 µg/mL) and ethyl acetate (IC_{50} = 1.54 µg/mL) extracts possess potent DPPH radical scavenging activity, which was even better than standard antioxidants (trolox (IC_{50} = 6.17 µg/mL), *n*-propyl gallate (IC_{50} = 2.31 µg/mL) and gallic acid (IC_{50} = 2.38 µg/mL)). In ABTS decolorization assay ethyl acetate extract (IC_{50} = 1.64 µg/mL) showed better potential than others fractions and standards such as trolox (IC_{50} = 3.86 µg/mL) and *n*-propyl gallate (IC_{50} = 1.71 µg/mL). Hexane extract was most protective against lipid peroxidation (IC_{50} = 0.52 mg/mL). Maximum total antioxidant activity was shown by chloroform extract (5.11 m mol g^{-1} ascorbic acid equivalent). All the extracts showed variable magnitude of phenolic and flavonoids content. In conclusion, aqueous and all organic extracts of *Terminalia bellerica* Roxb. bark possess considerable free radical scavenging and antioxidant potential, but chloroform, ethyl acetate and hexane extracts are among the potent ones. These antioxidant activities most probably contribute to the therapeutic benefits of the traditional claims of *Terminalia bellerica* Roxb..

Key Words: Antioxidant activity, DPPH assay, ABTS assay, Antilipid peroxidation, *Terminalia bellerica* bark.

INTRODUCTION

Reactive oxygen species (ROS) are associated with cellular oxidative damage in biological systems. They can initiate lipid peroxidation of poly unsaturated fatty acids of biological membranes or may denature proteins, important enzymes and DNA[1-4]. Natural antioxidants in living systems, such as enzymes and antioxidant nutrients capture these free radicals, thus block their damaging properties[5]. If reactive oxygen species are produced in bulk amount, a living system is unable to neutralize them on its own. Persistence of this accumulation of ROS leads to many degenerative diseases including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer[6,7].

Recent strategy to undertake these disorders involves exogenous supply of antioxidants. Different antioxidant compounds such as butylated hydroxy toluene (BHT), propyl gallate (PG), *tert*-butyl hydroquinone (TBHQ) *etc*., are synthesized for this purpose. However, they are not so much popular due to their involvement in liver damaging and cancer initiation as shown by the experimental animal models[8]. In comparison to this natural antioxidants such as, vitamins, tocopherol, ascorbic acid, carotenoids and phenolic phytochemicals act as chemopreventive agents *in vivo* without serious side effects and help to fight against oxidative stress. Antioxidants also play an important role in medicine and food chemistry. Lipid peroxidation of oily food cause rancidity. In order to avoid these changes, antioxidants are added as food additives to extend its shelf life and maintain the nutritional quality. Natural additives are preferred worldwide by manufacturers and consumers. This starts a new era of search for potential sources of natural antioxidants in different types of plant materials such as fruits, leaves, barks, roots, spices and herbs[9]. *Terminalia bellerica* Roxb. commonly known as belleric myrobalan and locally known as 'bahera' belongs to family Combretaceae. It is a large deciduous tree with thick grey brownish bark having longitudinal fissures and is found in different parts of Pakistan and India. It is a very popular herb in Ayurvedic medicinal system. Triditionally, the bark of *T. bellerica* is used in the treatment of anemia, jaundice, leucoderma and dysentery. Phytochemical reviews show the presence of belleric acid, bellericoside, arjungenin, arjunglucoside, sapogenols and their glycosides[10].

At present, scientific information reported on antioxidant activity of *T. bellerica* bark is very limited and indicates the

antioxidant activity of methanol extract only[11]. In the present study the antioxidant and free radical scavenging activities of aqueous and organic extracts of *T. bellerica* bark were evaluated for the presence of compounds with antioxidant activity. The evaluation of antioxidant power was performed *in vitro* by ABTS, DPPH, antilipid peroxidation and total antioxidant capacity assays. Total phenolic and flavonoid content was also determined.

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, extraction and fractionation: The mature bark of *T. bellerica* was collected from Bagh-e-Jinnah Lahore, Pakistan. These were identified and confirmed with morphological techniques by Prof. Mohammad Zaheer-Ud-Din, Government College University, Lahore, Pakistan. A voucher specimen was deposited at the Herbarium of the Government College University, Lahore, Pakistan.

Dried bark (1.0 kg) was powdered and extracted twice with methanol. The extract was concentrated under reduced pressure to yield methanol extract. This crude extract was suspended in deionized water and partitioned with hexane, chloroform, ethyl acetate and 1-butanol. Each extract was concentrated under reduced pressure to yield respective extract. Remainder was called aqueous extract. All extracts were stored in refrigerator until further use.

Antilipid peroxidation assay: The antilipid peroxidation activity of various extracts of *Terminalia bellerica* and antioxidant standards was evaluated according to the method of Halliwell *et al.*[12] with slight modifications. 1.5 mL of 1.15 % KCl and 1.0 mL egg yolk (10 %) were added to different concentrations of samples. 0.5 mL of 0.2 mM ferric chloride was added to initiate lipid peroxidation. After incubation at 37 ºC for 1 h, the reaction was stopped by adding 2.0 mL of ice cold HCl (0.25 N) containing 15 % trichloroacetic acid (TCA), 0.38 % thiobarbituric acid (TBA) and 0.5 % butylated hydroxytoluene (BHT). The reaction mixture was heated at 80 ºC for 1 h. The samples were cooled and centrifuged at 3000 rpm. Pink adduct of malondialdehyde (MDA) and thiobarbituric acid (TBA) was detected spectrophotometrically at 532 nm.

Radical scavenging activity: Free radical-scavenging ability of different samples was determined by using a stable free radical, DPPH, according to the method of Blois[13] with certain modifications. The reaction mixture contained 0.5 mL sample solution (in methanol) and 2.5 mL DPPH radical solution (1×10^4 M). The mixture was incubated at 37 ºC for 0.5 h. Absorbance was recorded at 517 nm by UV-VIS spectrophotometer (Hitachi, U2800). The scavenging percentage of DPPH was calculated according to the following equation:

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

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract, A_2 was the absorbance without DPPH.

ABTS decolorization assay: $ABTS^{\cdot+}$ decolorization potential was evaluated by applying an improved ABTS radical cation decolorization assay by Re *et al.*[14]. Oxidation of ABTS stock solution (7 mM) was carried out with 2.45 mM potassium persulphate to generate ABTS radical cation ($ABTS^{\cdot+}$). For the study of antioxidant activity, the solution was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm. Percentage inhibition was calculated by using the following equation:

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

Total antioxidant capacity: Total antioxidant capacity of each extract was evaluated by method describe by Prieto *et al.*[15]. A green phosphate molybdenum/Mo(V) complex at acidic pH is formed by reduction of Mo(VI) to Mo(V) by the plant extract. 0.5 mL of each extract was combined with 4 mL of reagent solutiuon (0.6M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate). The reaction mixture was incubated at 95 ºC for 90 min. The absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Total antioxidant activity is expressed as mM equivalence of ascorbic acid per gram dry weight.

Determination of total phenolics: Amount of total phenolics was determined by Folin-Ciocalteu reagent (FCR) procedure as described by Cliffe *et al.*[16]. Briefly, 20 µL of sample was mixed with 100 µL of FCR and 1.58 mL deionized water. 300 µL of 25 % sodium carbonate solution (w/v) were added after 10 min awaiting. The mixture was incubated at 40 ºC for 0.5 h and then cooled. Finally absorbance was measured at 765 nm. Gallic acid was used for the preparation of callibration curve and results were mentioned as gallic acid equivalent (mM g^{-1} dry mass).

Determinatioin of total flavonoids: Total flavonoids content was assessed using a colorimetric method described by Dewanto *et al.*[17]. In short 0.25 mL of the extract was mixed with 1.50 mL deionized water followed by the addition of 90 µL $NaNO_3$ solution (5 %). After 6 min, 180 µL of $AlCl_3$ solution (10 %) was added and allowed to stand for another 5 min before 0.6 mL of 1M NaOH was mixed. Final volume was made to 3 mL with deionized water and mixed well. Absorbance was measured at 510 nm against blank. A callibration curve was prepared using quercetin as standard and the results were expressed as quercetin equivalent (mM g^{-1} dry mass).

Statistical analysis: Tests were carried out in triplicate for each experiment. A positive control was used for each experiment. Values are mean and represented as ± SD. The concentration of each extract to give 50 % inhibition was calculated graphically using a non-linear regression algorithm.

RESULTS AND DISCUSSION

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 scavenging or acting as suppressing

agents, it is very unrealistic to use one chemical assay for overall quantification of antioxidant potential of natural products[18]. Taking this fact into account, antilipid peroxidation, DPPH radical scavenging, ABTS decolorization and total antioxidant capacity assays are used as antioxidant potential assessing tools for different extracts of *Terminalia bellerica* bark.

DPPH radical scavenging assay: The DPPH method is an easy and economical method to equate the antioxidant potential of plant extracts due to the sensitivity of DPPH radical to active phytochemicals at very low concentration. It provides a rapid tool to analyze a large number of plant extract samples in a very short time. DPPH is a stable free organic radical with one odd electron of nitrogen atom. Antioxidants reduce DPPH by a hydrogen atom donation to form corresponding hydrazine. The whole reaction results in the change of colour of solution from purple to yellow which is measured colourimetrically[19]. DPPH radical scavenging activity and hence antioxidant potential of samples usually increases with the concentration of phenolic compounds or degree of hydroxylation of the phenolic compounds.

Results showed that all *Terminalia bellerica* bark extracts exhibited appreciable scavenging activity. Fig. 1 shows that all samples reacted directly and neutralized DPPH radicals to different degrees with increase activities at higher concentrations. At concentration 1-32 µg/mL *Terminalia bellerica* bark showed scavenging rate ranging from 11.52-89.79 % for methanol extract, 5.18-53.7 % for *n*-hexane extract, 25.3-95.5 % for ethyl acetate extract, 14.5-92.6 % for butanol extract and 27.2-82.4 % for aqueous extract. It is clear from the Fig. 1 that chloroform extract is strongest scavenger among all, accompanied by ethyl acetate extract with almost parallel potential to eradicate free radicals. Aqueous and *n*-butanol extracts showed moderate activities, whereas, methanol and hexane extracts were quite weak in protection against free radicals. IC_{50} values of the extracts confirmed these results. Table-1 shows that scavenging effect of different extracts on the DPPH radical decreased in the order of chloroform (IC_{50} = 1.0 µg/mL) > ethyl acetate (IC_{50} = 1.54 µg/mL) > aqueous extract (IC_{50} = 4.54 µg/mL) > *n*-butanol extract (IC_{50} = 6.51 µg/mL) > methanol extract (IC_{50} = 14.37 µg/mL) > hexane extract (IC_{50} = 20.00 µg/mL). An interesting observation made was that, both chloroform and ethyl acetate extracts are much more potent than standard

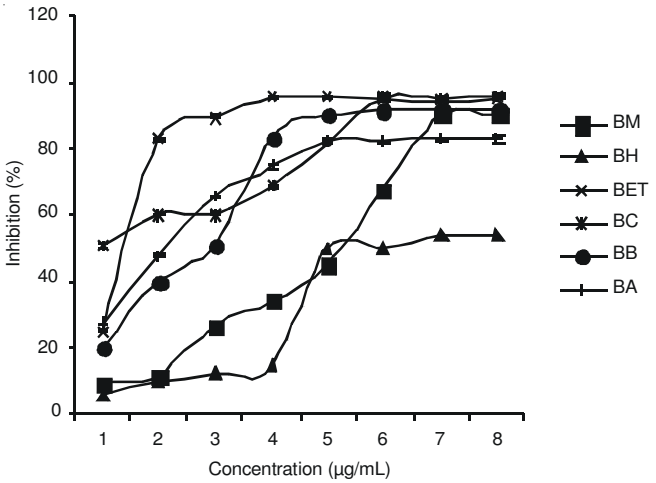

Fig. 1. DPPH radical scavenging activity of different *Terminalia bellerica* bark extracts at multiple concentrations, BM: methanol extract; BH: hexane extract; BET: ethyl acetate extract; BC: chloroform extract; BB: butanol extract; BA: aqueous extract, values are means ± SD of triplicate determinations

antoxidants such as Trolox (IC_{50} = 6.17 µg/mL), gallic acid (IC_{50} = 2.38 µg/mL) and *n*-propyl gallate (IC_{50} = 2.31 µg/mL) (Table-1). This may be attributed to the synergistic effect of mixture of different phytochemicals in plant extracts which may have more therapeutic value than the single antioxidants alone.

Antilipid peroxidation assay: In this assay, antioxidant potential is measured by evaluating the ability of an antioxidant to retard the oxidation of poly unsaturated fatty acids into thiobarbituric acid reactive substances (TBARS). During peroxidation, lipid peroxides are formed followed by the formation of peroxyl radicals. These are decomposed to aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal. Malondialdehyde 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 asses *in vitro* lipid peroxidation due to its simplicity and reproducibility.

The inhibitory effects of *Terminalia bellerica* bark's aqueous and organic extracts, as well as standard antioxidants against lipid peroxidation is shown in Table-1. In initial screening (5 mg/mL) hexane and chloroform extracts showed maximum activity (Fig. 2). When IC_{50} (half efficiency concentration)

TABLE-1
IN VITRO FREE RADICAL SCAVENGING, TOTAL ANTIOXIDANT AND ANTILIPID PEROXIDATION ACTIVITIES OF DIFFERENT EXTRACTS OF *T. bellerica* Roxb. BARK AND STANDARD ANTIOXIDANTS

Samples	Half efficiency concentration (IC_{50})[a]		
	Free radical scavenging activity (µg/mL)	Antilipid peroxidation activity (mg/mL)	ABTS decolorization assay (µg/mL)
Methanol extract	14.37 ± 0.04	2.0 ± 0.01	4.56 ± 0.08[b]
Hexane extract	14.87 ± 0.09	0.52 ± 0.03	15.92 ± 0.04
Ethyl acetate extract	1.54 ± 0.01	2.76 ± 0.06	1.64 ± 0.07
Chloroform extract	1.00 ± 0.08	0.73±0.04	34.31±0.3
n-butanol extract	6.51 ± 0.05	6.00 ± 0.04	4.55 ± 0.07
Aqueous extract	4.54 ± 0.09	8.41 ± 0.09	8.03 ± 0.09
n-propyl gallate	2.31 ± 0.04	0.42 ± 0.02	1.71 ± 0.03
Trolox	6.17 ± 0.02	0.08 ± 0.03	3.86 ± 0.03
Gallic acid	2.38 ± 0.04	2.22 ± 0.02	0.60 ± 0.08

[a]IC_{50} is the efficient concentration of the test samples that decreases 50 % of initial DPPH, TBARS or ABTS concentration. [b]Data are mean (n = 3) ± SD (n = 3, $p < 0.05$).

values were calculated, least amount was required for hexane extract (0.52 mg/mL) indicating that it is most efficient against lipid peroxidation. Chloroform extract (0.73 mg/mL) was also active than other extracts. These values are comparable with standard antioxidants such as propyl gallate (IC_{50} = 0.42 mg/mL) or gallic acid (IC_{50} = 2.22 mg/mL) as shown in Table-1. Methanol and ethyl acetate extracts showed moderate activities, whereas, butanol and aqueous extracts were quite weak (Table-1). From these results we can infer that compounds effective against lipid peroxidation were extracted in hexane and chloroform extract more than in others.

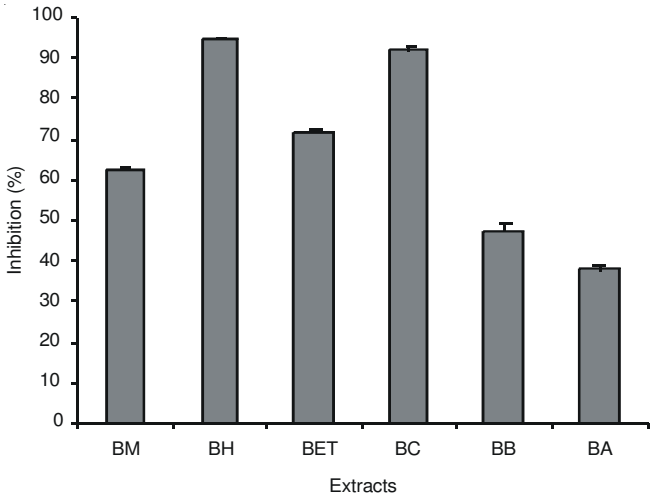

Fig. 2. Antilipid peroxidation capacity of various *Terminalia bellerica* bark extracts, BM: methanol extract; BH: hexane extract; BET: ethyl acetate extract; BC: chloroform extract; BB: butanol extract; BA: aqueous extract, values are means ± SD of triplicate determinations

$ABTS^{\cdot+}$ decolorizing assay: This is an inhibition assay which measures the extend of antioxidant potential by electron donation of antioxidants to pre-formed $ABTS^{\cdot+}$ radical cations. The antioxidant components of plant extracts having a redox potential lower than that of $ABTS^{\cdot+}$ radical cations decolorize the colour of the radical proportionate to their amount [21]. The method is rapid, sensitive and avoids undesired reactions, especially interference by endogenous peroxidase in samples, thus determination of antioxidant activity of hydrophilic plant extracts is more accurate.

Trolox equivalent antioxidant capacity (TEAC) values were obtained by calculating the percentage inhibition of each sample and comparing with Trolox, a standard antioxidant. Fig. 3 presents that each extract showed varied proportions of TEAC values. Highest level was achieved by the ethyl acetate extract where as chloroform and hexane extracts are among the low achievers. The same pattern of antioxidant potential was obtained in IC_{50} values. Results showed that ethyl acetate extract showed maximum antioxidant activity (IC_{50} = 1.64 µg/mL), which had a value parallel to *n*-propyl gallate (standard antioxidant, IC_{50} = 1.71 µg/mL) and much better than Trolox (standard antioxidant, IC_{50} = 3.86 µg/mL). Methanol and *n*-butanol extracts exhibited similar level of antioxidant potential (IC_{50} = 4.55 µg/mL). Contrary to good performance in DPPH and antilipid peroxidation asssays, chloroform and hexane extracts were very poor in scavenging $ABTS^{\cdot+}$ radical cation (Table-1).

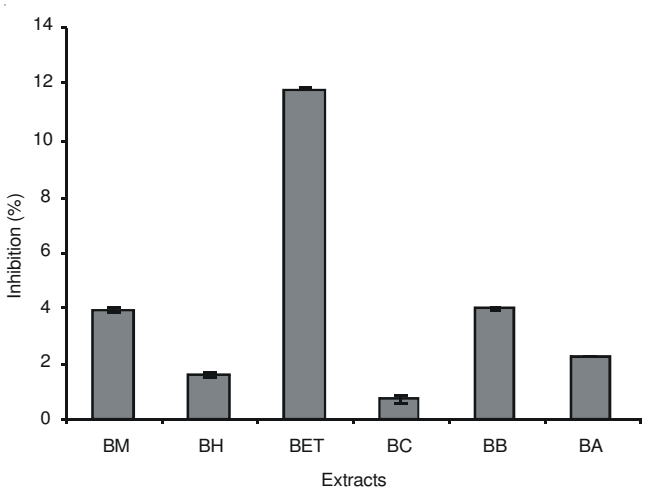

Fig. 3. TEAC values (m mol Trolox eq/g dry wt.) of different *Terminalia bellerica* bark extracts, BM: methanol extract; BH: hexane extract; BET: ethyl acetate extract; BC: chloroform extract; BB: butanol extract; BA: aqueous extract, values are means ± SD of triplicate determinations

Total antioxidant capacity assay: The total antioxidant activities of different extracts were determined by the formation of phosphomolybdenum complexes. The antioxidants in the sample reduce Mo(VI) to Mo(V) resulting in the formation of green Mo(V) complex with maximum absorption at 695 nm[15]. The different *Terminalia bellerica* bark extracts showed great diversity among the antioxidant potential level. Highest antioxidant index was achieved by chloroform extract (5.11 mM AAE/g). Total antioxidant activity of chloroform extract was much better than Trolox and *n*-propyl gallate, used as standard antioxidants. Methanol and aqueous extracts also showed good total antioxidant capacities (Table-2). However, hexane extract exhibited quite less antioxidant activity as compared to other extracts (Table-2).

TABLE-2
IN VITRO TOTAL ANTIOXIDANT ACTIVITIES OF DIFFERENT EXTRACTS OF *T. bellerica* Roxb. BARK AND STANDARD ANTIOXIDANTS AS ASCORBIC ACID EQUIVALENTS[a]

Extracts	Total antioxidant activity (µmol/g)
Methanol extract	4.54 ± 0.8
Hexane extract	1.58 ± 0.4
Ethyl acetate extract	2.27 ± 0.7
Chloroform extract	2.12 ±0.3
n-Butanol extract	5.11 ± 0.2
Aqueous extract	3.40 ± 0.09
n-Propyl gallate	4.54 ± 0.3
Trolox	3.97 ± 0.6
Gallic acid	11.35 ± 0.8

[a]Data are mean (n = 3) ± SD (n = 3, $p < 0.05$).

Total phenolic and flavonoid contents: Plants phenolics have become popular natural antioxidants due to their strong free radical neutralization potential. Phenolic compounds may be simple one with single aromatic ring, bearing one or more hydroxyl groups or polyphenols with two phenolic subunits (flavonoids) or three or more phenol subuints (tannins)[22]. Flavonoids, constitute a special class of polyphenols with C6-C3-C6 flavone skeleton. It is reported that they are strong antioxidant due to their ability to scavenge free radicals. They

also interact with other antioxidants to facilitate the localization and mobility of antioxidants at the microenvironment[23].

The total phenol assay by Folin-Ciocalteu reagent (FCR) is used to quantify total phenolics. Under basic conditions, phenolic compounds dissociate to form phenolate ion which reduces Folin-Ciocalteu reagent. The blue compound is formed between phenolate and Folin-Ciocalteu reagent is measured spectrophotometrically. Chloroform extract contained highest phenolic content (2.57 mM GAE/g, Table-3). Maximum amount of flavonoid was also present in this extract as shown by the results (2.94 mM GAE/g). Ethyl acetate extract also grabed decent amount of phenolic and flavonoid content. Least quantities of phenolics and flavonoids were analysed in butanol and aqueous extracts. There is positive correlation between phenolic and flavonoid content and antioxidant activities of these extracts. It has been reported that phenolic and flavonoid compounds are the main antioxidant compounds of plants[24]. In most of the assays performed, ethyl acetate and chloroform extracts had highest antioxidant activities which may be related to their high phenolic and flavonoid contents.

TABLE-3
CONTENTS OF TOTAL PHENOLICS AND FLAVONOIDS EXPRESSED AS GALLIC ACID AND QUERCETIN EQUIVALENTS, RESPECTIVELY

Samples	Total phenolics (mM GAE/g)[a]	Total flavonoids (mM QE/g)[b]
Methanol extract	1.20 ± 0.04[c]	1.09 ± 0.04
Hexane extract	1.47 ± 0.02	2.51 ± 0.07
Ethyl acetate extract	1.97 ± 0.01	2.68 ± 0.06
Chloroform extract	2.57 ± 0.08	2.94 ± 0.04
n-Butanol extract	1.07 ± 0.01	0.44 ± 0.03
Aqueous extract	1.14 ± 0.03	0.231 ± 0.02

[a]Milli mole gallic acid equivalents per gram. [b]Milli mole quercetin equivalents per gram. [c]Data are mean (n = 3) ± SD (n = 3, $p < 0.05$). All calculations are made on dry mass basis. GAE, gallic acid equivalents; QE, quercetin equivalents.

Conclusion

The present study has shown for the first time that organic and aqueous extracts of *Terminalia bellerica* bark possess potent free radical scavenging and antioxidant activity. High phenolic and flavonoid contents value suggests that they have a prominent role to the antioxidant index and hence therapeutic benefits of *Terminalia bellerica* bark. These results make *Terminalia bellerica* bark a competent source of natural antioxidants.

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