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ORIGINAL ARTICLE

Assessment of Antioxidant Potential, Total Phenolics and Flavonoids of Different Solvent Fractions of *Monotheca Buxifolia* Fruit

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Abstract
Objectives: This study was conducted to investigate the antioxidant potential of methanol extract and its derived fractions (hexane, ethyl acetate, butanol, and aqueous) of fruits of *Monotheca buxifolia* (Falc.) Dc., a locally used fruit in Pakistan.
Methods: Dried powder of the fruit of *M. buxifolia* was extracted with methanol and the resultant was fractionated with solvents having escalating polarity; *n*-hexane, chloroform, ethyl acetate, *n*-butanol and the residual soluble aqueous fraction. Total phenolic and total flavonoid contents were estimated for the methanol and various fractions. These fractions were also subjected to various *in vitro* assays to estimate the scavenging activity for 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), superoxide, hydroxyl, hydrogen peroxide and reductive ability for ferric ions and phosphomolybdate assay.
Results: The *n*-butanol, aqueous and methanol fractions possessed high amount of phenolics and flavonoids compared with other fractions, and subsequently showed a pronounced scavenging activity on DPPH, ABTS, superoxide, hydroxyl and hydrogen peroxide radicals and had a potent reductive ability on ferric ion and phosphomolybdate assay. There was a found significant correlation between total phenolic and flavonoid contents and EC_{50} of DPPH, superoxide, hydrogen peroxide radical and phosphomolybdate assays, whereas a nonsignificant correlation was found with the hydroxyl radical and ABTS radical assay.
Conclusion: *M. buxifolia* fruit can be used as natural antioxidant source to prevent damage associated with free radicals.

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1. Introduction

The role of free radicals in many disease conditions has been well established. Oxidative stress due to the production of free radicals such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical (•OH), peroxide radical (ROO•), and nitric oxide radical, is the major cause of a variety of pathological conditions including coronary heart diseases, reperfusion injury, inflammation, diabetes, drug toxicity, carcinogenesis and neurodegenerative diseases such as Parkinson and Alzheimer diseases [1].

Antioxidant substances can block the harmful action of the free radicals by scavenging the free radicals and detoxify the organism. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly used in food processing and preservation but have been found to have side effects and have been proved carcinogenic [2]. Thus there has been increased interest in natural antioxidants, especially those of plant origin. In the past decade there have been many reports of plant extracts and different types of phytochemicals particularly polyphenols, the secondary metabolites from plants, which were shown to have antioxidant activity [3,4]. Therefore, phenolics and other natural compounds are capable of protecting against reactive oxygen species-mediated damage with possible application in avoidance and/or curing of diseases. As safe sources of antioxidants, fruits and vegetables have been investigated for their antioxidant properties, for example blueberry (*Vaccinium corymbosum* L.) [5] and *Launaea procumbens* [6]. Fruits have been also associated conversely with aging and mortality from cardiovascular and neurodegenerative diseases [7].

Monotheca buxifolia is a broad-leaved evergreen small tree belonging to the family Sapotaceae. This species is found in the hilly regions of Afghanistan and in Northern Pakistan. It is used as fuel, fodder, wood lumber, roof thatching materials, and particularly used as hedge around cultivated fields due to its barbed nature. This species bears small fruits, locally called Gurgura, sold in the local markets as fresh and dried food [8,9]. Medicinally, fruits have laxative and digestive properties, and are used in the treatment of urinary tract diseases. They are also used to reduce temperature in fevers and as a vermifuge [8,10,11].

There have been no reports on the antioxidant activities of this plant previously in the literature. Hence, the present work investigates the potential antioxidant free radical scavenging effects of a methanol extract of the powder of dried fruit pulp of *M. buxifolia* and its derived solvent fractions, in order to understand the usefulness of this plant as a foodstuff as well as in medicinal preparations. The antioxidant capacity was evaluated for different *in vitro* models such as scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydroxyl, hydrogen peroxide (H_2O_2), superoxide radicals, and total antioxidant activity by the phosphomolybdate method and reducing power. Because of the effectiveness of the phenolics and flavonoids as antioxidants, the amounts of total phenolics and total flavonoids in the extracts/fractions were also determined.

2. Materials and Methods

2.1. Chemicals

Aluminum chloride, ascorbic acid, ferric chloride ($FeCl_3$), ABTS, potassium persulfate, gallic acid, rutin, linoleic acid, DPPH, Folin-Ciocalteu's phenol reagent, phenazine methosulfate (PMS), nitro blue tetrazolium (NBT), trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were acquired from Sigma Co. (St. Louis, MO, USA). Riboflavin, sulfuric acid, deoxyribose, sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), disodium hydrogen phosphate (Na_2HPO_4), sodium nitrite ($NaNO_2$) and H_2O_2 were purchased from Wako Co. (Osaka, Japan). Ferrous chloride ($FeCl_2$), sodium dihydrogen phosphate (NaH_2PO_4), potassium ferricyanide ($K_3Fe(CN)_6$), and solvents used were of analytical grade were purchased from Merck Co. (Darmstadt, Germany). Distilled deionized water (dd. H_2O) was prepared using the Ultrapure water purification system (Lotus Co., Ltd., Taipei, Taiwan).

2.2. Plant material and extract preparation

The fruit was collected in April 2010 from KPK province of Pakistan and the plant was identified by its local name and later identified by Dr Mir Ajab Khan, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. A specimen was kept at the Herbarium of the Pakistan Museum of Natural History, Islamabad. The fruits (5.0 kg fresh) of uniform size at maturity were collected and dried under shade to obtain 1.0 kg dry sample, excluding the seeds. The dried samples were powdered in a Willy Mill to 60-mesh size and used for solvent extraction. For extract preparation, 1.0 kg of dried sample was extracted twice with 2.0 L of 95% methanol at 25 °C for 48 h. The extracts were filtered with Whatman No. 1 filter paper and evaporate to dry the filtrate by using rotary evaporator. The extract was suspended in distilled water and partitions were made with increasing polarity of solvents i.e., *n*-hexane, ethyl acetate, chloroform, butanol, and water. Then all fractions were dried by using rotary evaporator, and preserved at 4 °C. The dry extract was weighed and the yield was determined as the percentage of air-dried weight of plant material.

2.3. Estimation of total polyphenolic contents

The total polyphenolic content was estimated using Folin-Ciocalteu reagent [12]. Folin-Ciocalteu reagent (400 μL) was mixed with 200 μL of fractions (1.0 mg/mL) in a volumetric flask. The solution was heated to 25 °C for 5−10 min and mixed with 0.2 mL of 7% Na_2CO_3 solution,

and finally the mixture was diluted with deionized distilled water and made up to 10.0 mL in a volumetric flask. Before taking the absorbance at 725 nm, the mixture was held for 2 hours at 25 °C. A calibration curve was plotted for the standard of gallic acid. Total phenolics were calculated as equivalent of per mg gallic acid (GAE) per gram of dried sample (mg/g).

2.4. Estimation of total flavonoids

Total flavonoid content was estimated according to the method of Park et al [13]. Fifty milligrams of each fraction was suspended in 10 mL of 80% methanol and filtered through Whatman filter paper No. 42 (125 mm). In a test tube (10 mL), 0.3 mL of extracts, 3.4 mL of 30% methanol, 0.15 mL of 0.5M $NaNO_2$ and 0.15 mL of 0.3M $AlCl_3.6H_2O$ was mixed. Five minutes after the addition of 1 mL of 1M NaOH, the absorbance was measured at 506 nm. Rutin was used to plot the calibration curve. Total flavonoids were calculated as mg rutin equivalents per gram of dried sample (mg/g).

2.5. Antioxidant activity assays

For the antioxidant assays, all fractions were dissolved (1.0 mg/mL of fractions) in 95% methanol and a series of concentration-dependent dilutions were made. For all antioxidant assays standard chemicals were used for comparison.

2.5.1. DPPH assay for radical scavenging activity

The DPPH assay was performed following the method of Blois [14]. DPPH (2.4 mg) was dissolved in 100 mL methanol to prepare the stock solution and then stored at 20 °C until needed. The DPPH solution was diluted with methanol to achieve an absorbance of 0.980 (±0.02) at 517 nm with the spectrophotometer. A 500-µL aliquot of the above mixture was mixed with 500 µL of the samples at different concentrations (25−250 µg/mL). The mixture was incubated in the dark for 15 minutes and the absorbance was measured at 517 nm. The DPPH scavenging activity of various fractions was calculated by the following equation:

$$\text{Percentage inhibition}(\%) = [(\text{Absorbance of control} - \text{Absorbance of sample})/(\text{Absorbance of control})] \times 100$$

EC50 values calculated to determine the 50% inhibition of DPPH radicals. Ascorbic acid and rutin were used as standards.

2.5.2. Superoxide radical scavenging activity

The scavenging activity assay for superoxide anion radical was performed according to the method of Beauchamp and Fridovich [15]. The reaction mixture consisted of 500 µL of 50 mM PO_4 buffer (pH 7.6), 300 µL of 50 mM riboflavin, 250 µL of 20 mM phospho-methozine sulphate (PMS), and 100 µL of 0.5 mM nitroblue tetrazolium (NBT) before adding up of 1.0 mL of various fractions. Triggering of reaction was done by illuminating the above solutions using a fluorescent lamp. After 20 minutes the absorbance was measured at 560 nm. The percentage of scavenging superoxide anion generation was calculated as:

$$\text{Percentage inhibition}(\%) = (1 - \text{Sample absorbance}/\text{Controlabsorbance}) \times 100$$

EC50 values calculated to determine the 50% inhibition of superoxide radicals. Ascorbic acid was used as standards.

2.5.3. Phosphomolybdate assay (total antioxidant capacity)

The total antioxidant capacity assay of samples was carried out by the phosphomolybdenum method [16]. A 0.1-ml aliquot of the sample solution was shaken with 1 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were covered and incubated in a water bath at 95 °C for 90 min. After the samples were cooled, the absorbance of the mixture was measured at 765 nm. Ascorbic acid was used as standard. The antioxidant capacity was estimated using the following formula:

$$\text{Total antioxidant capacity}(\%) = [(\text{Abs. of control} - \text{Abs. of sample})/(\text{Abs. of control}] \times 100$$

2.5.4. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of samples was determined according to the method of Halliwell and Gutteridge [17]. The reactive mixture was consisted of: 100 µL of pre-mixed ferric chloride (100 mM), 250 µL of 2.8 mM 2-deoxyribose in 50 mM phosphate buffer (pH 7.4), and 100 mM EDTA solution (1:1; v/v); 200 mM H_2O_2 (50 µL) without or with the 50 µL extract solution. The reaction was triggered by adding 50 µL of ascorbate (300 mM) and heated for 60 min at 37 °C. A solution of 1% thiobarbituric acid (TBA) in 500 µL of NaOH (50 mM) and 500 µL of 2.8%TCA was added. Then the mixture was placed in a boiling water bath for 15 minutes. After cooling, the absorbance was taken at 532 nm. Hydroxyl radical scavenging was calculated as:

$$\text{Percentage inhibiting activity} = \times(1 - \text{Abs. of sample}/\text{Abs. of control}) \times 100$$

2.5.5. ABTS radical scavenging activity

The ABTS radical scavenging activity was determined by calculating the disappearance of the ABTS radical cation, following the method with of Re et al [18]. ABTS (7 mM) and potassium persulfate (2.4 mM) was mixed to make the stock solution and placed in the dark for 12−16 hours at room temperature. Then the

solution was diluted by mixing 1 mL of $ABTS^+$ solution with 60% methanol to obtain an absorbance of 0.708 ± 0.001 units at 734 using the spectrophotometer. For each assay $ABTS^+$ solution was made fresh. Then 1 mL of plant extracts was added to react with 1 mL of the $ABTS^+$ solution and the absorbance was measured at 734 nm. The decrease in absorbance was taken after 1 minute up to 6 minutes. Then the final absorbance was noted. The percentage inhibition was calculated using following formula:

$$\text{Percentage inhibiting activity} = \times(1 - \text{Abs. of sample}/\text{Abs. of control}) \times 100$$

The antioxidant capacity of test samples was given by EC_{50}, the concentration necessary for a 50% reduction of ABTS.

2.5.6. H_2O_2-scavenging activity

The H_2O_2 radical scavenging activity of extracts was determined according to the method of Ruch et al [19]. H_2O_2 solution (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). An aliquot (0.1 mL) of the extract sample was placed into a test tube and the volume made up to 0.4 mL with 50 mM phosphate buffer (pH 7.4). After addition of 0.6 mL H_2O_2 solution, tubes were mixed and the absorbance of the H_2O_2 at 230 nm was determined. The ability to scavenge the H_2O_2 was calculated using the following equation:

$$\text{Percentage inhibiting activity} = \times(1 - \text{Abs. of sample}/\text{Abs. of control}) \times 100$$

The EC_{50} value is the effective concentration that is required to scavenge 50% H_2O_2 radicals. EC_{50} values calculated to determine the 50% inhibition of DPPH radicals. Ascorbic acid and rutin were used as standards.

2.5.7. Reducing power assay

The reducing power of extracts was estimated following the method of Oyaizu [20]. Extract solution (2 mL), phosphate buffer (2 mL, 0.2M, pH 6.6) and potassium ferricyanide (2 mL, 10 mg/mL) were added, and then kept at 45 °C for 30 minutes. TCA (2 mL, 100 mg/L) was added to the reaction mixture. A 2-mL aliquot of the above mixtures was added to 2 mL of distilled water and 0.4 mL of 0.1% (w/v) ferric chloride in a test tube, the absorbance was measured after 10 minutes, at 700 nm. Increased absorbance of the reaction mixture suggests a high reducing power.

2.6. Statistical analysis

Readings for all antiradical scavenging assays were taken in triplicate. Graph Pad Prism 5 software (H.J. Motulsky, Prism5 Statistics Guide, GraphPad Software Inc., San Diego, CA, USA, www.Graph Pad.com) was used to calculate the EC_{50} values. Standard deviation and ANOVA was employed to investigate the differences among EC_{50} of different fractions for different antiradical assays. The Pearson correlation coefficient for phenolic and flavonoids was also employed. All the assays findings were subjected to the Student t test ($p < 0.05$; $p < 0.01$) to determine their significance.

3. Results and Discussion

3.1. Extraction yield, total phenolics and flavonoid contents

The percentage yield, total phenolic content, and flavonoids of the methanol extract and solvent fractions obtained from *M. buxifolia* fruits are shown in Table 1. The recovery percentage of extractable compounds varied from 4.56 ± 1.25 to 24.18 ± 3.22. The highest yield was given by the methanol extract (24.18 ± 3.22), whereas the aqueous fraction gave the lowest (4.56 ± 1.25). Although the active components of the medicinal plants compounds are not known, polyphenols have received growing attention because of some exciting new findings concerning their biological activities. Pharmacologically, the antioxidant potential of polyphenolic compounds, particularly free radical scavenging and inhibition of lipid peroxidation, are the most important. The total phenolic compounds as recorded in Table 1 in *M. buxifolia* fruit fractions (determined as gallic acid equivalents or GAE), ranged between 59.13 ± 2.6 mg and 16.66 ± 1.3 mg/g dry weight of fraction. The butanol extract showed the highest total phenolics (59.13 ± 2.6 mg GAE/g fraction), whereas the phenolic contents of *n*-hexane were much smaller (16.66 ± 1.3 mg GAE/g), which is in agreement with other similar reports [4,21]. The antioxidant property of the compounds was well correlated with the content of their phenolic compounds [22]. Phenols contain good antioxidant, antimutagenic, and anticancer properties [23]. Flavonoids are

Table 1. Total phenolic and flavonoid content and extraction yield of *M. buxifolia* fractions

Plant fractions	Total phenolics (mg GAE/g)	Total flavonoids (mg rutin equivalent/g)	Extraction yield (%)
Methanol extract	48.54 ± 2.9	42.045 ± 3.1	24.18 ± 3.2
n-Hexane fraction	16.66 ± 1.3	4.110 ± 0.51	8.85 ± 1.1
Ethyl acetate fraction	31.48 ± 2.4	28.89 ± 2.1	6.74 ± 0.7
Butanol fraction	59.13 ± 2.6	36.51 ± 2.2	10.83 ± 0.9
Aqueous fraction	42.91 ± 2.3	48.68 ± 2.8	4.56 ± 1.2

Each value in the table is represented as mean ± SE ($n = 3$).Means not sharing the same letter are significantly different (LSD) at $p < 0.01$ probability level in each column.

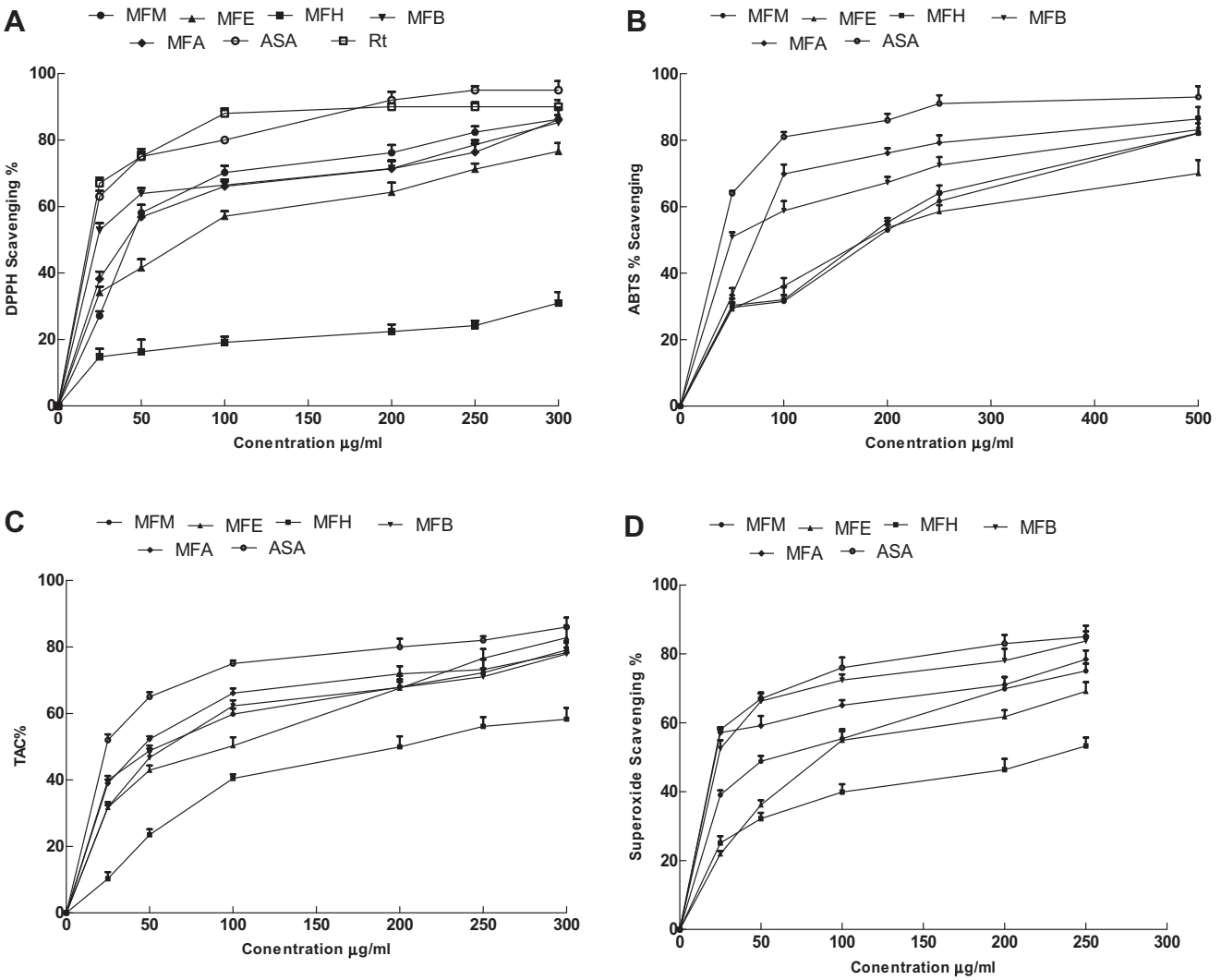

Figure 1. Antioxidant activities of different solvent fractions from the methanol extract of *M. buxifolia* at different concentrations. Each value represents a mean ± SE ($n = 3$): (A) DPPH radical scavenging activity, (B) ABTS radical scavenging activity, (C) total antioxidant capacity, (D) superoxide scavenging activity. ASA = ascorbic acid; MFA = aqueous fraction of *M. buxifolia*; MFB = butanol fraction of *M. buxifolia*; MFE = ethyl acetate fraction of *M. buxifolia*; MFH = *n*-hexane fraction of *M. buxifolia*; MFM = methanol fraction of *M. buxifolia*; Rt. = rutin.

the naturally occurring polyphenolic compounds representing one of the most prevalent classes of compounds in vegetables, nuts, fruits, and beverages such as coffee, tea, and red wine [24]. The present study showed flavonoid contents in the range of 4.11 ± 0.51 to 48.68 ± 2.8 mg as rutin equivalents/g fraction. The highest amount was observed in the aqueous fraction (48.68 ± 2.8 mg/g) followed by the methanol extract (42.045 ± 3.1 mg/g). Halliwell [25] reported that plants rich in flavonoids are potential sources of natural antioxidants that would add to the overall antioxidant capacity of an organism and inhibit lipid peroxidation. Therefore, the result suggested that phenolic acids and flavonoids may be the major contributors for the antioxidative properties and inhibitory actions toward the oxidative reaction *in vitro* and *in vivo*.

3.2. DPPH radical scavenging activity

The DPPH radical has been used widely to test the antioxidant activities of plant extracts and foods. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant, bringing about a color change from purple to yellow, which is measured at 517 nm [14]. Figure 1A shows the scavenging effect of plant fractions on DPPH radical was in the following order methanol > butanol > ethyl acetate > aqueous > *n*-hexane fractions. From the analysis of EC_{50} values (Table 2), the DPPH radical scavenging activity of the butanol fraction (24.1 ± 1.02) was found to be significantly higher ($p < 0.001$) followed by methanol extract (42.7 ± 1.62) and aqueous fractions (56.5 ± 3.82). The EC_{50} value for *n*-hexane fraction was found to be greater than 300 µg/mL. The EC_{50} value for butanol fraction was close to that of ascorbic acid (20.4 ± 0.62) and rutin (19.5 ± 1.33) used as positive controls. It shows that the *M. buxifolia* fruits act as antioxidants since they possess hydrogen-donating properties. The scavenging activity of the extract increased in a concentration-dependent manner.

3.3. Phosphomolybdate assay

This assay is based on the reduction of phosphomolybdate ion in the presence of an antioxidant resulting in the formation of a green phosphate/MoV complex which is measured spectrophotometrically [26]. Figure 1C shows antioxidant capacity of fractions of *M. buxifolia* fruit in the order of aqueous > methanol > butanol > ethyl acetate > *n*-hexane fractions. The aqueous fraction with an EC_{50} value of (45.2 ± 2.53) showed high antioxidant capacity followed by the methanol fraction (56.4 ± 2.06). However, the antioxidant activity of ascorbic acid, a known antioxidant used as the positive control, was comparatively more effective than that of *M. buxifolia* fractions.

Table 2. Antioxidant effect (EC_{50}) on DPPH radicals, superoxide radicals, total antioxidant capacity and hydroxyl radicals of methanol extract and soluble fractions of *M. buxifolia* fruit

	EC_{50}					
Plant extracts	Scavenging ability on DPPH radicals	Scavenging ability on superoxide radicals	Phosphomolybdate assay	Scavenging ability on hydroxyl radicals	Scavenging ability on hydrogen peroxide radicals	Scavenging ability on ABTS radicals
Methanol fraction	42.7 ± 1.62	53.2 ± 2.19	56.4 ± 2.06	103 ± 2.96	123.5 ± 2.17	186 ± 2.87
n-Hexane fraction	>300	242.6 ± 3.61	203.2 ± 4.11	109 ± 3.21	>300	177 ± 3.26
Ethyl acetate fraction	178.5 ± 3.25	88.5 ± 2.73	99.1 ± 2.36	260 ± 3.68	125.7 ± 2.83	179 ± 3.43
Butanol fraction	24.1 ± 1.02	24.4 ± 0.89	58.2 ± 1.74	64.1 ± 3.15	89.3 ± 1.91	52.2 ± 2.57
Aqueous fraction	56.5 ± 3.82	22.3 ± 1.35	45.2 ± 2.53	159.6 ± 2.55	69.2 ± 1.29	73.1 ± 3.26
Ascorbic acid	20.4 ± 0.62	21.1 ± 0.86	24.8 ± 1.07	33.3 ± 1.34	24.8 ± 1.16	34.7 ± 1.51
Rutin	19.5 ± 1.33	–	–	–	28.2 ± 0.93	33.3 ± 1.08

Each value in the table is represented as mean ± SE ($n = 3$).Means not sharing the same letter are significantly different (LSD) at $p < 0.01$ probability level in each column.

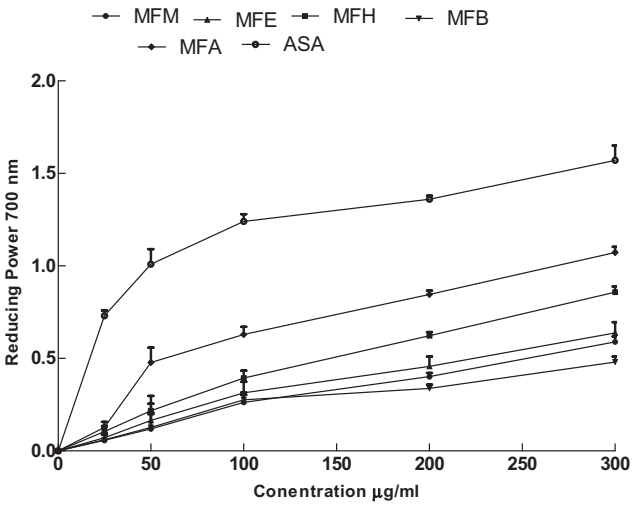

Figure 2. Reducing power of different solvent fractions from the methanol extract of *M. buxifolia* fruits at varying concentrations. Each value represents a mean ± SE (n = 3). ASA = ascorbic acid; MFA = aqueous fraction of *M. buxifolia*; MFB = butanol fraction of *M. buxifolia*; MFE = ethyl acetate fraction of *M. buxifolia*; MFH = *n*-hexane fraction of *M. buxifolia*; MFM = methanol fraction of *M. buxifolia*.

3.4. ABTS radical scavenging activity

The ABTS assay is an excellent tool to determine the antioxidant activity of hydrogen donating and chain-breaking antioxidants. Pietta et al [27] investigated the antioxidant activity of frequently used medicinal plants and verified that the phenolic compounds are important scavengers of ABTS. Figure 1B shows that all the fractions of *M. buxifolia* fruit exhibited a strong scavenging activity against ABTS radicals. The butanol and aqueous fractions with respective EC_{50} values of (52.2 ± 2.57) and (73.1 ± 3.26) exhibited higher antioxidant activity. The butanol and aqueous extracts of *M. buxifolia* fruits indicate that they could cease the oxidation process by reducing free radicals. This is due to the presence of high content phenols in these extracts, as polyphenols play a vital role as antioxidants in living systems due to the presence of hydroxyl groups in *ortho-* and *para-* positions [28].

Table 3. Correlations between the EC_{50} values of antioxidant activities and phenolic and flavonoid content of *M. buxifolia* fruit

	Correlation R^2	
EC_{50}	Phenolics	Flavonoids
EC_{50} of scavenging ability on DPPH radicals	0.9295*	0.8554*
EC_{50} of scavenging ability on superoxide	0.7947†	0.9202*
EC_{50} of phosphomolybdate assay	0.7732†	0.9648*
EC_{50} of scavenging ability on hydroxyl radicals	0.1661	0.004
EC_{50} of scavenging ability on hydrogen peroxide radicals	0.6779†	0.8995*
EC_{50} of scavenging ability on ABTS radicals	0.3992	0.2406

*Indicates significance at $p < 0.05$; †Indicates significance at $p < 0.01$. *M. buxifolia* fruit methanolic extract and its soluble fractions were used in the correlation.

3.5. Hydroxyl radical scavenging activity

The hydroxyl radical is one of the most reactive oxygen species in living systems. It damages the cell by reacting with the polyunsaturated fatty acid of cell membrane phospholipids [25]. Thus, removing OH radicals is very important for the protection of biological systems. In this study all the samples generally registered good hydroxyl radical scavenging activity in a concentration-dependent manner (25−300 µg/mL). Among them, the butanol fraction showed the highest OH scavenging potential (EC_{50} value of 64.1 ± 3.15). The capability of *M. buxifolia* fractions to eliminate hydroxyl radicals appears to directly relate to the inhibition of lipid peroxidation, and acts as scavengers of active oxygen species by breaking free radical chains.

3.6. H_2O_2 radical scavenging activity

Our environment contains H_2O_2 at low concentration levels in the air, water, human body, plants, microorganisms, food, and beverages. It enters the human body through inhalation or skin contact. H_2O_2 is rapidly decomposed in the body into oxygen and water resulting in hydroxyl radicals (OH•) that can begin lipid peroxidation and cause damage to cell membrane and DNA. The scavenging ability of extracts of *M. buxifolia* fruit extracts

on H_2O_2 radicals was in the order of aqueous > *n*-butanol > methanol > ethyl acetate > *n*-hexane fractions. Data obtained from EC_{50} values (Table 2) shows that the fractions showed moderate H_2O_2-scavenging activity. The percentage scavenging activity increased with increasing concentration of the fractions. However, the scavenging activities of ascorbic acid and rutin, used as positive controls for comparison, were relatively more evident than those of the *M. buxifolia* fractions.

3.7. Superoxide radical scavenging activity

Figure 1D shows the superoxide radical ($O_2^{\bullet -}$) scavenging activity of the samples, as measured by the riboflavin−NBT−light system *in vitro*. Reduction of flavins in the presence of light generates superoxide radicals which reduce NBT, forming a blue-colored formazan [15]. The fractions evaluated were found to be potent scavengers of superoxide radicals produced in the riboflavin−NBT−-light system. The fractions inhibited the formation of the blue formazan in a concentration-dependent pattern and the scavenging potential was in the following order aqueous > *n*-butanol > ethyl acetate > methanol > *n*-hexane fractions. The EC_{50} values of aqueous and butanol fractions were 22.3 ± 1.35and 24.4 ± 0.89. These results indicated that *M. buxifolia* fruit fractions had a notable effect on inhibition of superoxide when compared with ascorbic acid (EC_{50} values 21.1 ± 0.86), which was used as positive control.

3.8. Reducing power activity

Figure 2 shows the reducing power of sample extract compared to ascorbic acid the reductive capability was investigated by measuring the Fe_3^{+}−Fe_2^{+} transformation in the presence of *M. buxifolia* fruit fractions, following the method of Oyaizu [20]. The reducing power of extracts is usually associated with the occurrence of reductants, which exert antioxidant action by donating a hydrogen atom and breaking the free radical chain. In our study the aqueous fraction showed highest reductive ability followed by the *n*-hexane fraction. The reducing power of *M. buxifolia* fractions suggest that it is likely to add considerably towards the overall antioxidant effect. However, the antioxidant activity of plant extracts have been recognized to have various mechanisms of action, such as binding of heavy metal ion catalysts, breakdown of peroxides, inhibition of chain initiation, reductive capacity on metals, and radical scavenging [29]. Like the antioxidant activity, the reducing power of *M. buxifolia* fruit fractions increased with increasing concentration of extract. However, the reducing power of ascorbic acid was comparatively more effective than that of our fractions.

3.9. Correlation with EC_{50} values of antioxidant activities and phytochemical contents

Through correlation analysis for phytochemical contents with EC_{50} values of radical scavenging activities of various soluble fractions of *M. buxifolia* fruits, the contents of phenolics and flavonoids exhibited good correlation with phosphomolybdate assay, DPPH, superoxide, and H_2O_2 radical scavenging activities (Table 3). However, correlation in the case of ABTS and hydroxyl radical scavenging activity was found to be nonsignificant. The results indicate that phenolic acids and flavonoids are the major contributors to the antioxidant and free radical scavenging activities of fractions of *M. buxifolia* fruits, and enhanced the importance of phenolic compounds in the antioxidant property of plant extracts. The perceptible correlation among different investigations exhibited that the antioxidant assays selected in the present investigation are feasible and complementary to the antioxidant activities in natural environment. Our results are in agreement with other reports of a strong correlation of antioxidant activities and total polyphenols [4,30].

4. Conclusion

The methanol extract of *M. buxifolia* fruits and its solvent fractions performed varied levels of antioxidant activity in all the *in vitro* models of antioxidant assays studied. The results from various free radical-scavenging systems revealed that the *M. buxifolia* had significant antioxidant activity and free radical-scavenging activity. The major antioxidative components appeared to be to be phenolics and flavonoids. *M. buxifolia* can be suggested as a potential natural source of antioxidants appropriate for utilization in nutritional/pharmaceutical fields. However, further evaluation of their bioactive compounds, antioxidant activities in living models is required.

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