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Antioxidant Studies on Various Fractions of Methanol Extract of *Terminalia bellerica* Robx

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Potential *in vitro* antioxidant activities of various fractions of crude methanol extract of *Terminalia bellerica* Roxb. fruits were evaluated using1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging, 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^+$) radical cation inhibition and antilipid peroxidation assays. The total phenolic and flavonoid content was also assessed. Results showed that ethyl acetate fraction (IC_{50}, 3.09 µg/mL) possess potent DPPH radical scavenging activity, which was better than crude methanol extract (IC_{50}, 7.72 µg/mL) and reference antioxidant, Trolox (IC_{50}, 6.17 µg/mL). It showed better $ABTS^+$ inhibition potential (IC_{50}, 1.18 µg/mL) than crude extract (IC_{50}, 2.27 µg/mL) and Trolox (IC_{50}, 3.86 µg/mL). Highest antioxidant activity, as measured by the inhibition of lipid peroxidation was also offered by ethyl acetate fraction (IC_{50}, 0.78 mg/mL). Maximum amounts of total phenolics and flavonoids were found in ethyl acetate fraction which correlates well with its antioxidant activity.

Key Words: *Terminalia bellerica* Roxb., Antioxidant activity, DPPH radical scavenging, Lipid peroxidation, $ABTS^+$ radical cation, Total phenolic.

INTRODUCTION

Oxidative stress is among the major cause of many degenerative diseases including atherosclerosis, heart disease, ageing, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases, liver disorders and others[1]. Antioxidants are the most effective entities to eliminate free radicals by scavenging them or promoting their decomposition and suppressing such disorders[2,3]. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly advocate this idea that plant constituents with high antioxidant potential are capable of exerting protective effects against oxidative stress in biological systems[4,5]. Recently, natural antioxidants of herbal resources have been a point of interest, as they have traditionally played a major role in the management of human health in many countries[6]. It has been established by many studies that antioxidant potentials of medicinal plants are attributed to the redox properties of phenolic compounds that allow them to act as reducing agents, hydrogen donors and free radicals scavengers[7].

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Terminalia bellerica Roxb. (family Combretaceae), is an important plant in Indian traditional medicine system. It is an essential component of Ayurvedic herbal formulation 'Triphala', which has been reported to possess antiinflammatory, antimutagenic, cytoprotectiive, antioxidant, gastroprotective, myocardial necrosis, hepatoprotective, antibacterial, hypolipdemic and anticancer activity[8]. Recently many biological activities of *T. bellerica* fruits, such as antidiabetic[9], antidepressant[10], antimicrobial[11], antispasmotic and bronchodilatory[12] activities have been reported in the literature. It is suggested that, most likely it exerts its biological activities through its potent antioxidant activities. So it is necessary to evaluate the antioxidant potential of *T. bellerica* fruits to confirm the therapeutic role of antioxidants in prevention or management of these ailments. Unfortunately, reports on antioxidant potential of *T. bellerica* fruits are scanty and they merely reveal the antioxidant activity of crude aqueous or alcoholic extracts[13]. A comprehensive evaluation of antioxidant activity of various fractions of crude extract was required in assessing antioxidant activity of endogenous compounds. The aim of this study is to investigate the *in vitro* antioxidant activities of various fractions of crude methanol extract in a view to find out most active fraction.

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), 6-hydroy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) 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, dried fruits of *T. bellerica* were collected from the local market of Lahore, Pakistan. These were identified and confirmed with morphological techniques by 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 fruits along with seeds (2 kg) were powdered and extracted twice with methanol. The extract was concentrated under reduced pressure to yield crude extract. This crude extract was suspended in deionized water and partitioned with hexane, ethyl acetate and 1-butanol. Each fraction was concentrated under reduced pressure to yield respective fractions. Remainder fraction was called aqueous fraction. All fractions were stored in refrigerator until further use. Percentage yield was 14.3 %.

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[14] 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 % of DPPH was calculated according to the following equation:

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

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

Total antioxidant activity: Total antioxidant activity was evaluated applying an improved ABTS radical cation decolorization assay by Re *et al.*[15]. ABTS radical cation ($ABTS^+$) was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulphate and allowing the solution to stand in the dark at room temperature for 12-14 h before use. For the study of total 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:

$$\text{Percentage inhibition} = [1 - \text{absorbance}_{\text{sample}}/\text{absorbance}_{\text{control}}] \times 100$$

Antilipid peroxidation assay: The antilipid peroxidation activity of various fractions of *T. bellerica* and antioxidant standards was evaluated according to the method of Halliwell *et al.*[16] with slight modifications. Different concentrations of samples were mixed with 1.5 mL of 1.15 % KCl and 1.0 mL egg yolk (10 %). Peroxidation was initiated by adding 0.5 mL of 0.2 mM ferric chloride. After incubation at 37 ºC for 60 min, the reaction was stopped by adding 2 mL of ice cold HCl (0.25 N) containing 15 % trichloroacetic acid (TCA), 0.38 % thiobarbituric (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. Peroxidation of lipid produces malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) to form pink colour adduct, detected spectrophotometrically at 532 nm.

Determination of total phenolics: Amount of total phenolics was determined by Folin-Ciocalteu reagent procedure as described by Cliffe *et al.*[17]. Briefly, 20 µL of sample was mixed with 100 µL of Folin-Ciocalteu reagent and 1.58 mL deionized water. The mixture was kept at room temperature for 10 min and then 300 µL of 25 % sodium carbonate solution (w/v) were added. 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 (mg g^{-1} dry mass).

Determinatioin of total flavonoids: Total flavonoids content was assessed using a colorimetric method described by Dewanto *et al.*[18]. 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 1 M 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 (mg g^{-1} dry mass).

Statistical analysis: The mean value and standard deviation were calculated from the data obtained with three separate experiments.

RESULTS AND DISCUSSION

In the present study we focused on the evaluation of antioxidant activity of various fractions of crude methanol extract of *T. bellerica* fruits using three different testing systems including DPPH free radical scavenging, ABTS radical cation decolonization and anti-lipid peroxidation assays. Moerover, correlation between antioxidant activity and total phenolics and flavonoids was also investigated.

Scavenging activity for DPPH radicals: DPPH is a very stable organic free radical, which has been widely employed to assess the free radical scavenging ability of antioxidants. Each fraction and standard antioxidants were assayed to measure their radical scavenging potential. As shown in Table-1, all fractions quenched DPPH radicals to various degrees. Among all fractions, ethyl acetate extract showed higher activity than did other extracts with an IC_{50} value of 3.09 µg/mL, followed by methanol extract with an IC_{50} value of 7.72 µg/mL. Hexane and butanol fractions showed mediocre scavenging effect with similar range of values (IC_{50}, 23.02 and 21.55 µg/mL). Aqueous extract showed a relatively low inhibitory effect with an IC_{50} value of 60.00 µg/mL. It is evident from the results that highest level of radical scavenging activity was achieved by ethyl acetate fraction, indicating its stronger therapeutic value than crude extract. Interestingly, when compared with reference antioxidants, its activity was more than Trolox (IC_{50}, 6.17 µg/mL) and comparable to propyl gallate and gallic acid (IC_{50}, 2.31 and C50, 2.38 µg/mL), revealing good protection against free radical injury.

TABLE-1
IN vitro FREE RADICAL SCAVENGING, TOTAL ANTIOXIDANT AND ANTILIPID PEROXIDATION ACTIVITIES OF DIFFERENT FRACTIONS OF *T. bellerica* AND STANDARD ANTIOXIDANTS

Samples	Half efficiency concentration (IC_{50})[a]		
	Total antioxidant activity (µg/mL)	Free radical scavenging activity (µg/mL)	Antilipid peroxidation activity (mg/mL)
Methanolic extract (crude)	2.27 ± 0.02[b]	7.72 ± 0.09	3.90 ± 0.01
Hexane extract	10.00 ± 0.04	23.02 ± 0.05	1.67 ± 0.02
Ethyl acetate extract	1.18 ± 0.01	3.09 ± 0.01	0.78 ± 0.04
n-Butanolic extract	7.36 ± 0.06	21.55 ± 0.07	12.88 ± 0.02
Aqueous extract	11.03 ± 0.02	60.00 ± 0.06	12.80 ± 0.03
n-Propyl gallate	1.71 ± 0.03	2.31 ± 0.04	0.42 ± 0.02
Trolox	3.86 ± 0.03	6.17 ± 0.02	0.08 ± 0.03
Gallic acid	0.60 ± 0.08	2.38 ± 0.04	2.22 ± 0.02

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

ABTS radical cation decolorization assay: An improved version of ABTS radical cation decolonization assay was used to measure the total antioxidant activity of various fractions of *T. bellerica* and different reference antioxidants. The ABTS assay measures antioxidant activity of samples by the decolorization of the $ABTS^+$

through reduction of radical cation and presented as percentage inhibition of absorbance at 734 nm. Table-1 shows that strongest antioxidant activity was shown by ethyl acetate fraction with IC_{50} value 1.18 µg/mL. Methanolic extract also showed potent antioxidant activity with IC_{50} value of 2.27 µg/mL. However, hexane and aqueous fractions were less effective in decolorizing ABTS radical cation and exhibited IC_{50} values of 10.00 and 11.03 µg/mL, respectively. Results indicated that ethyl acetate fraction was even more active than Trolox (IC_{50}, 3.86 µg/mL). It can be inferred that this fraction may serve as the source of natural antioxidants in food and pharmaceutical industry.

Antilipid peroxidation potential: Lipid peroxidation in biological systems has long been thought to produce many pathological disorders. Unsaturated lipids in cell membrane get oxidized by hydroxyl radicals. Malonaldehyde (MDA) produced as a result of lipid peroxidation, takes part actively in cross linking with DNA and proteins and damages hepatocytes[19]. It was reported that crude extract of *T. bellerica* inhibited lipid peroxidation. In present experiments, ethyl acetate fraction showed stronger antilipid peroxidation activity as compared to crude methanol extract. Results showed that ethyl acetate extract was most effective against the production of thiobarbituric acid reacting species (TBARS) with IC_{50} value 0.787 mg/mL. Second highest protection against lipid peroxidation was shown by hexane fraction with IC_{50} value of 1.673 mg/mL. Methanol extract showed mediocre effect (IC_{50}, 3.9 mg/mL). Lowest activity was shown by aqueous and butanol extrct with almost similar IC_{50} value.

Total phenolic and flavonoid content: Phenolic compounds in the medicinal plants extracts are frequently reported to be responsible for the antioxidant status[20]. The major known active compounds of *T. bellerica* fruits are polyphenolic including gallic acid, ellagic acid, ethyl gallate, galloyl glucose and chebulagic acid[21]. Taking this into account, total phenolics and flavonoids content of all the samples was assayed (Table-2). Ethyl acetate fraction appeared with remarkably higher concentration of total phenols (422 mgGAE/g). Methanol extract and hexane fraction showed a mediocre range of 146 and 103 mgGAE/g, respectively. Phenolics content of very low value was detected in aqueous and butanol fraction. A significant positive correlation was shown by total phenolics content and free radical scavenging potential of crude extract and all fractions. A high correlation between DPPH radical scavenging activities and total phenolics has been reported in literature[7]. Antilipid peroxidation activity of crude extract and all fractions was also correlated well with total phenolics. The same scenario was presented by ABTS radical cation decolorization assay. This clearly manifested a prominent role of phenolics in the preventive effects of *T. bellerica* fruits. Results confirmed that greater antioxidant activity of ethyl acetate fraction in all antioxidant testing systems, compared to crude, was probably due to highest amount of polyphenolic compounds as shown in Table-2.

TABLE-2
CONTENTS OF TOTAL PHENOLICS AND FLAVONOIDS EXPRESSED AS GALLIC ACID AND QUERCETIN EQUIVALENTS, RESPECTIVELY

Samples	Total phenolics (mg GAE/g)[a]	Total flavonoids (mg QE/g)[b]
Methanolic extract (crude)	146.00 ± 0.04[c]	478.30 ± 0.04
Hexane extract	103.00 ± 0.02	388.00 ± 0.07
Ethyl acetate extract	422.00 ± 0.01	460.00 ± 0.06
n-Butanolic extract	30.00 ± 0.01	143.33 ± 0.03
Aqueous extract	60.00 ± 0.03	105.00 ± 0.02

[a]: milligram gallic acid equivalents/gram; [b]: milligram quercetin equivalents/gram; (All calculations are made on dry mass basis); [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.

Flavonoids, another class of phenolic compounds, also act as antioxidants. Complex structure and the position of hydroxyl groups in it play an important role in determining their free radical scavenging potential[22]. Methanolic crude extract showed highest value of flavonoid content (478.3 mgQE/g). Ethyl acetate fraction showed almost parallel value (460.0 mgQE/g). Significant flavonoids quantity was extracted in hexane fraction (388 mgQE/g). However, aqueous and butanolic fractions showed poor values as compared to other fractions.

Conclusion

We investigated antioxidant activities of various fractions of crude methanol extract of *T. bellerica* fruits. In this study it was demonstrated for the first time that ethyl acetate fraction of *T. bellerica* fruits possessed potent free radical scavenging and antioxidant activities, which were better than crude extract or some standard antioxidants. Considerable good content of polyphenols and flavonoids was found in this fraction, which most probably, contributed towards its antioxidant potential.

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