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Original Article

IN VITRO ANTIOXIDANT ACTIVITY AND GC-MS ANALYSIS OF THE ETHANOLIC EXTRACTS OF *TERMINALIA BELLERICA* ROXB (BAHEDA)

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ABSTRACT

Objective: To investigate the antioxidant activity of ethanolic extract of *Terminalia bellerica* fruit pulp (TBFP), seed (TBS) and bark (TBB), and identification of phytochemical constituents of said extracts.

Methods: The antioxidant potential of *T. bellerica* (TB) parts was evaluated by free radical scavenging activity (FRSA), superoxide anion radical scavenging activity (SARSA), hydroxyl radical scavenging activity (HRSA) and compared with reference standard quercetin. Lipid peroxidation (LPO), ferric thiocyanate activity (FTC) and reducing power (RP) of the plant extracts were also examined. The phytochemical constituents of said extracts have been quantified by gas chromatography-mass spectrometer (GC-MS) method.

Results: Ethanolic extract of TBFP exhibited high phenolic content (254.33) followed by TBS (227.33) and TBB (185) mg/g of GAE. TBFP showed lowest IC_{50} for FRSA (62 µg/ml), SARSA (39 µg/ml) and HRSA (27 µg/ml) and highest RP (3.39 ascorbic acid equivalents/ml). TBB showed lowest IC_{50} for LPO (48 µg/ml) whereas TBS showed lowest IC_{50} for FTC (109 µg/ml). Predominant phyto-compounds present in TB extracts were quinic acid, gallic acid, ethyl galate, 9, 12 octadecadienoic acid and glucopyranose in varying concentrations as analyzed by GC-MS.

Conclusion: On the basis of correlation of antioxidant studies with total phenolic content (TPC) and GC-MS analysis of different part of TB, it may be concluded that high concentration of total phenolics and other bioactive phytochemicals of TB extracts make it a potential source of nutraceutical antioxidants.

Keywords: Reactive oxygen species, Antioxidant, GC-MS, Phenolics, Phytochemicals

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INTRODUCTION

Terminalia bellerica (TB) Roxb (Combretaceae), is a large deciduous tree and is commonly known as beleric mycobalane. It has been valued in Ayurvedic and traditional system of medicine for treatment of wide range of diseases having many pharmacological properties such as immunomodulatory, anti-inflammatory, anticancer, hepatoprotective and antimicrobial activities [1-5]. It is an integral part of the traditional laxative formulation, *Triphala* which is used for a variety of ailments in Ayurvedic medicines since ancient times. Fruits of TB are purgative, analgesic, anthelmintic and antipyretic, and are also useful in bronchitis, asthma, dyspepsia, piles, diarrhea and cough. Ethanolic extract of TB fruits was reported to enhance glucose uptake over the control group of rats in various muscles cell lines e. g. Vero, L6 and 3T3 [6]. Methanolic extract of TB bark has shown thrombolytic and cytotoxic activities in human blood [7]. Leaf extracts of TB showed promising free radical scavenging activity with inhibitory concentration $IC_{50,}$ (concentration of a drug that is needed for 50% inhibition *in vitro*) of 58 µg/ml [8]. The seed oil is utilized to cure skin diseases, premature graying of hair and can be applied on painful swollen parts. Overall, TB enhances the body resistance against diseases and is used as herbal medicine to get remedies from all the above ailments [9].

Oxidative stresses occur when the concentration of reactive oxygen species (ROS) increases to a level that exceeds the cell's defense capacity. ROS involves both free radicals as superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), per hydroxy ($HO_2^{\bullet}$) and alkoxy ($RO^{\bullet}$) radicals and non-radical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are produced either extracellular in the normal metabolism of mammalians cells by NADPH-oxidase, myelo-peroxidase and nitric oxide synthase enzymes or intracellular as a byproduct of metabolic processes. The controlled generation of ROS in the extracellular space was developed evolutionarily as part of the innate immune response against bacteria and other pathogens. However, excessive release of ROS may also induce deleterious effects; causing damage to host DNA, RNA, proteins and lipids which ultimately result in oxidative stress [10, 11]. Thus oxidative stress is an imbalance situation between oxidants and antioxidants potentially leading to cell damage. It is believed to significantly contribute to the development of a number of diseases like cancers, liver injury, cardiovascular and age-related diseases [12].

Many synthetic antioxidants are currently in use that may cause cellular toxicity, however, there is a growing consumer preference for natural antioxidants because of their good efficacy and lower toxicity. Natural antioxidants have the capacity to improve medicinal values of plants, and acts as nutraceuticals to terminate free radical chain reactions in biological systems, thus may provide additional health benefits to consumers [13]. *T. bellerica*, having shown the potential to cure many diseases, is a fit case for investigation of its phytochemicals which impart antioxidant activity to this plant. The present study has been planned to explore the antioxidant potential of ethanolic extracts of TB fruit pulp, seed and bark along with quantification of important biomolecules in these parts using GC-MS technique. These experiments are expected to provide an insight in to the scientific basis of the healing potential of TB.

MATERIALS AND METHODS

Plant materials

Plant materials were collected from herbal garden of Narendra Dev University of Agriculture and Technology Kumarganj, Faizabad, U. P., India and identified with the help of Dr. MN Srivastava, Senior Scientist, 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) were purchased from Sigma-Aldrich, St. Louis, USA. Ascorbic acid, Folin Ciocalteau's phenol reagents were the product of E. Merk, 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 SRL India. All other reagents and chemicals used were of analytical grade.

Extraction procedure

Twenty grams of the dried and powdered plant sample of *T. bellerica* fruit pulp (TBFP), seed (TBS) and bark (TBB) were extracted with 70% ethanolic solvent (in distilled water) for overnight at room temperature in an orbital shaker. The extracts were separated from the residues by filtering through Whatman No. 1 filter paper. The residues were extracted until discoloration 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 and GC-MS analysis.

Antioxidant studies

Total phenolic content (TPC)

TPC of powdered plant material was extracted with 50% methanol+1% HCl, filtered and made up to 10 ml each with water. TPC was measured with the method of Ragazzi and Veronese [14]. To 0.1 ml plant extract, 0.5 ml of Folin's reagent (1 N) and 1.0 ml of sodium carbonate were added subsequently. The test mixture was mixed properly and kept at room temperature for 30 min and volume was made up to 12.5 ml with distilled water. The absorbance of this solution was measured at 720 nm. 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 by using DPPH stable radical according to the method of Yen and Duh [15]. Each extract (0.1 ml) was added to freshly prepared DPPH solution (6×10^{-5} M in HPLC grade 2.9 ml methanol) and mixed vigorously. The reduction of the DPPH radical was measured by continuous monitoring of the decrease in absorbance at 515 nm until a stable value was obtained.

$$\text{Inhibition (\%)} = [(\text{blank absorbance-sample absorbance})/\text{blank absorbance}] \times 100$$

The 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 NBT by the method of Nishikimi *et al.* [16]. Three milliliters reaction mixture containing a different aliquot of plant extracts (50, 100, 150 and 200 µl) with 0.1 M phosphate buffer (pH 7.8), 60 µM PMS, 468 µM NADH and 150 µM NBT was incubated for 5 min at ambient temperature. Absorbance was read after 6 min at 560 nm using UV-Vis spectrophotometer. The percentage inhibition of $O_2^{\bullet-}$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 extracts was determined by using a slightly modified method of ferric reducing antioxidant power assay [17]. Each extract (1.0 ml) was mixed with 2.5 ml of phosphate buffer (0.1 M, pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide and was incubated at 50 °C for 20 min. After completion of the incubation period, 2.5 ml of 10% (w/v) TCA was added to terminate the reaction. The upper layer (2.5 ml) was diluted with equal volume of deionized water. Finally, 0.5 ml of 0.1% (w/v) $FeCl_3$ was added and after 10 min the absorbance was measured at 700 nm against a blank. RP was expressed as ascorbic acid equivalents (1 ASE = 1 mmol 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.* [18] was applied to measure the LPO formation, using egg homogenate as lipid rich media. Egg homogenate (10% in 0.2 M PBS, 0.5 ml), test extract (0.1 ml) and distilled water (0.85 ml) were mixed in a test tube. Finally, $FeSO_4$ (0.07 M, 0.05 ml) was added to the reaction mixture and incubated at 37 °C temperature for 30 min to induce LPO. Thereafter, acetic acid (20%, 1.5 ml), TBA (0.8% prepared in 1.1% SDS, 1.5 ml) and TCA (20%, 0.05 ml) were added, vortexed and then heated in a boiling water bath for 60 min. After cooling, butanol (5 ml) was added to each tube and centrifuged for 10 min at 3000 rpm. The absorbance of the upper organic layer was measured at 532 nm by UV-Vis spectrophotometer (Labtronics, model LT-2910).

Hydroxyl radical scavenging activity (HRSA)

Hydroxyl radicals were generated by a mixture of Fe^{3+}-EDTA, H_2O_2 and ascorbic acid and, scavenging activity was assessed by monitoring the degraded fragments of deoxyribose, through malondialdehyde (MDA) formation [19]. The reaction mixtures contained ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (2 mmol), H_2O_2 (1.42 mmol), deoxyribose (2.8 mmol) with different concentrations of the plant extracts in a final volume of 1 ml, was incubated at 37 °C for 1 hour and then 1 ml of 2.8% TCA (w/v in water) and 1 ml of 1% TBA (w/v) were added. The mixture was heated in a boiling water bath for 30 min. It was cooled and absorbance was taken at 532 nm.

Ferric thiocyanate assay (FTC)

The reaction mixture containing 400 µl of different concentration of ethanolic plant extracts, 200 µl of diluted linoleic acid (25 mg/ml in 99% ethanol) and 400 µl of 50 mmol phosphate buffer (pH 7.4) was incubated for 15 min at 40 °C. A 100 µl aliquot of this was then mixed with a reaction mixture containing 3 ml of 70% ethanol, 100 µl of ammonium thiocyanate (300 mg/ml in distilled water) and 100 µl of $FeSO_4$. Red color developed was measured at 535 nm [20].

Gas chromatography-mass spectrometry (GC-MS)

Plant samples were further processed for GC-MS (Trace GC ultra TSQ quantum XLS mass spectrometer, Thermo, USA) analysis [21] to identify bioactive phytoconstituents responsible for antioxidant activity. Interpretation on mass-spectrum was conducted using the database of the National Institute of Standards and Technology (NIST). The spectrum of the unknown components was compared with the spectrum of known components stored in the NIST library.

Statistical analysis

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

RESULTS

Total phenolic content

Phenolic compounds may contribute directly to antioxidant potential [22, 23]. Therefore, it would be worthwhile to determine the TPC of TB extracts. The TPC in TBFP, TBS and TBB was 254.33, 227.33 and 185 mg/g of GAE (fig. 1). The highest value of TPC in TBFP showed its high antioxidant potential than TBS and TBB.

Free radical scavenging activity

TBFP, TBS and TBB extracts were examined for their potential to scavenge free radicals and measured as percentage inhibition. TBFP was considered to be most potent significant free radical scavenger

than TBS and TBB and its value of inhibition ranged from 31.66 to 84.16% in a concentration-dependent manner (50-200 µg/ml) (fig. 2). FRSA values were in following order TBFP (84.16%)>TBS (81.45%)>TBB (75.62%) in comparison to standard quercetin (90.25%). The IC_{50} value of TBFP was 62 µg/ml whereas, in the case of TBS and TBB, it was 73 and 106 µg/ml, respectively.

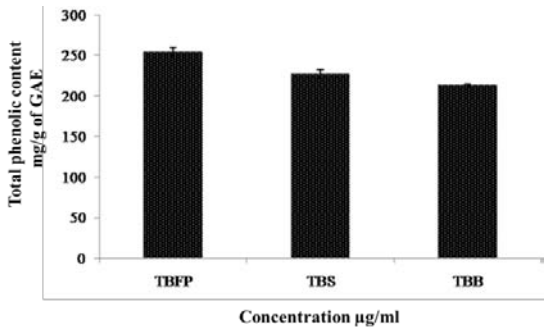

Fig. 1: Total phenolic content of ethanolic extracts of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB). Values are mean±SD of three replications (n=3)

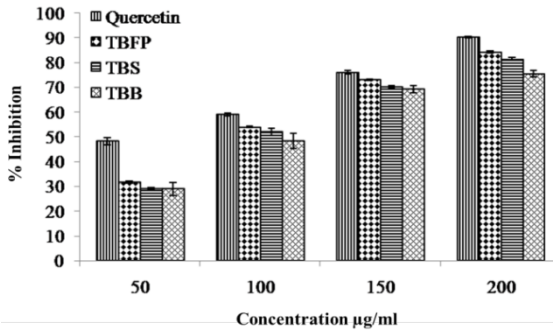

Fig. 2: Free radical scavenging activity of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB) and standard quercetin against DPPH radicals at varying concentrations. Values are mean±SD of three replications (n=3)

Superoxide anion radical scavenging activity

The antioxidant potential of TBFP, TBS and TBB was considered to be significant in comparison to the reference standard quercetin. The result presented in fig. 3 shows that TBFP and TBB extracts inhibits NBT reduction significantly higher than TBS. The TBFP extract inhibited production of $O_2^{\bullet-}$ by 20.69, 39.40, 61.50 and 78.39%, respectively when 50, 100, 150, 200 µg/ml of extract were used in the reaction. The order of SARSA was TBFP (78.39%)>TBB (77.14%)>TBS (71.51%) in comparison to standard quercetin (91.77%).

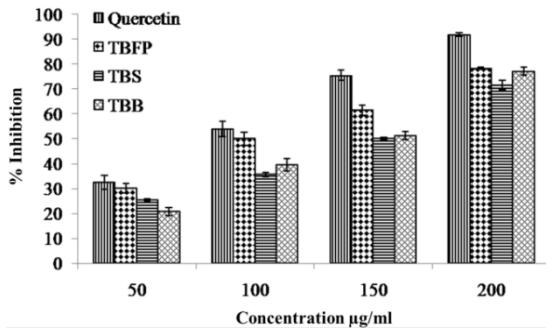

Fig. 3: Inhibitory effects of ethanolic extracts of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB) and standard quercetin on superoxide anion radical at varying concentrations. Values are mean±SD of three replications (n=3)

Reducing power

The RP of a compound may act as a significant indicator of its potential antioxidant activity [24]. With regards to RP, higher reducing capacity might be attributed to the higher amount of phenolic compounds. TBFP had significantly high $Fe^{3+}$to Fe^{2+}transformation capacity (3.39) than TBS (5.02) and TBB (5.12) in comparison to standard quercetin (1.12) ASE/ml (fig. 4) showing high phenolic content in TBFP.

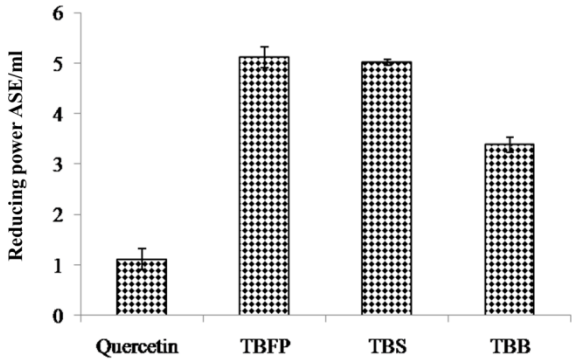

Fig. 4: Reducing power (ASE/ml) of ethanolic extracts of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB) and standard quercetin. Values are mean±SD of three replications (n=3)

Lipid peroxidation

Studies on the inhibition of LPO in the presence of extracts were carried out and expressed as percent inhibition. The TBFP, TBS and TBB extracts prevent LPO induced by FeSO4 and percentage inhibition varied from 74.36 to 84.41%, respectively. Maximum LPO inhibition was shown by TBFP than TBS and TBB in a concentration dependent manner at 100-400 µg/ml in comparison to standard (95.39%). Antioxidant potential was in the order of TBFP>TBB>TBS (fig. 5).

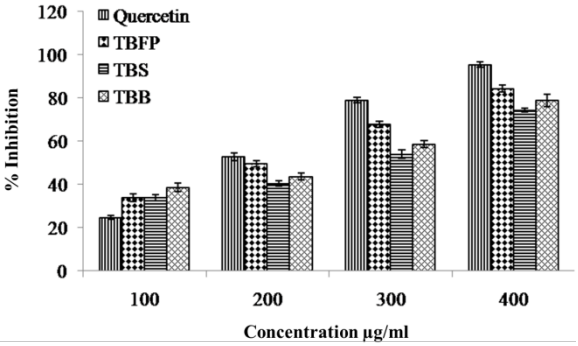

Fig. 5: Inhibitory effects of ethanolic extracts of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB) and standard quercetin on LPO using egg homogenate as a lipid-rich source at varying concentrations. Values are mean±SD of three replications (n=3)

Hydroxyl radical scavenging activity

TB extracts were further studied for their ability to chelate iron and/or to scavenge $OH^{\bullet}$ by using deoxyribose degradation assay. The TBFP was found to be most potent $OH^{\bullet}$ scavenger with inhibition of 40.46 to 78.87% in increasing extracts concentration (50-200 µg/ml) in comparison with quercetin (91.12%). The biochemical studies revealed that TBFP, TBS and TBB caused a concentration-dependent inhibition of deoxyribose oxidation. Based on their inhibitory potential the order of $OH^{\bullet}$scavenging was as follows; TBFP>TBB>TBS (fig. 6).

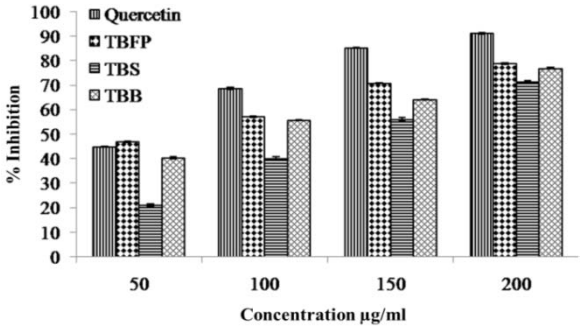

Fig. 6: Inhibitory effects of ethanolic extracts of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB) and standard quercetin on hydroxyl radical mediated deoxyribose degradation at varying concentrations. Values are mean±SD of three replications (n=3)

Ferric thiocyanate assay

In tested extracts, TBS showed maximum inhibition (83.19%) than TBFP (80.19%) and TBB (79.90%) to inhibit production of free radicals which initiate oxidation of lipids and proteins. Quercetin showed 92.12% inhibition to inhibit production of peroxides. The inhibition is increased with increasing concentration of the extracts from 50 to 200 µg/ml in the reaction mixture (fig. 7).

GC-MS analysis

In order to find out phytoconstituents responsible for the antioxidant activity, GC-MS analysis of the TB extracts was conducted. GC-MS chromatogram showed the presence of 8 peaks in TBFP, 12 in TBS and 13 in TBB (fig. 8). Mass spectra of the detected compounds were compared with the already available spectra with NIST library and the phyto-compounds were characterized and identified (table 1). Of all identified compounds in TB extracts, the most prevailing compounds were: quinic acid (chlorogenic acid), gallic acid, ethyl galate, 9, 12 octadecadienoic acid and glucopyranose.

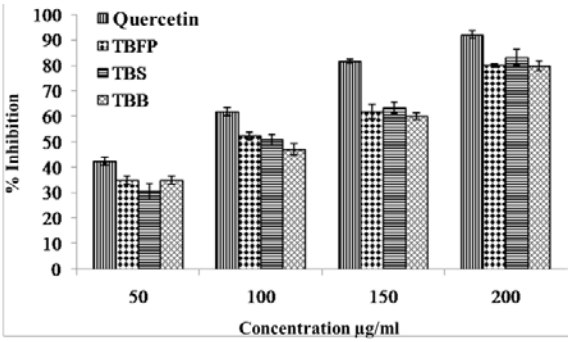

Fig. 7: Inhibitory effects of ethanolic extract of *T. bellerica* fruit pulp (TBFP), seed (TBS), bark (TBB) and standard quercetin on ferric ion chelation by ferric thiocyanate assay method at varying concentrations. Values are mean±SD of three replications (n=3)

Correlation between total phenolic content and antioxidant activity

Phenolics are the major contributors to the antioxidant activity. The correlation between TPC and FRSA of different plant extracts had a correlation coefficient of $R^2 = 0.9935$ (y =0.1245x+52.751) (fig. 9 a.). A good correlation also exists in between TPC and SARSA ($R^2 = 0.9501$), LPO (R^2= 0.9662) and HRSA ($R^2 = 0.9788$) (fig. 9 b,c,d) whereas FTC assay showed less correlation ($R^2 = 0.8795$) with TPC (fig. 9 e).

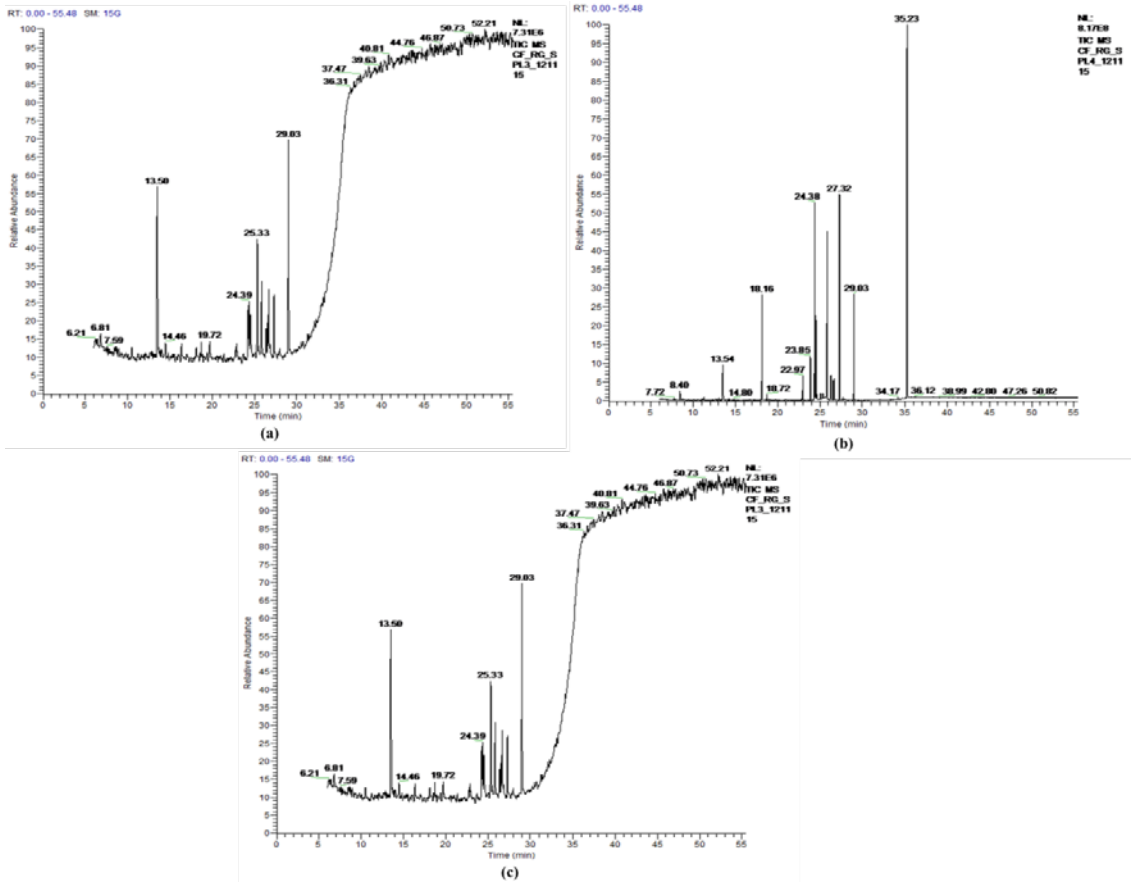

Fig. 8: GC-MS chromatogram of ethanolic extracts of *T. bellerica* (TB). (a) fruit pulp (TBFP); (b) seed (TBS); (c) bark (TBB)

Table 1: Compounds found in real samples by GC-MS as per NIST library

S. No.	RT	Compound name	Peak area Percentage (%)	MW	Fragmentation pattern values (m/z)
***T. bellerica* fruit pulp**					
1	13.50	Methyl 7-(2,4,6 trimethylphenyl)-5H furo (2,3c)thiopyran-4-corboxylate	24.85	314	299, 300, 301, 314, 315
2	24.39	D-fructose 1,3,4,5,6 pentakis-O-(trimethylsilyl)	5.44	540	73, 204, 217, 437, 447
3	25.33	Quinic acid	6.75	552	73, 147, 255, 345, 346
4	25.85	Glucopyranose	6.14	540	73, 147, 191, 204, 205
5	26.36	3,4,5 trimethoxy benzoic acid ethyl ester	29.8	414	45, 73, 281, 282, 414
6	26.69	3,4,5 tris (trimethylsiloxyl) benzoic acid	12.52	458	73, 281, 447, 458, 459
7	27.33	Glucopyranose	3.86	540	73, 147, 191, 204, 205
8	29.03	Inositol-TMS	10.63	612	7, 147, 217, 305, 318
***T. bellerica* seed**					
1	13.54	Silanol-trimethyl phosphate	5.14	314	73, 299, 300, 301
2	18.16	9,12 Octadecadienoic acid	7.94	442	41, 55, 67, 91, 105
3	22.97	9,12 Octadecadienoic acid	1.01	442	41, 55, 67, 91, 105
4	23.85	3,4,5 Tris (trimethylsiloxyl)-1-cyclohexene-1-corboxylic acid	3.48	462	73, 204, 255, 357, 372
5	24.38	Hexopyranose 1,2,3,4,6 pentakis-O-trimethylsilyl	12.41	540	73, 147, 191, 204, 217
6	25.85	Alfa-D-Galactopyranose	15.28	420	73, 75, 117, 147, 191
7	26.31	Quinic acid	1.96	552	73, 147, 255, 345, 346
8	26.56	Alfa-D-galactopyranose	1.43	420	73, 75, 117, 147, 151
9	26.69	3,4,5-trihydroxy benzoic acid ethyl ester	0.93	414	45, 73, 281, 282
10	27.32	L-fructose-trimethylsilyl ether	16.44	452	73, 147, 191, 204, 205
11	29.03	Ethyl galate	9.07	198	125, 153, 154, 170, 198
12	35.23	D-fructose 1,3,4,5,6 pentakis-O-(trimethylsilyl)	24.93	540	73, 204, 217, 437, 447
***T. bellerica* bark**					
1	13.5	3,7-Dioxa-2,8-disilanone	23.95	308	73, 103, 117, 147, 205
2	18.13	L-mallic acid-O-trimethylsilyl	0.86	350	73, 133, 147, 233, 245
3	19.72	D-Xylopyranose	1.44	438	73, 147, 191, 204, 217
4	24.26	D-Xylofuranose	3.89	230	43, 59, 100, 129, 215
5	24.38	D-fructose 1,3,4,5,6 pentakis-O-(trimethylsilyl)	3.04	540	73, 147, 204, 217, 437
6	24.52	Galactopyranose	2.84	540	73, 191, 204, 205, 217
7	25.33	Acrylic acid 2,3 bis-trimethylsilyl-ester	12.38	320	45, 73, 147, 221, 305
8	25.85	Benzoic acid 3,4,5 tristrimethylsilyl ester	7.75	458	73, 281, 447, 458, 459
9	26.37	Glucopyranose	2.97	540	73, 147, 191, 204, 205
10	26.56	Inositol 1,2,3,4,5,6-hexakis-O-trimethylsilyl	2.59	612	73, 147, 217, 305, 318
11	26.7	D-fructose 1,3,4,5,6 pentakis-O-(trimethylsilyl)	5.51	540	73, 147, 204, 217, 361
12	27.33	Glucopyranose	6.78	540	73, 143, 191, 204, 205
13	29.03	Inositol	26.01	612	73, 147, 191, 217, 305

RT: retention time; MW: molecular weight

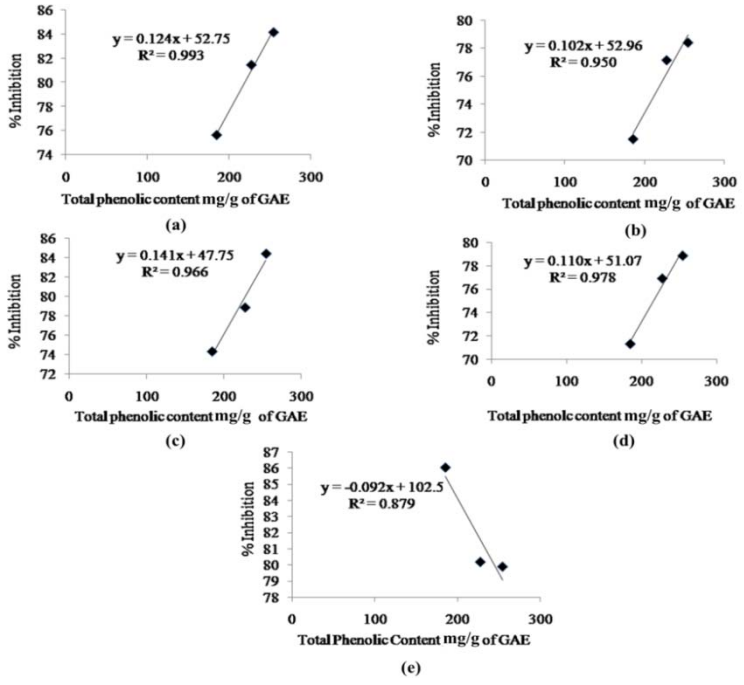

Fig. 9: Linear correlation between TPC (x axis) in the plant extracts in relation to their antioxidant activity (y axis). (a) TPC versus FRSA; (b) TPC versus SARSA; (c) TPC versus LPO; (d) TPC versus HRSA; (e) TPC versus FTC assay

DISCUSSION

Recently, phenolic compounds received considerable attention for their effective antioxidant properties, and their beneficial effects are attributed to their RP and FRSA affinity [22]. Plants are good sources of phenolic compounds. Therefore, the plant which contains a high concentration of phenolic compounds could be a good source of natural antioxidant. Numerous investigations of the antioxidant activity of plant extracts confirmed a linear correlation between the total phenolic content and particular antioxidant activity [25]. In earlier studies, the TPC in methanolic and acetone extracts of TB fruits were reported as 108.692 and 186.288 mg/g GAE [26[, respectively, which is lower than the ethanolic extract values reported by us. This is in agreement with a study by Taso and Deng [27] which showed that phenolic compounds are generally better extracted by using alcoholic solvents.

DPPH is a stable nitrogen-centered, lipophilic free radical widely used in evaluating antioxidant activities in a relatively short time compared to some other methods. The odd electron in DPPH becomes paired with hydrogen from a free radical scavenging antioxidant to form reduced DPPH-H. The resultant decolorization from purple to yellow can be detected spectrophotometrically at 517 nm. We have already reported that TB leaf inhibits DPPH* radical at IC_{50} of 58 µg/ml [8]. These results were in agreement with earlier findings where methanolic extracts of TB fruits significantly suppressed free radicals at IC_{50} of 213.11 µg/ml [26]. Singhal *et al.* [28] and Venkatalakshmi *et al.* [29] reported that aqueous extract of TB bark and *Terminalia catappa* had 35.29% and 30.71% scavenging potential to DPPH* radical at 200 and 250 µg/ml which was lower than the value reported by us in ethanolic extract showing superiority of the ethanolic extraction process.

The $O_2^{\bullet-}$is primary ROS among the free radicals and is often associated with the regulation of inflammatory pathways. SARSA of TB extracts was studied by using a non-enzymatic method known as PMS-NADH-NBT reduction system. In this method, $O_2^{\bullet-}$derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which was measured spectrophotometrically at 560 nm. It was found that plant samples containing antioxidant molecules are able to inhibit the formation of blue formazan complex [30, 31]. The decrease in absorbance at 560 nm with plant extract indicates the consumption of $O_2^{\bullet-}$in the reaction mixture. Earlier studies showed SARSA of aqueous extracts of TB fruits at IC_{50} of 892.85 µg/ml [32] which is very high in comparison to IC_{50} of ethanolic extract TBFP (39 µg/ml) and TBS (92 µg/ml) reported by us. Venkatalakshmi *et al.* [29] showed an aqueous extract of *T. catappa* bark had 63.46% $O_2^{\cdot-}$ scavenging potential at 1000 µg/ml whereas ethanolic extract had 77.14% at 200 µg/ml reported by us.

RP was measured by direct electron donation in the reduction of $Fe^{3+}(CN\text{-})_6$-$Fe^{2+}(CN\text{-})_6$ to reduce ferricyanide to ferrocyanide, which then reacts with $FeCl_3$ to form blue colored complex (Perl's Prussian blue) $(Fe^{3+})_4[Fe^{2+}(CN)_6]_3$ that was measured at 700 nm. Depending on the reducing ability of tested extracts, the yellow colour of test solution changes to various shades of green or blue [33]. Earlier study by Tupe *et al.* [34] showed the strong RP potential of methanolic extracts of *Terminalia chebula* fruit (4.29) and *Terminalia arjuna* bark (4.26), which is in agreement with the present study. Our data on the RP of the tested extracts suggested that it is such as to contribute significantly towards the observed antioxidant effect of TB in comparison to standard quercetin.

LPO is an oxidative degradation of polyunsaturated fatty acids present in the biological membranes which produce a variety of secondary products including several aldehydes such as malondialdehyde (MDA). MDA may react with adenine and guanine in DNA, forming DNA adducts which may be mutagenic [35]. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism [36]. According to past research finding aqueous fruit extract of *Terminalia chebula* exhibited anti-LPO activity at IC_{50} of 163 µg/ml [37] in comparison to our reported IC_{50} values of TBFP (51 µg/ml) and TBS (60 µg/ml). On the basis of reported studies, it may be concluded that ethanolic extracts of TB had significantly high potential to inhibit initiation and progression of LPO in membranes. The high anti-LPO activity of TBFP may be attributed to the high content of phenolic compounds. Therefore, the inhibition of LPO could be caused by the chelation of Fe^{2+}or by trapping of free radicals [8, 36].

The $OH^{\bullet}$is the nonspecific mediator of many of the oxidative damage to cells and are involved in various neurodegenerative and cardiovascular diseases [38]. HRSA of plant extracts was determined by its ability to compete with deoxyribose for $OH^{\bullet}$. In this assay, 2-deoxy-2-ribose was oxidized when exposed to $OH^{\bullet}$ 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 (thiobarbituric acid reactive species) with a maximum absorbance at 532 nm [39]. Tested extracts compete with deoxyribose and diminish chromogen formation in a dose-dependent response. Hazra *et al.* [25] reported that the IC_{50} of methanolic extract of TB fruit that inhibits 50% DNA damage was 203.25 µg/ml, which is much higher in comparison to ethanolic extract IC_{50} of TBFP (27 µg/ml) and TBS (58 µg/ml). Based on these scientific data we can conclude that ethanolic extracts of TB plant parts contain potent compounds that may have a better capability of reducing free radical induced DNA damage.

FTC assay is used to assess the production of peroxides at the initial stage of oxidation, while TBA test is used to measure the secondary products of oxidation such as aldehydes and ketones. The advantage of using ammonium thiocyanate over other coloring reagents is that the specific binding of thiocyanate ion to iron only. Our results with ammonium thiocyanate experiments showed that the TBS extract is an active scavenger of Fe^{3+}ion which is in agreement with the work done on known natural Fe^{3+}scavengers [8, 24, 36]. In this method, the concentration of peroxide decreases as the antioxidant potential of plant extract increases.

To identify the responsible phytochemicals for antioxidant potential of TB extracts, we further carried out GC-MS analysis of fruit pulp, seed, and bark of TB. Quinic acid (chlorogenic acid), gallic acid, ethyl galate, 9, 12 octadecadienoic acid and glucopyranose were found to be most abundant compounds in TB extracts. They were designated by comparing their MS spectra to those of standard spectra from the NIST library. Gallic acid (3, 4, 5-trihydroxybenzoic acid), a naturally occurring low molecular weight tri phenolic compound, has emerged as a strong antioxidant and an efficient apoptosis inducing agent [40]. Gallic acid has been reported to have antimutagenic, antioxidant, anti-inflammatory, antitumor, anticancer and apoptotic properties [41, 42]. Gallic acid derivatives such as ethyl galate have also been found in a number of phytomedicines with antioxidant, apoptotic and anticancer activities [43]. Reported studies on the biological function of gallic acid and its derivatives show that it is a versatile natural antioxidant with promising therapeutic and industrial applications. In plant extracts, quinic acid can occur as an ester with caffeic acid, forming chlorogenic acid, a major component in coffee. Quinic acid is known to induce nutritional efficiency of aromatic amino acids that might be responsible for its antioxidant activity [44]. TBS extract is a rich source of essential fatty acids linoleic and linolenic (9, 12 octadecadienoic acid) that play a very important role in the formation of lipid raft during cell signaling. Earlier studies by Amala and Jeyaraj [45] on GC-MS analysis revealed the presence of various phytochemical compounds in 95% methanolic fruit extracts of TB. These findings suggest that TB plant is a rich source of many bioactive phyto compounds with significant antioxidant activity.

It is a known fact that phenolic compounds are potential antioxidants and free radical scavengers; hence, there should be a close correlation between the content of phenolic compounds and antioxidant activity [46]. The correlation coefficient (R^2) between TPC and FRSA (R^2=0.99) suggests that 99% of the antioxidant capacity of plant extracts results from the contribution of the phenolic compounds. Also, only 1% of the activity is attributable to non-phenolic compounds. Activity may also come from the presence of other antioxidant secondary metabolites such as volatiles oils, terpenes, metalloproteins, vitamins, etc. It has been proposed that the antioxidant activity of plants may be due to their phenolic compounds [47, 48]. These findings suggest that the phenolic content of TB plant is highly attributed to their antioxidant activity.

CONCLUSION

Oxidative stress, caused by an imbalance between antioxidant systems and the production of oxidants (ROS), seems to be associated with various acute and chronic diseases. Fruits and vegetables which contain significant quantities of antioxidants are believed to have health benefits by counteracting oxidative stress thus reducing the risks of chronic diseases. The results revealed that plant extracts containing higher phenolic compounds showed maximum antioxidant activity. GC-MS analysis revealed the presence of various bioactive constituents in ethanolic extracts of TB plant parts which might be responsible for their antioxidant activity. The present study, for the first time, reports that seed of TB is a very effective Fe^{3+}chelator. The antioxidant activities of extracts of TB plant parts may be attributed to their strong hydrogen donating and metal chelation ability, reducing potential, effective hydroxyl and free radical scavenging activities. Overall, all tested parts of TB could be a potential source of natural antioxidants for food and pharmaceutical applications. Further studies may be focused on the mechanistic analysis of antioxidant and anti-inflammatory capacities of TB.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

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