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Antioxidant, DNA protective and antibacterial activities of *Terminalia bellerica* extracts

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Terminalia bellerica Roxb., commonly known as Beleric myrobalan, is a well-known large deciduous tree with various pharmaceutical properties. However, scientific information on *T. bellerica*, growing in India, as regards to its phytochemical constituents and pharmacological properties is very limited. With this in view, this study investigated the antioxidant, DNA protective and antibacterial activities of different parts (fruit pulp, seed and bark) of *T. bellerica*. Methanol (70%) and water (100% distilled water) were used for the extraction and analysis of total phenolic contents (TPC). Plant extracts were further screened for antibacterial activity against both gram (+) and gram (-) bacteria and minimum inhibitory concentration (MIC) values were calculated. Amongst the tested extracts, methanolic extract contained more TPC than aqueous extract. Methanolic fruit pulp (MEFP) showed lower IC_{50} (118.7 μg/ml) for free radical, (77.65 μg/ml) superoxide anion radical, (73.76 μg/ml) hydroxyl radical scavenging activity, (115.6 μg/ml) lipid peroxidation and (184.98 μg/ml) ferric thiocyanate assay. Methanolic extract also exhibited more potential DNA protective and antibacterial activity than aqueous extract. Furthermore, the correlation between TPC and antioxidant studies revealed that phenolics are mainly responsible for antioxidant, DNA protective and antibacterial activities of *T. bellerica*. This study suggests that the methanolic extract of *T. bellerica* could be a potential source of natural antioxidants.

Key words: *Terminalia bellerica*, antioxidative, DNA protective, antibacterial.

INTRODUCTION

Reactive oxygen species (ROS) cause severe damage to the cells of the body. This damage can be to the DNA, proteins and other macromolecules. Oxidative stress, caused by an imbalance between antioxidant systems and the ROS, seems to be associated with many multifactorial diseases, especially cancers, cardiovascular diseases and inflammatory disorders (Reuter et al., 2010; Balmus et al., 2016). The increase in ROS generation or decreased antioxidant availability results in a net increase in intracellular oxidative damage. The mechanism of action of many synthetic antioxidants involves free radical scavenging property, which protects against oxidative damage, but has adverse side effects (Yazdanparast and Ardestani, 2007). The synthetic antioxidant may cause cellular toxicity; however, the alternative is the consumption of natural antioxidants from various food

supplements and traditional medicines (Yazdanparast et al., 2008).

Terminalia bellerica Roxb (Combretaceae), is a large deciduous tree found throughout India. It is an integral part of ancient formulation, *Triphala* which is used for a variety of ailments. It has been valued in Ayurvedic and traditional system of medicine because of its many pharmacological properties such as anti-inflammatory, immunomodulatory, anticancer, hepatoprotective and antioxidant activities (Rashed et al., 2014; Saraphanchotiwitthaya and Ingkaninan, 2014; Ghate et al., 2014; Gupta et al., 2015; 2016). Gas chromatography mass spectrometry (GC-MS) analysis revealed that *T. bellerica* contains various polyphenolic and other bioactive compounds such as gallic acid, quinic acid (chlorogenic acid), ethyl galate, 9,12 octadecadienoic acid, glucopyranose, sitosterol, protein, tannins, galactose, glucose, mannitol, fructose, ramnose etc. (Gupta et al., 2016). Antioxidant activities increase proportionally with the phenolic content, primarily because of their redox properties (Gupta et al., 2015, 2016). Polyphenolic compounds provide protection against oxidative damage and lower the risk of various oxidative stress associated degenerative diseases by acting as potent free-radical scavengers (Pandey and Rizvi, 2009). This study is focused on the evaluation of the antioxidant, DNA damage protective and antibacterial activities of methanolic and aqueous extracts of *T. bellerica* and correlation of antioxidant studies with total phenolic content (TPC).

MATERIALS AND METHODS

Plant materials

Plant materials were collected (November, 2014) from herbal garden of Narendra Dev University of Agriculture and Technology Kumarganj, Faizabad, India and identified at the Botany Division, CSIR-Central Drug Research Institute, Lucknow, India and the voucher specimens (2322 CSIR-CDRI) were submitted in CDRI herbarium.

Chemicals and reagents

Quercetin, gallic acid, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and thiobarbituric acid (TBA), ethidium bromide was purchased from Sigma-Aldrich, St. Louis, USA. Ascorbic acid, Folin Ciocalteau's phenol reagents were the product of E. Merck, Mumbai, India. Nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$) and sodium dodecyl sulphate (SDS) were purchased from Sisco Research Laboratories (SRL), India. All other reagents and chemicals used were of analytical grade.

Extraction procedure

Twenty grams of dried and powdered plant sample of *T. bellerica* from fruit pulp, seed and bark were extracted using 70% methanol (in distilled water) and water (100% distilled water) for overnight shaking at room temperature. The methanolic fruit pulp (MEFP), seed (MES), bark (MEB) and aqueous fruit pulp (AQFP), seed (AQS) bark (AQB) extracts were separated from the residues by filtering through Whatman No. 1 filter paper. The residues were extracted until decoloration with the same fresh solvent and extracts combined. The combined extracts were concentrated and freed of solvent under reduced pressure at 40°C by using a rotary evaporator and lyophilized till dryness. The dried crude concentrated extracts were stored at -4°C and used for the antioxidant activity determination (Gupta et al., 2015).

Antioxidant studies

Total phenolic content (TPC)

TPC of powdered plant material was measured by the method of Ragazzi and Veronese (1973). The TPC was reported as mg of gallic acid equivalent (GAE)/g of dry weight.

Free radical scavenging activity (FRSA)

FRSA of the extracts was measured using DPPH stable radical according to the method of Yen and Duh (1994). The reduction of the DPPH radical was measured by decrease in absorbance at 515 nm until a stable value was obtained.

Inhibition (%) = [(blank absorbance-sample absorbance)/blank absorbance] × 100

The inhibitory concentration (IC_{50}) which represents the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, representing a parameter widely used to measure the antioxidant activity, was calculated from a calibration curve by linear regression.

Superoxide anion radical scavenging activity (SARSA)

This assay was based on the capacity of the extract to inhibit the reduction of nitro blue tetrazolium (NBT) (Nishikimi et al., 1972). The percentage inhibition (PI) of superoxide ($O_2^{\cdot-}$) generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample.

Reducing power (RP)

RP of the extract was determined using method of ferric reducing-antioxidant power assay (Apati et al., 2003). RP was expressed as ascorbic acid equivalents (1 ASE = 1 mM ascorbic acid). ASE value is inversely proportional to RP.

Lipid peroxidation (LPO)

A modified thiobarbituric acid-reactive species (TBARS) assay method of Ohkawa et al. (1979) was applied to measure the LPO formation. The absorbance was measured at 532 nm by UV-Vis spectrophotometer (Labtronics, model LT-2910).

Hydroxyl radical scavenging activity (HRSA)

$OH^{\cdot}$ was measured by the method of Halliwell et al. (1987). The reaction mixtures (50 µM ascorbic acid, 20 µM FeCl3, 2 mM EDTA,
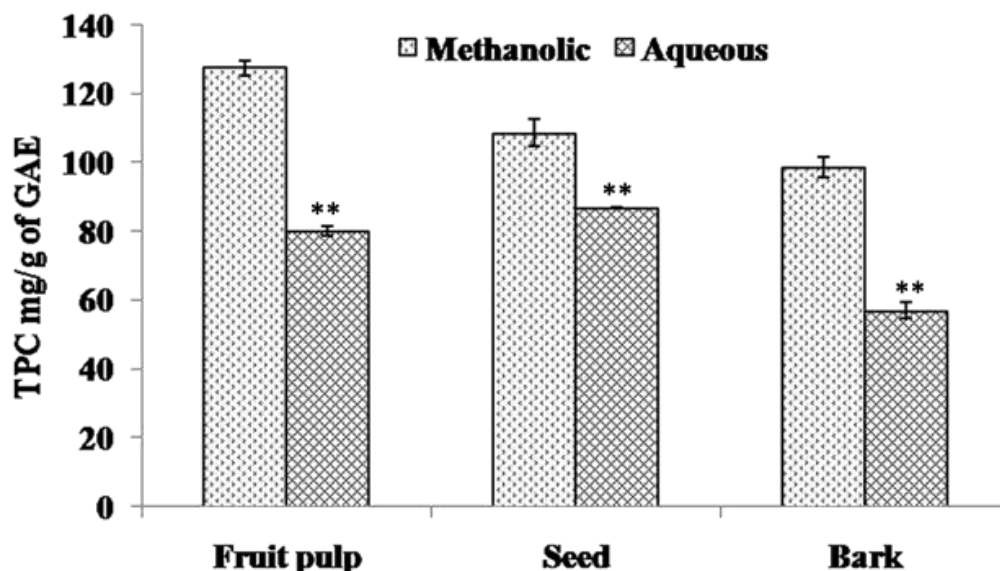
Figure 1. Total phenolic content of methanolic and aqueous extracts of *T. bellerica.* Values are mean±SD of three replications (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. **, Results were considered significant at P < 0.01, when compared with methanolic extracts.

1.42 mM H_2O_2 and 2.8 mM deoxyribose) were prepared with different concentrations of the plant extracts. The mixture was heated in a boiling water bath for 30 min. It was cooled and the absorbance was taken at 532 nm.

Ferric thiocyanate assay (FTC)

The reaction mixture (different concentrations of plant extracts, linoleic acid (200 µl) and phosphate buffer 400 µl, pH 7.4) was incubated at 40°C for 15 min. Red color developed was measured at 535 nm (Tsuda et al., 1994).

DNA damage assay

The DNA damage assay was performed using supercoiled pBR322 plasmid DNA by method of Lee et al. (2002).

Antibacterial study

Bacterial culture

Clinical isolates of bacterial strains were obtained from the Department of Microbiology, Dr. Ram Manohar Lohia Avadh University, Faizabad, Uttar Pradesh, India. Bacterial strains were confirmed by using staining and biochemical reactions (Gaur et al., 2015). All the test strains were maintained at 4°C on nutrient agar (Hi-media, Mumbai, India).

Antibacterial activity assay

Broth microdilution method

The broth microdilution method was carried out in a 96-well microtiter plate to determine the minimum inhibitory concentration

(MIC). The different concentrations of compounds (200, 150, 100 and 50 µg/ml) were diluted in Mueller Hinton broth and the final volume was maintained upto 200 µl. The final concentration of DMSO was less than 1%. 5 µl of an overnight grown bacterial culture was added to the test medium to bring the final inoculum size to 1×10^5 cfu/ml (Kannan et al., 2006). The agar plates were incubated at 37°C for 24 h and the absorbance was read at 600 nm. The percent growth inhibition was calculated by comparison with a control using the formula indicated below. The lowest concentration of the compound that inhibits the complete growth of the bacterium was determined as the MIC.

$$\% \text{ of growth inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Statistical analysis

All analytical data were subjected to an analysis of variance (ANOVA). Each value is mean ± standard deviation (SD) of three replications (n=3). Statistical analysis was conducted using prism software (GraphPad prism software version 3.0, USA). The results obtained were considered statistically significant at *P<0.05 and **P<0.01. The regression equation and R^2 value were calculated by plotting a graph showing the TPC on the x axis and the antioxidant deciding parameters on the y axis, using MS office excel 2007.

RESULTS AND DISCUSSION

Total phenolic content (TPC)

The TPC in methanolic and aqueous extracts of *T. bellerica were* in the range of 98.68 to 127.60 mg/g GAE and 56.97 to 80.11 mg/g GAE (Figure 1). The highest value of TPC was present in MEFP (127.60 mg/g GAE)
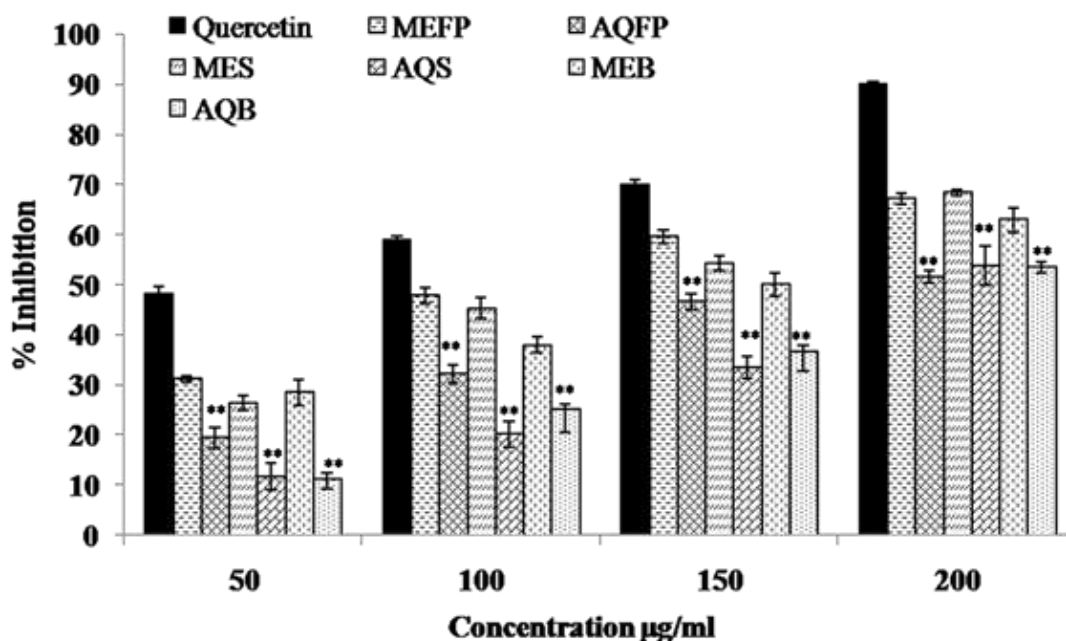
Figure 2. Free radical scavenging activity of methanolic and aqueous extracts of *T. bellerica* and standard quercetin against DPPH radicals at varying concentrations. Values are mean ± SD of three replications (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. **, Results were considered significant at P < 0.01 when compared with methanolic extracts.

followed by MES (108.58 mg/g GAE) and MEB (98.68 mg/g GAE) which signifies its high antioxidant activity than aqueous extracts.

Phenols and polyphenols are the most abundant chemical constituents in plants. The antioxidant properties of phenolic compounds originate from their properties of donating e^- to free radicals to stabilize them. Therefore, the quantity of TPC in methanolic and aqueous extracts of *T. bellerica* was determined (Figure 1) in order to determine the antioxidant capacity of plant extracts (Jang et al., 2010; Sampath Kumara et al., 2012). According to Shahriar et al. (2012) and Venkatesan et al. (2017) hexane and chloroform extracts of *Terminalia arjuna* fruit (73.00 and 61.72 mg/g of GAE) and *Terminalia chebula* bark (28.68 and 62.68 mg/g of GAE) exhibited lower TPC in comparison to the reported values of methanolic extracts in this study.

**Free radical scavenging activity (FRSA)**

Methanolic and aqueous extracts of *T. bellerica* were examined for their potential to scavenge free radicals and measured as percentage inhibition (PI). Methanolic extracts were considered to be most potent free radical scavenger than aqueous extracts and its value of inhibition ranged from 62.95 to 68.34% (Figure 2). FRSA values of methanolic extracts were in the following order MES (68.34%) > MEFP (68.34%) > MEB (62.95%) while

for the aqueous extracts the order was AQS (53.82%) > AQB (53.50) > AQFP (51.58%). The IC_{50} value of MEFP was 118.7 μg/ml whereas, in the case of MES and MEB, it was 130 and 146.7 μg/ml, respectively.

The $DPPH^{\bullet}$, at its maximum wavelength at 517 nm, can easily receive an electron or hydrogen from antioxidant molecules to become a stable diamagnetic molecule as DPPH-H (Soares et al., 1997). Owing to the $DPPH^{\bullet}$ ability to bind H, it is considered to have a radical scavenging property. Discoloration occurs due to the decreasing quantity of $DPPH^{\bullet}$ into reaction mixture which reflects the FRSA of tested extract (Guo et al., 2007). The radical scavenging activity of the methanolic extract was found significantly higher in comparison to the aqueous extract.

Superoxide anion radical scavenging activity (SARSA)

The antioxidant potential of methanolic extracts against $O_2^{\bullet-}$ was considered to be significant in comparison to the standard quercetin. The result presented in Figure 3 shows that methanolic extracts inhibited NBT reduction higher than aqueous extracts. The order of SARSA of methanolic and aqueous extracts were MEFP (75.86%) > MEB (73.86%) > MES (72.99%) and AQFP (62.91%) > AQS (56.82%) > AQB (56.19) in comparison to standard quercetin (91.77%) at 200 μg/ml concentration. The IC_{50} values at which methanolic extracts showed significant
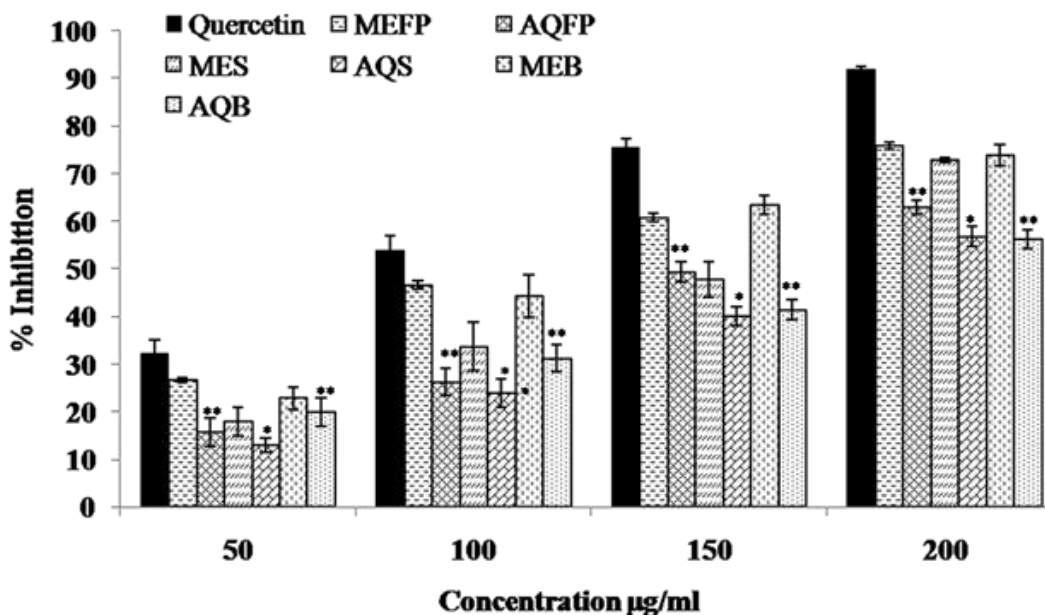
Figure 3. Inhibitory effects of methanolic and aqueous extracts of *T. bellerica* and standard quercetin on superoxide anion radical at varying concentrations. Values are mean±SD of three replicates (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. *,**, Results were considered significant at P < 0.05 and P < 0.01, respectively when compared with methanolic extracts.

SARSA were found as: 77.65 (MEFP), 155.53 (MEB) and 191.75 (MES) µg/ml.

The $O_2^{\cdot-}$ is a precursor to active free radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage (Halliwell and Gutteridge, 1984). It has been implicated in several pathophysiological processes due to its transformation into more ROS such as $OH^{\cdot}$, H_2O_2, $^1O_2$ and oxidizing agents that initiate LPO (Wickens, 2001; Liu et al., 1997) and damage to protein and DNA (Pietta, 2000). $O_2^{\cdot-}$ derived from dissolved oxygen by PMS-NADH coupling reaction and reduces NBT in this system. In this method, $O_2^{\cdot-}$ reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm. Plant extracts containing antioxidants are able to inhibit the formation of blue tetrazolium complex (Cos et al., 1998; Parejo et al., 2002). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of $O_2^{\cdot-}$ in the reaction mixture. Figure 3 clearly indicates that *T. bellerica* is a potent $O_2^{\cdot-}$ scavenger. According to Venkatesan et al. (2017) methanolic extracts of *T. bellerica* bark showed $O_2^{\cdot-}$ inhibition at IC_{50} 166.29 µg/ml which is almost equal to the study reported value of 155.53 µg/ml.

Lipid peroxidation (LPO)

Studies on the inhibition of LPO in the presence of extracts were carried out and expressed as PI. The

methanolic and aqueous extracts prevented LPO induced by $FeSO_4$ and PI varied from 63.02 to 71.51 and 54.40 to 60.75%, respectively. Maximum LPO inhibition was shown by MEFP (71.51%) than MES (64.70%) and MEB (63.02%) in a concentration dependent manner at 100 to 400 µg/ml in comparison to standard (95.39%). Methanolic extracts exhibited anti-LPO activity with lower IC_{50} values 115.6 (MEFP), 130.3 (MES) and 144.5 (MEB) µg/ml compared to aqueous extracts 290 (AQFP), 300 (AQS) and 331 (AQB) µg/ml (Figure 4).

In LPO assay, antioxidant potential is measured by evaluating the capability of the test sample to hamper the oxidation of polyunsaturated fatty acids (PUFA) into TBARS. Peroxidation generates peroxyl radicals which decompose to MDA. It forms a stable product with TBA, which serve as a mean to quantify the level of peroxidation (Lugasi, 1997). This assay is a very useful mean to assess LPO *in vitro* due to its simplicity and reproducibility. According to Sherin et al. (2015) hexane, chloroform and ethyl acetate extracts of *T. bellerica* leaf showed 50% inhibition at concentrations of 0.350, 0.280 and 0.520 mg/ml which was much higher than the reported values for methanolic extracts.

Hydroxyl radical scavenging activity (HRSA)

Methanolic and aqueous extracts were further studied for their ability to chelate iron and/or to scavenge $OH^{\cdot}$ by using deoxyribose degradation assay. The methanolic
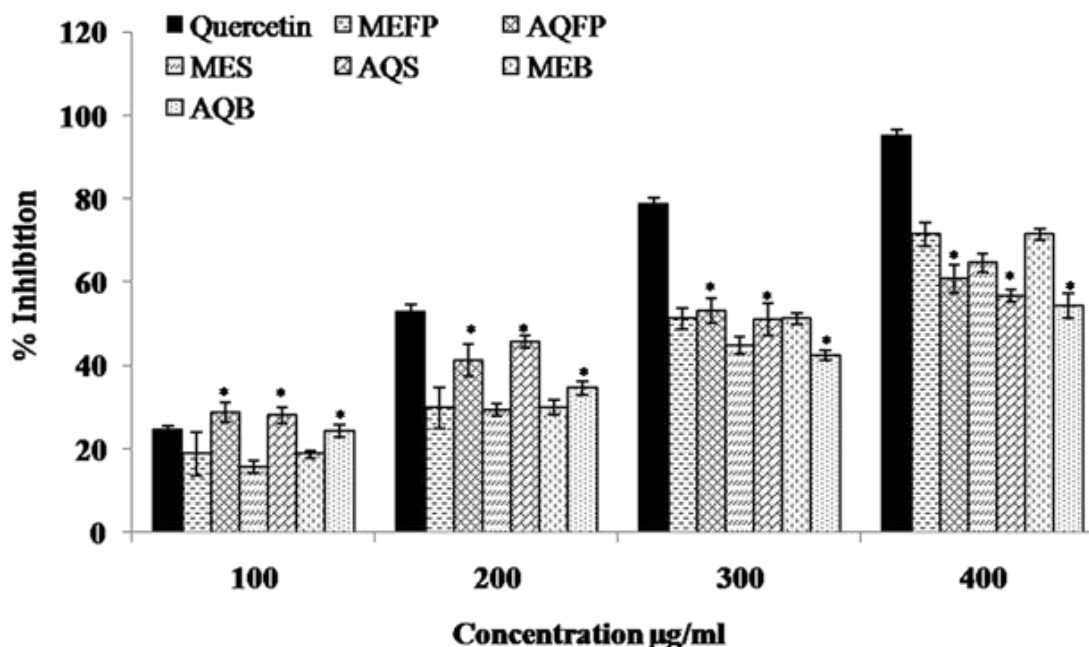
Figure 4. Inhibitory effects of methanolic and aqueous extracts of *T. bellerica* and standard quercetin on LPO using egg homogenate as a lipid-rich source at varying concentrations. Values are mean±SD of three replicates (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. **, Results were considered significant at P < 0.05 when compared with methanolic extracts.

extracts were found to be most potent OH$^•$ scavenger with inhibition of 70.51 to 74.86% than aqueous extracts of 52.70 to 56.23% (Figure 5). The biochemical studies revealed that methanolic extracts caused a concentration-dependent (50 to 200 µg/ml) inhibition of deoxyribose oxidation. The IC_{50} value at which methanolic and aqueous extracts showed HRSA was found to be 73.76 (MEFP), 81.99 (MES), 90.76 (MEB) µg/ml and 425.9 (AQFP), 488.0 (AQS) and 548.0 (AQB) µg/ml, respectively.

The OH$^•$ induced oxidative damage to DNA, lipids and proteins are involved in various neurodegenerative and cardiovascular diseases (Spencer et al., 1994). The HRSA of the extracts was determined by its ability to compete with deoxyribose for OH$^•$. In this assay, 2-deoxy-2-ribose was oxidized when exposed to OH$^•$ generated by the fenton-type reaction. The oxidative degradation can be detected by heating the products with TBA under acidic conditions to develop a pink chromogen with a maximum absorbance at 532 nm (Marnett, 1999).

Ferric thiocyanate (FTC)

In tested extracts, methanolic extracts showed maximum inhibition (76.08 to 79.78%) than aqueous extracts (53.00 to 54.80%) to inhibit the production of free radicals which initiate the oxidation of lipids and proteins. The inhibition was increased with increasing concentration of the

extracts from 50 to 200 µg/ml in the reaction mixture and the maximum inhibition was shown by MES (79.78%) in comparison to quercetin (92.12%) (Figure 6). The IC_{50} of methanolic extracts was found to be in the range 184.98 (MEFP) > 212.5 (MES) > 221.4 (MEB).

The antioxidant activity of plant extracts was further determined by the inhibition of peroxidation of linoleic acid system using thiocyanate method (Yen et al., 2000). Linoleic acid is a PUFA which upon oxidation forms peroxides that oxidize Fe^{2+} to Fe^{3+}. Fe^{3+} forms complex with thiocyanate ion (SCN$^-$), whose concentration is determined spectrophotometrically by measuring the absorbance at 535 nm. Higher absorbance denotes higher concentration of peroxides formed during reaction; consequently lower will the antioxidant activity. According to Sultana et al. (2007) methanolic extracts of *T. arjuna* bark exhibited 44.4% inhibition at 200 µg/ml concentration which was found much lower than the bark extracts tested in the study.

Reducing power (RP)

The RP of a compound may act as a significant indicator of its potential antioxidant activity. With regards to RP, higher reducing capacity might be attributed to the higher amount of phenolic compounds. Methanolic extracts exhibited significantly high Fe^{3+} to Fe^{2+} transformation capacity (6.73 to 4.68 ASE/ml) compared to aqueous
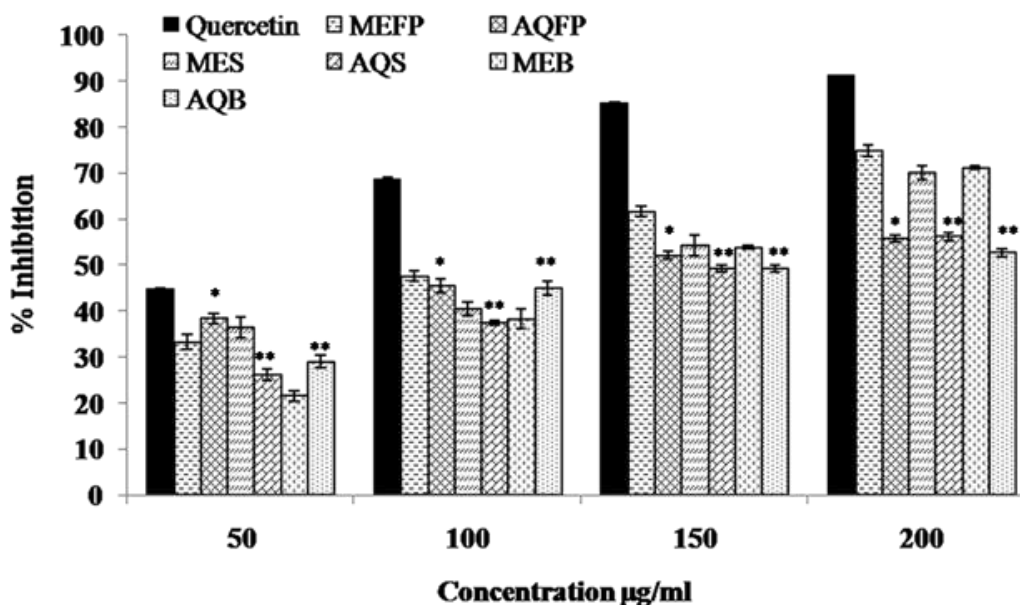
Figure 5. Inhibitory effects of methanolic and aqueous extracts of *T. bellerica* and standard quercetin on hydroxyl radical mediated deoxyribose degradation at varying concentrations. Values are mean ± SD of three replicates (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. *,**, Results were considered significant at P < 0.05 and P < 0.01, respectively when compared with methanolic extracts.
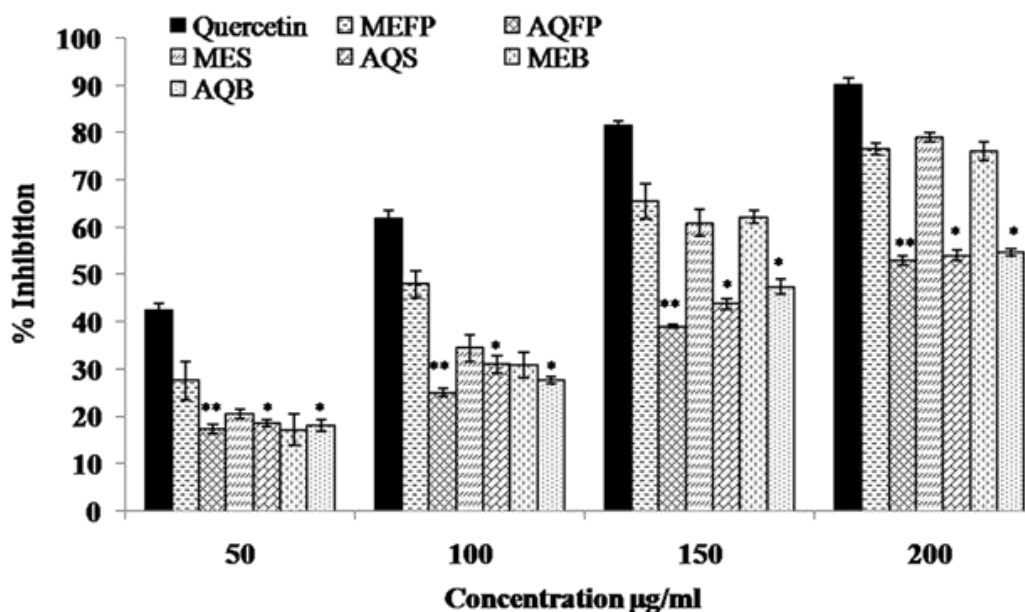

Figure 6. Inhibitory effects of methanolic and aqueous extract of *T. bellerica* and standard quercetin on ferric ion chelation by ferric thiocyanate assay method at varying concentrations. Values are mean ± SD of three replicates (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. *,**, Results were considered significant at P < 0.05 and P < 0.01, respectively when compared with methanolic extracts.

extracts (10.03 to 7.25 ASE/ml (Figure 7). In the RP assay, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{3+} reduction can be then monitored by measuring the formation of $(Fe^{3+})_4[Fe^{2+}(CN)_6]_3$ complex (pearl's Prussian blue) at 700 nm, indicates an
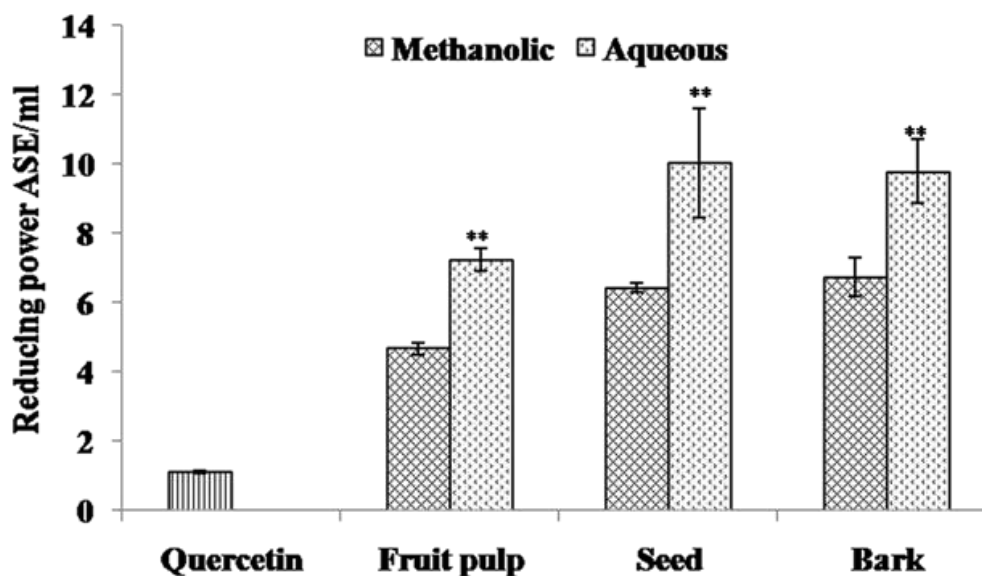
Figure 7. Reducing power (ASE/ml) of methanolic and aqueous extracts of *T. bellerica* and standard quercetin. Values are mean ± SD of three replicates (n=3). MEFP: Methanolic fruit pulp; MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp; AQS: Aqueous seed; AQB: Aqueous bark. **Results were considered significant at P < 0.01 when compared with methanolic extracts.
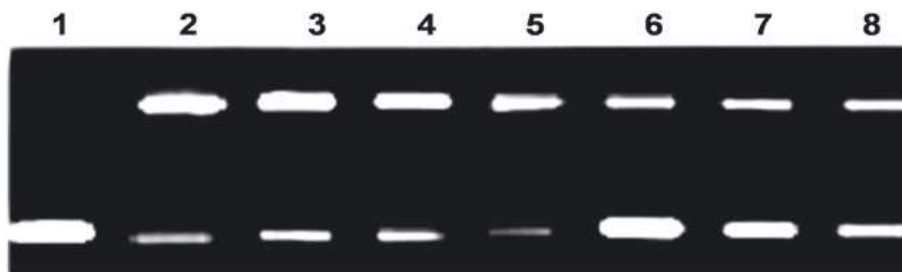
Figure 8. DNA damage protection offered by methanolic (ME) and aqueous (AQ) extracts of *T. bellerica* fruit pulp, seed and bark on native pBR322 DNA nicking caused by hydroxyl radicals. Lane 1: DNA (pBR322 plasmid DNA alone), Lane 2: DNA + Fenton reagent, Lane 3: DNA + Fenton reagent + 50 µg/ml AQFP, Lane 4: DNA + Fenton reagent + 50 µg/ml AQS, Lane 5: DNA + Fenton reagent + 50 µg/ml AQB, Lane 6: DNA + Fenton reagent + 50 µg/ml MEFP, Lane 7: DNA + Fenton reagent + 50 µg/ml MES, Lane 8: DNA + Fenton reagent + 50 µg/ml MEB.

increase in reductive ability (Djeridane et al., 2006). Fe^{3+} reduction is often used as a significant indicator of electron donating activity which is an important mechanism of phenolic antioxidant action (Nabavi et al., 2009).

DNA damage protective activity

Hydroxyl radical scavenging activity of *T. bellerica* extracts was further explored by the protection of plasmid pBR322 DNA against Fenton reagent. When DNA is exposed to Fenton reaction, H_2O_2 will be generated to hydroxyl radicals (OH·), and then the supercoiled form of DNA would cleave to give rise to a linear form. The DNA

damage protective activity of the plant extracts showed a significant protection of supercoiled form compared to open circular form, at 50 µg/ml. Addition of Fenton reagent to a mixture containing DNA and *T. bellerica* extracts showed a significant reduction in the formation of open circular and linear forms and increased supercoiled or native forms of plasmid DNA as shown in Figure 8.

OH· can be formed by the Fenton reaction, capable of damaging DNA and protein in living cells (Rollet-Labelle et al., 1998). In the present experiment, plasmid pBR322 DNA was treated with Fenton reagent. It interacts with the supercoiled DNA and cleaved into open and nicked circular forms. The results were obtained through gel electrophoresis (Figure 8) showed that the methanolic

Table 1. Minimum inhibitory concentration (MIC) (µg/ml) of methanolic and aqueous extracts of *Terminalia bellerica*.

Bacteria	Extract (µg/ml)	Plant extract							
		MEFP	MES	MEB	AQFP	AQS	AQB	CN (30 µg/disc)	TE(30 µg/disc)
Gram (+)									
Sa	200	19±0.5	19±0.1	21±0.1	19±0.2	16±0.5	20±0.5	15±0.5	17±0.3
	150	22±0.4	23±0.6	28±0.2	23±0.3	20±0.2	24±0.3	21±0.3	22±0.4
	100	29±0.3	28±0.5	34±0.4	31±0.4	25±0.3	29±0.5	26±0.2	27±0.5
	50	34±0.2	33±0.5	39±0.2	35±0.3	31±0.2	33±0.5	31±0.2	34±0.5
Se	200	09±0.3	16±0.3	14±0.3	15±0.3	14±0.1	17±0.1	12±0.2	12±0.1
	150	12±0.5	28±0.2	25±0.4	23±0.0	20±0.2	24±0.2	17±0.3	17±0.3
	100	15±0.1	33±0.1	30±0.1	30±0.1	27±0.5	29±0.5	20±0.4	24±0.4
	50	30±0.5	38±0.1	35±0.1	36±0.0	33±0.1	34±0.3	24±0.4	27±0.4
Bc	200	36±0.1	54±0.1	73±0.1	76±0.1	28±0.1	38±0.1	23±0.4	22±0.3
	150	49±0.1	61±0.1	89±0.1	82±0.1	45±0.2	47±0.2	28±0.2	26±0.4
	100	78±0.5	109±0.2	108±0.1	98±0.0	57±0.5	67±0.5	33±0.3	31±0.2
	50	94±0.5	124±0.2	128±0.2	119±0.0	76±0.1	86±0.1	38±0.3	36±0.2
Gram (-)									
Pa	200	31±0.1	37±0.1	39±0.1	31±0.1	34±0.1	34±0.1	25±0.5	28±0.4
	150	35±0.1	45±0.1	56±0.1	48±0.1	50±0.2	48±0.2	33±0.3	36±0.3
	100	52±0.5	63±0.5	71±0.0	67±0.0	63±0.5	65±0.5	39±0.5	41±0.3
	50	74±0.1	85±0.1	88±0.1	89±0.1	82±0.1	82±0.1	48±0.5	53±0.2
Ec	200	08±0.1	09±0.1	10±0.1	11±0.1	10±0.1	11±0.1	08±0.3	08±0.5
	150	17±0.1	19±0.1	20±0.1	19±0.1	18±0.2	20±0.2	12±0.3	15±0.3
	100	23±0.5	29±0.1	28±0.0	24±0.0	25±0.5	28±0.5	19±0.2	21±0.4
	50	29±0.5	36±0.2	39±0.0	37±0.0	34±0.1	38±0.1	28±0.2	28±0.4

Values are mean ± SD of three replicates (n=3). CN: Gentamicin; TE: Tetracycline; MEFP: Methanolic fruit pulp, MES: Methanolic seed; MEB: Methanolic bark; AQFP: Aqueous fruit pulp, AQS: Aqueous seed; AQB: Aqueous bark; Sa: *S. aureus*; Se: *S. epidermidis*; Bc: *B. cereus*; Pa: *P. aeruginosa*; Ec: *E. coli*

extract of *T. bellerica* significantly protected DNA compared to aqueous extracts. As observed in Figure 8, where lane 1 in gel pictures is the positive control with only plasmid (pBR322) DNA in the fully supercoiled form and lane 2 is a negative control containing Fenton's reagent without plant extract and having only the open circular form. The lanes 3 to 8 in the corresponding gel picture signify DNA protective activity with different extracts of 50 µg/ml. Thus, results suggested that methanolic extracts of *T. bellerica* was better than aqueous extracts in retaining the supercoiled form of DNA, thus protecting DNA effectively against oxidative damage. The result was significant in comparison to earlier reported study by Singh et al. (2016).

Antibacterial activity

Minimum inhibitory concentration (MIC)

MIC is defined as a highest dilution or lowest concentration of a chemical that inhibits the growth of bacteria and thereby estimates its antimicrobial activity.

The methanolic and aqueous extract of *T. bellerica* was further subjected to the broth microdilution method to determine the MIC (Table 1). The lowest MIC was observed against *S. aureus, S. epidermidis* and *E. coli* at a concentration of 200 µg/ml. As antimicrobial concentration increases, the turbidity decreases until the MIC is reached and microbes no longer survive. Antimicrobials with low MICs are more effective than those with high MICs, as only a low dosage is necessary to eradicate microbes. This result is in agreement with the report of Kannan et al. (2009) and Bag et al. (2012) studied on *T. chebula* fruits extracts, respectively. According to Bag et al. (2012), *T. chebula* aqueous extracts showed MIC 0.866 mg/ml and 1.65 mg/ml against *E. coli* and *S. aureus* which were much higher than that reported in the tested extracts.

Correlation between antioxidant activities and TPC

Phenolics are the major contributors to the antioxidant activity. The correlation between TPC and FRSA of methanolic and aqueous extracts had a correlation
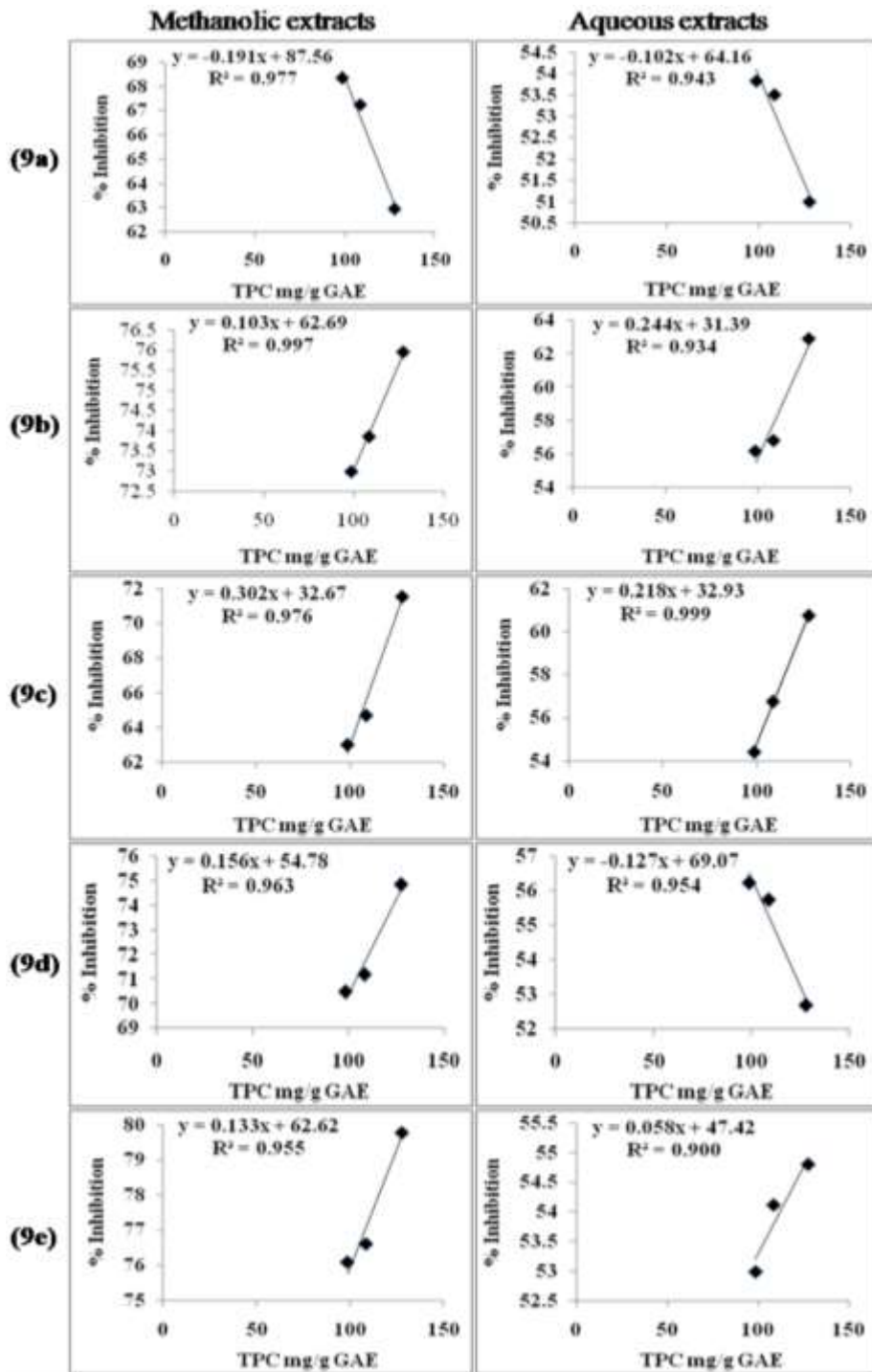
Figure 9. Linear correlation between TPC (x axis) in the plant extracts in relation to their antioxidant activity (y axis). (9a) TPC versus FRSA; (9b) TPC versus SARSA; (9c) TPC versus LPO; (9d) TPC versus HRSA; (9e) TPC versus FTC assay.

coefficient of R^2 = 0.977 (y = -0.191x + 87.56) and R^2 = 0.943 (y = -0.102x + 64.16). A good correlation also exists between TPC and other antioxidant activities (Figure 9).

It was observed that the FRSA of the methanolic and aqueous extracts was positively correlated with their total amount of phenolic compounds (R^2 = 0.977 and 0.943). Many studies in the literature present positive correlations

between the quantity of phenolic compounds and the DPPH[·] scavenging effect (Sagar and Singh, 2011; Liu et al., 2009). In this study as well, a positive correlation was observed between the antioxidant activities and the content of total phenolics.

Conclusion

This study revealed that *T. bellerica* possesses potent free-radical scavenging activities. Overall, methanolic extracts were found to be a good free-radical scavenger and showed high antioxidative, DNA protective and antibacterial activities compared to aqueous extracts. The resulting activities may be attributed to the presence of more phenolic compounds in the methanolic extracts compared to aqueous extracts. Furthermore, it is concluded that *T. bellerica* is a potential candidate for natural antioxidants in food and pharmaceutical industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

ROS, Reactive oxygen species; **PI,** percentage inhibition; **LPO,** lipid peroxidation; **TBARS,** Thiobarbituric acid-reactive species; **MDA,** Malondialdehyde; IC$_{50}$, inhibitory concentration; **O$_2^{\cdot-}$,** superoxide; **OH$^{\cdot}$,** hydroxyl radical; **PUFA,** polyunsaturated fatty acids.

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