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Antioxidant and Antimicrobial Attributes of Different Solvent Extracts from Leaves of Four Species of Mulberry

[1,2,3]Farooq Anwar, [3]Shamsa Kanwal, [4]Ghulam Shabir, [5,6]Khalid M. Alkharfy and [7]Anwar H. Gilani
[1]Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, 11942, Saudi Arabia
[2]Department of Chemistry, University of Sargodha, Sargodha, 40100, Pakistan
[3]Department of Chemistry and Biochemistry, University of Agriculture Faisalabad, 38040, Pakistan
[4]Environmental Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box 577, Jhang Road, Faisalabad, Pakistan
[5]Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
[6]College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, 11942, Saudi Arabia
[7]Pakistan Council for Science and Technology, Shahrah-e-Jamhuriat, G-5/2, Islamabad

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Corresponding Author:
Farooq Anwar
Department of Chemistry,
University of Sargodha, Sargodha,
40100, Pakistan
Tel: +92-48-923-0546
Fax +92-48-322-2121

ABSTRACT
The current study appraises the antioxidant and antimicrobial activities of the leave extracts from four different species of Mulberry (*Morus nigra* L., *Morus alba* L., *Morus macroura* Miq. and *Morus laevigata* W.). In addition to estimation of total phenolic and total flavonoids contents, the antioxidant activity of the extracts was evaluated by measuring the reducing power, inhibition of linoleic acid peroxidation and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activity. The yield of the extractable components from leaves ranged from 4.41-14.53 g/100 g Dry Weight (DW). The tested leave extracts exhibited widely variable amounts of total phenolics (3.14 to 11.38 g GAE/100g DW) and total flavonoids (0.53-5.83 g CE/100g DW). Reducing power of extracts at concentration 2.5 to 12.5 mg mL^{-1} ranged from 0.40-1.52. The leave extracts considerably inhibited linoleic acid peroxidation up to the level of 60.23-88.51% and also showed remarkable DPPH radical scavenging activity with contribution 43.83-77.51%. The antioxidant and antimicrobial potential of the tested leave extracts varied considerably in relation to the species and extraction solvents employed. Overall, the aqueous-methanolic extract of *Morus nigra* exhibited the superior antioxidant and antimicrobial activities among others. The present results advocate that mulberry leaves are a potential candidate for isolation of antioxidant and antimicrobial agents for potential nutraceutical and pharmaceutical applications.

Key words: Bioactives, antioxidant, extracts, aqueous alcohols, phenolics, microbes, inhibition diameter

INTRODUCTION

Lipid oxidation not only deteriorates the quality of food products, nevertheless, the reactive oxygen species and free radicals generated during this self-sustained process lead to damage bio-molecules and thus enhance the incidence of oxidative stress related diseases such as aging, inflammation and certain cancers (Sarikurkcu *et al*., 2008; Georgettia *et al*., 2008). Currently, there is a growing interest in finding the naturally occurring safer antioxidant for uses as ingredients in

functional food, pharmaceutical and cosmo-nutraceutical products so as to replace the synthetic antioxidants (Riaz *et al*., 2012; Naseer *et al*., 2014; Sultana *et al*., 2014; Saeed *et al*., 2014). On the other hand infectious diseases caused by pathogenic microorganisms are a serious threat for public health not only in developing countries but also in developed countries. The growing microbial resistance against synthetic antimicrobial drugs has further intensified the problem of infectious diseases (Sokmen *et al*., 2004; Chopra, 2007). The plant derived antimicrobial extracts have shown broad-spectrum bioactivity against infection causing agents such as bacteria, fungi, viruses and yeasts (Burt, 2004; Saeed *et al*., 2014; Sultana *et al*., 2014).

Selected plant bioactives exhibit multiple biological activities. Especially, plant phenolics including phenolic acids and flavonoids have been reported to possess remarkable antioxidant and antimicrobial activities and medicinal health functions and hence, play a preventive role against different diseases (Saleem *et al*., 2001; Cai *et al*., 2004; Baris *et al*., 2006; Saeed *et al*., 2014).

Although a huge number of medicinal plants have demonstrated antioxidant and antimicrobial potential with multiple applications in foods, drinks, cosmetics, perfumes, sanitary and pharmaceutical industries (Burt, 2004), however, due to increasingly growing demand of natural food additives, still the need exists to search for different sources of such functional components.

One of the potential sources of bioactives is Mulberry belonging to the genus *Morus* of the family Moraceae. This plant is distributed in temperate to subtropical and tropical regions of the world and can grow in a wide range of climatic, topographical and soil conditions (Ercisli *et al*., 2010). Mulberry fruits have been reported as a worming agent and a remedy for dysentery, as well as have a laxative, odontalgic, anthelmintic, expectorant, hypoglycaemic and emetic role (Weiguo *et al*., 2005). The fruits are also a potential source of purple and red colored natural dyes (Boschini, 2002). On the other hand, mulberry leaves are ecologically important as a dietary source for silkworm (Weiguo *et al*., 2005). The leaves have rarely been investigated for the biological principles. As the need for widely usable and easily available natural antioxidants and antimicrobial agents continues to grow, it is important to evaluate anti-oxidative and antimicrobial activities of Mulberry leaves. Therefore, the present research was designed to investigate the antioxidant and antimicrobial activities of leaves from different species of Mulberry.

MATERIALS AND METHODS

Collection of samples: The leave samples of different Mulberry species (Mulberry: *Morus nigra* L., *Morus alba* L., *Morus macroura* Miq. and *Morus laevigata* W.) were collected from the vicinity of University of Agriculture, Faisalabad, Pakistan following a random sampling protocol. The specimens were further identified and authenticated by the Department of Botany, University of Agriculture Faisalabad. Mulberry leaves were ambient-dried, ground and stored in polythene bags for further experiment.

Reagents and standards: Linoleic acid, BHT (99.0%), DPPH, catechin, gallic acid and Folin-Ciocalteu reagent (2 N) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Preparation of extracts: The ambient dried samples of Mulberry leaves were ground using a commercial blender (TSK-949, Westpoint, France). The material that passed through 80- mesh sieve was used for extraction purposes. The ground leaves material (20 g) was extracted with each of the solvents including aqueous methanol (methanol:water 80:20 v/v), absolute methanol, aqueous ethanol (ethanol:water 80:20) and absolute ethanol (solvent: solid ratio 10:1 v/w) for 8 h at room temperature in an orbital shaker (Gallenkamp, UK). The extract was separated from the residues by filtering through Whatman No. 1 filter paper. The residues were extracted twice with the fresh solvent and the extracts (filtrates) recovered were combined. The combined extracts were freed of solvent under reduced pressure at 45°C, using a rotary evaporator (EYELA, SB-651, Rikakikai Co. Ltd. Tokyo, Japan). The solvent freed, crude concentrated extract was weighed to calculate the yield and stored at 4°C, until used for further testing.

Determination of Total Phenolic Contents (TPC): TPC were estimated using Folin-Ciocalteu reagent. Briefly, 50 mg of crude extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL deionized water. The mixture was placed at room temperature for 10 min and then 1.5 mL of 20% sodium carbonate (w/v) added. The mixture was heated in a water bath at 40°C for 20 min and then cooled in an ice bath; absorbance was measured at 755 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). The TPC were calculated using gallic acid calibration curve within range of 10-100 ppm ($R^2 = 0.9986$). The results were expressed as gallic acid equivalents (GAE) g/100 g of DW. All samples were analyzed thrice and the averaged results were reported on dry weight basis.

Determination of Total Flavonoid Contents (TFC): TFC were measured following a previously reported method (Dewanto *et al*., 2002). Briefly, 1 mL extract (containing 0.1 mg mL^{-1} of extract) was taken in a 10 mL volumetric flask and diluted with 4 mL water. Initially, 0.3 mL of 5% $NaNO_2$ was added to volumetric flask; at 5 min, 0.3 mL of 10% $AlCl_3$ was added; at 6 min, 2 mL of 1.0 M NaOH was added. Water (2.4 mL) was then added to the reaction flask and mixed well. Absorbance of the reaction mixture was measured at 510 nm.

TFC were calculated as catechin equivalents (g/100 g of DW). Three readings were taken for each sample and the results were averaged.

DPPH radical scavenging assay: The antioxidant activity of leaves extract was assessed by measuring their free radical scavenging ability using 2, 2-diphenyl-1-picrylhydrazyl as free radical (DPPH˙). The DPPH assay was performed as described by Bozin *et al*. (2006). The samples (from 0.5-100 μg L^{-1}) were mixed with 1 mL of 90 μM DPPH solution and made up with 95% Methanol, to a final volume of 4 mL. Synthetic antioxidant, BHT was used as control. After 1 h incubation period at room temperature, the absorbance was recorded at 515 nm using spectrophotometer (U-2001, model 121-0032 Hitachi, Tokyo, Japan).

Inhibition of free radical by DPPH in% was calculated using following formula:

$$I (\%) = 100\times(A_b–A_s/A_b)$$

where, A_b is the absorbance of the control reaction (containing all reagents except the test compounds) and A_s is the absorbance of the test compounds.

Assessment of antioxidant activity in linoleic acid system: The antioxidant activity of Mulberry leaves extract was investigated by measuring the inhibition of peroxidation of linoleic acid (Iqbal *et al*., 2005). A weighed amount (5 mg) of leaves extract was mixed with a solution of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 10 mL of 0.2 M sodium phosphate buffer (pH 7). The mixture was made up to 25 mL with distilled water and incubated at 40°C up to 360 h. The extent of oxidation was measured by peroxide value applying thiocyanate method as described by Yen *et al*. (2000). Briefly, 10 mL of ethanol (75% v/v), 0.2 mL of aqueous solution of ammonium thiocyanate (30% w/v), 0.2 mL of sample solution and 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl; v/v) were added sequentially. After 3 min of stirring, the absorbance was measured at 500 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). A control contained all the reagents but without the extracts. A synthetic antioxidant, butylated hydroxytoluene (BHT) was used as a positive control. Percent inhibition of linoleic acid oxidation was calculated using the following equation:

100-[(Abs. increase of sample at 360 h/Abs. increase of control at 360 h)×100]

Determination of reducing power: The reducing power of Mulberry leaves extract was determined according to the procedure described by Yen *et al*. (2000) with slight modification. The extract (2.5-12.5 mg) was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%); the mixture was incubated at 50°C for 20 min. Then 5 mL of 10% trichloroacetic acid was added and the mixture centrifuged at 980 g for 10 min at 5°C in a refrigerated centrifuge (CHM-17; Kokusan Denki, Tokyo, Japan). The upper layer of the solution (5.0 mL) was taken and diluted with 5.0 mL of distilled water and ferric chloride (1.0 mL, 0.1%) and absorbance was noted at 700 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). All the measurements were made in triplicate and the results averaged.

Assessment of antimicrobial activity

Microbial strains: Pure bacterial and fungal strains were obtained from the Animal Sciences Division of the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The purity and identity of the strains were verified by the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. Bacterial strains were cultured overnight at 37°C in nutrient agar (Oxoid, Hampshire, UK) while fungal strains were cultured overnight at 30°C using potato dextrose agar (Oxoid). Mulberry leaves extracts were individually tested against a panel of microorganisms, including four bacteria, *Escherichia coli* B10, *Bacillus subtilis* SPS2, *Pasteurella multocida* and *Staphylococcus aureus* and four pathogenic fungi, *Aspergillus niger* ATCC (10-575), *Aspergillus flavis*, *Alternaria alternata* and *Rhizopus solani.*

Disc diffusion method: The antimicrobial activity of Mulberry leaves extract was determined by the disc diffusion method (NCCLS., 1997). The discs (6 mm in diameter) were impregnated with 50 μL or 30 mg mL^{-1} extracts (300 μg $disc^{-1}$) placed on the inoculated agar. Rifampicin (30 μg $disc^{-1}$) (Oxoid) and Fluconazole (30 μg $disc^{-1}$) (Oxoid) were used as a positive reference for bacteria and fungi, respectively. Disc without samples was used as a negative control. Antimicrobial activity was evaluated by measuring the inhibition zone.

Microdilution broth method: For calculation of Minimum Inhibitory Concentration (MIC), which represents the concentration that completely inhibits the growth of microorganisms, a micro-dilution broth susceptibility assay was used (NCCLS., 1999). A series of dilutions of Mulberry leaves extract were prepared in a 96-well microtiter plate, including one growth control (NB/SDB+Tween 80) and one sterility control (NB/SDB+Tween 80+test oil). One hundred sixty microliter NB and SDB for bacteria and fungi, respectively, were added onto the microplates with 20 μL of the test solution. Then, 20 μL 5×10^5 CFU mL^{-1} (confirmed by viable count) of standard microorganism suspension was inoculated onto the microplates. The plates were incubated at 37°C for 24 h for bacteria and at 30°C for 48 h for fungi. Rifampicin was used as a reference compound for antibacterial whereas Fluconazole for antifungal activities. The growth was indicated by the presence of a white ‘pellet’ on the well bottom. The MIC was calculated as the highest dilution showing complete inhibition of the tested strains.

RESULTS AND DISCUSSION

Yield of extracts: The percentage yield of ethanolic and methanolic extracts from leaves of Mulberry ranged from 4.41-14.53% (Table 1). The highest extraction yield from leaves was achieved with 80% aqueous methanol while the lowest with absolute ethanol among others. Among species selected, *Morus nigra* L., showed the highest percentage yield with 80% methanol. Arabshahi-Delouee and Urooj (2007) reported 12.35% yield of Mulberry leaves extract with methanol which are lower than our findings. Whereas Iqbal *et al*. (2012) reported the percentage yield of Mulberry leaves methanolic extract to be 14.48%. This reported value is comparable with the present yields. The results showed that both the species and extraction solvent had exerted significant ($p<0.05$) effect on the extraction yields. The variations in extraction yields in relation to solvent and species might be linked to the varying chemicals nature of the extractable compounds as well as polarity of the solvents used.

Total phenolic and total flavonoid contents: The highest TPC and TFC were recovered with 80% methanol (5.02-11.38 g GAE/100 g DW, 2.60-5.83 CE/100 g DW) in *Morus nigra* L., whereas lowest was with absolute ethanol (3.14-5.02 g GAE/100 g DW, 0.53 to 2.01 g CE /100 g DW) in *Morus laevigata* W., leaves, respectively. Among solvents used in this study, 80% methanol and 80% ethanol showed the best recovery for phenolic components. It can also be observed that aqueous mixture of solvents (80% methanol and 80% ethanol) have greater efficacy towards extraction of TPC and TFC from mulberry leaves. The 80% methanol is known to be an efficient and widely used solvent to extract phenolics and flavonoids from plant materials, due to the fact that methanol-water mixtures are highly polar and thus show greater efficacy in the extraction of polar phytochemicals such as phenolics and flavonoids (Anwar *et al*., 2013).

Although the aqueous-methanol is found relatively more effective for extraction of mulberry phenolics, however, ethanol due to its bio-solvent and degradable nature, is a good choice for recovery of safer phenolics extract form this species. The contents of TP and TF of mulberry leaves noted to be varied significantly ($p<0.05$) in relation to species and extraction media depending upon nature of extractable chemical constituents as well as nature of the extraction solvent. An earlier study showed that phenolic compounds of methanol extract of Mulberry leaves ranged from 1.62-2.43 g GAE/100 g (Iqbal *et al*., 2012). The reported value of TPC is much lower than our findings. Total flavonoid compounds in mulberry leaves reported by Iqbal *et al*. (2012), from 2.64-3.13 rutin equivalent g/100 g DW are quite comparable with our present results.

Antioxidant activity of in the linoleic acid peroxidation system: All the tested leave extracts exhibited appreciable inhibition of peroxidation ranging from 60.23-88.51%. Aqueous methanol (80% methanol) extracts from *Morus nigra* leaves had the highest inhibition of peroxidation 88.51%

Table 1: Yields and antioxidant activity of different solvent extracts from leaves of different species of Mulberry

	Solvent extracts			
Species of Mulberry	Absolute methanol	80% methanol	Absolute ethanol	80% ethanol
***Morus nigra* L.**				
Yield (%)	9.43±0.19^{b}	14.53±0.23^{a}	4.60±0.26^{c}	10.53±0.32^{b}
TPC (g/100 g DW)	8.48±0.21^{a}	11.38±0.20^{a}	5.02±0.14^{bc}	7.16±0.18^{bc}
TFC (g/100 g DW)	3.62±0.12^{a}	5.83±0.07^{a}	2.60±0.08^{ab}	3.04±0.05^{ab}
DPPH (%)	70.80±1.40^{bc}	77.51±1.54^{a}	60.61±1.98^{bc}	65.81±1.38^{ab}
Inhibition of linoleic acid peroxidation (%)	78.45±2.50^{ab}	88.51±1.44^{a}	65.56±2.98^{ab}	77.54±1.40^{ab}
***Morus alba* L.**				
Yield (%)	8.62±0.26^{b}	13.70±00.21^{a}	4.41±0.28^{c}	12.20±0.20^{a}
TPC (g/100 g DW)	7.12±0.18^{ab}	9.07±0.17^{a}	4.38±0.22^{c}	5.98±0.13^{bc}
TFC (g/100 g DW)	3.06±0.09^{a}	3.30±0.09^{a}	1.89±0.08^{ab}	2.11±0.06^{b}
DPPH (%)	70.23±1.41^{ab}	75.72±1.51^{a}	58.72±2.32^{b}	63.71±2.04^{bc}
Inhibition of linoleic acid peroxidation (%)	61.42±1.25^{b}	80.24.±1.40^{a}	60.44±2.90^{b}	70.43±1.35^{b}
***Morus macroura* Miq.**				
Yield (%)	6.41±0.17^{bc}	10.32±0.22^{b}	5.62.6±0.22^{c}	6.60±0.20^{bc}
TPC (g/100 g DW)	6.21±0.14^{bc}	7.02±0.28^{b}	3.64±0.19^{c}	4.99±0.14^{bc}
TFC (g/100 g DW)	1.72±0.06^{ab}	2.13±0.08^{b}	0.90±0.06^{bc}	1.01±0.05^{bc}
DPPH (%)	67.51±2.01^{bc}	72.42±1.44^{b}	55.82±2.20^{c}	61.4±2.44^{b}
Inhibition of linoleic acid peroxidation (%)	66.32±2.20^{bc}	77.02±1.63^{ab}	55.34±1.42^{c}	65.63±1.38^{bc}
***Morus laevigata* W.**				
Yield (%)	6.21±0.20^{bc}	10.71±0.21^{b}	8.00±0.32^{ab}	10.32±0.31^{b}
TPC (g/100 g DW)	3.56±0.15^{bc}	4.66±0.15^{ab}	3.14±0.14^{c}	3.88±0.23^{c}
TFC (g/100 g DW)	1.60±0.08^{ab}	2.01±0.06^{b}	0.53±0.02^{c}	0.60±0.07^{c}
DPPH (%)	61.51±2.48^{b}	66.93±2.07^{b}	43.83±2.12^{c}	54.62±2.16^{c}
Inhibition of linoleic acid peroxidation (%)	50.65±1.85^{bc}	73.11±1.40^{ab}	60.23±1.08^{c}	65.22±1.39^{bc}

TPC: Total phenolic contents expressed as gallic acid equivalent, TFC: Total flavonoid contents expressed as catechin equivalent, Values are Mean±SD of three separate experiments. Different superscript letters within the same row indicate significant ($p<0.05$) differences of means within the extracting solvents

while the lowest (60.23%) was observed for ethanol extract of *Morus laevigata* leaves (Table 1). A positive control, BHT used for comparison purposes exhibited 90.82% inhibition of peroxidation. The potential of the tested leaves for inhibition of peroxidation can be attributed to presence of antioxidants such as phenolics (Yen *et al*., 2000). The percent inhibition of linoleic peroxidation in methanol extract of mulberry leaves reported by Arabshahi-Delouee and Urooj (2007) was 81.8% which was lower to our results (88.51%) using 80% methanol extract.

DPPH radical scavenging activity: The tested leaves extract exhibited appreciable DPPH radical scavenging activity ranging from 43.83-77.51%. In line with total phenolics contents, 80% methanol extract of *Morus nigra* L., showed the highest radical scavenging mounting to 77.51%, while pure ethanol extract from leaves of *Morus laevigata* had the lowest (43.83%) potential (Table 1). DPPH radical scavenging activity of the leave extracts was noted to be varied significantly ($p<0.05$) among different extraction solvents and species selected. A good radical scavenging activity of the leaves might be due to the presence of phenolic and flavonoids with potential hydrogen donors to quench DPPH radicals. The value of DPPH radical scavenging activity of methanolic extract of leaves (77.51%) is higher than previously reported values (61.00%) (Bhakta and Ganjewala, 2009). A comparison of different extracting solvents used in the present study revealed that DPPHº radical scavenging capacity of Mulberry leaves extracts was as follows: 80% methanol extract>absolute methanol extract >80% ethanol extract>absolute ethanol extracts.

Reducing power of the extracts: Measurement of reducing potential can be used to describe some aspects of antioxidant activity of the plant extracts. In this method, ferric (Fe^{3+}) ions are reduced to ferrous (Fe^{2}+) ions with change in color from yellow to bluish green. The intensity of color depends on the reducing potential of the compounds present in the extract medium. Greater the intensity of the color, greater will be the absorption; consequently, greater will be the antioxidant activity (Zou *et al*., 2004).

The data for the reducing potential of different leave extracts are presented in Table 2. The reducing potential of the leaves extract measured for the concentration varying from 2.5-12.5 mg mL^{-1} and showed a general increase in activity with increase in concentration of the extracts. Reducing potential of different leaves extracts at 12.5 mg mL^{-1} ranged from 0.40-1.52. There are no earlier reports available on the reducing potential of leaves of Mulberry plant with which to compare the present results.

Correlations among the results of different antioxidant assays: Assessment of antioxidant activity of plant materials using different methods is widely accepted. Taking into account the results of the different antioxidant assays such as inhibition of lipid peroxidation, DPPH radical scavenging

Table 2: Reducing Power (absorbance value at 700 nm) of different solvent extracts from leaves of different species of Mulberry

Species and concentration (mg mL^{-1})	Absolute methanol	80% methanol	Absolute ethanol	80% ethanol
***Morus nigra* L.**				
2.5	0.93±0.01	0.99±0.03	0.64±0.01	0.97±0.03
5	1.00±0.03	1.21±0.04	0.79±0.02	1.00±0.02
7.5	1.07±0.02	1.45±0.02	1.00±0.03	1.14±0.03
10	1.42±0.02	1.68±0.05	1.11±0.04	1.22±0.04
12.5	1.56±0.03	1.79±0.06	1.21±0.02	1.30±0.03
Mean	1.42±0.02^{b}	1.52±0.04^{a}	0.95±0.02^{c}	1.22±0.03^{ab}
***Morus alba* L.**				
2.5	0.67±0.02	0.90±0.03	0.59±0.03	0.62±0.04
5	0.88±0.01	1.09±0.01	0.71±0.02	0.76±0.04
7.5	0.98±0.04	1.20±0.01	0.90±0.02	1.01±0.02
10	1.07±0.02	1.30±0.05	1.02±0.03	1.18±0.02
12.5	1.52±0.03	1.60±0.03	1.17±0. 02	1.29±0.02
Mean	1.02±0.02^{b}	1.21±0.03^{a}	0.87±0.02^{ab}	0.97±0.03^{b}
***Morus macroura* Miq.**				
2.5	0.59±0.01	0.84±0.04	0.43±0.02	0.51±0.01
5	0.72±0.04	0.96±0.03	0.57±0.03	0.63±0.02
7.5	0.87±0.02	1.14±0.05	0.66±0.02	0.75±0.04
10	0.93±0.05	1.25±0.05	0.73±0.03	0.82±0.02
12.5	1.28±0.02	1.37±0.04	0.81±0.02	0.90±0.02
Mean	0.88±0.03^{b}	1.11±0.04^{a}	0.64±0.02^{bc}	0.72±0.02^{c}
***Morus laevigata* W.**				
2.5	0.58±0.01	0.67±0.03	0.49±0. 01	0.50±0.04
5	0.70±0.03	0.72±0.03	0.51±0.02	0.61±0.03
7.5	0.87±0.04	0.84±0.02	0.60±0.02	0.72±0.04
10	0.92±0.02	0.99±0.03	0.67±0.01	0.80±0.05
12.5	1.00±0.05	1.16±0.02	0.78±0.01	0.85±0.02
Mean	0.91±0.03^{a}	0.87±0.03^{a}	0.40±0.01^{c}	0.50±0.03^{b}

Values are Mean±SD of three separate experiments. Different superscript letters within the same row indicate significant ($p<0.05$) differences of means within the extracting solvents

Table 3: Comparison of results from different antioxidant assays as represented by Correlation coefficient (r)

Correlation variable	TPC	TFC	DPPH	% Inhib	RP
TPC	1				
TFC	0.916	1			
DPPH	0.854	0.786	1		
Inhibition (%)	0.865	0.765	0.717	1	
Reducing power	0.872	0.900	0.881	0.746	1

Table 4: Antimicrobial activity in terms of inhibition zones and minimum inhibitory concentration of *Morus nigra* L. leaves extracts against the selected strains of bacteria and fungi using different solvents

	Leaves extracts					
Tested organisms	Absolute methanol	80% methanol	Absolute ethanol	80% ethanol	Rifampicin	Fluconazole
Diameter of inhibition zone (mm)						
E. coli	19.3±0.5^{b}	22.40±0.5^{b}	12.20±0.7^{d}	14.6±0.7^{cd}	22.5±0.9^{b}	-
B. subtilis	13.6±0.4^{d}	14.20±0.3^{cd}	11.60±0.7^{d}	12.8±0.4^{d}	24.3±0.6^{b}	-
S. Aureus	16.7±0.4^{c}	18.70±0.3^{c}	14.40±0.4^{cd}	15.6±0.5^{cd}	26.2±0.7^{a}	-
P. multocida	15.4±0.7^{cd}	19.90±0.8^{b}	13.80±0.2^{cd}	15.7±0.5^{cd}	18.7±0.8^{c}	
A. niger	16.4±0.3^{c}	18.10±0.3^{c}	12.50±0.5^{d}	14.6±0.6^{cd}		28.5±0.6^{a}
A. flavus	17.5±0.4^{c}	24.50±0.4^{b}	12.60±0.3^{d}	15.4±0.6^{cd}		26.6±0.8^{a}
R. solani	17.9±0.3^{c}	24.50±0.4^{b}	12.40±0.3^{d}	16.3±0.7^{c}		28.9±0.5^{a}
A. alternata	18.3±0.3^{c}	25.60±0.5^{b}	14.30±0.3^{cd}	16.7±0.6^{c}		24.3±0.4^{b}
Minimum inhibitory concentration (mg mL^{-1})						
E. coli	29.9±0.9^{d}	24.40±0.2^{de}	61.80±0.6^{a}	47.9±0.5^{b}	32.5±0.7^{d}	-
B. subtilis	24.4±0.7^{de}	22.00±0.2^{e}	55.90±0.9^{ab}	49.5±0.4^{b}	25.2±0.2^{de}	-
S. aureus	24.7±0.7^{de}	19.00±0.1^{e}	57.70±0.9^{a}	47.9±0.4^{b}	29.3±0.8^{d}	-
P. multocida	27.5±0.9^{d}	20.18±0.6^{e}	31.80±0.9^{d}	19.5±0.1^{e}	34.5±0.8^{cd}	-
A. niger	39.5±0.8^{c}	32.50±0.1^{d}	59.20±1.5^{a}	41.7±0.9^{c}		35.5±0.9^{cd}
A. flavus	57.4±0.5^{a}	48.00±0.4^{b}	62.30±1.1^{a}	58.2±1.9^{a}		37.8±0.5^{cd}
R. solani	59.5±1.5^{a}	47.90±0.4^{b}	66.20±0.1^{a}	50.5±1.8^{b}		39.7±0.6^{c}
A. alternata	46.8±0.4^{ab}	24.20±0.2^{de}	45.60±0.9	35.6±0.9^{cd}		37.2±0.5^{c}

Values are Mean±SD of three separate experiments. Different superscript letters within the same row are significant (P< 0.05) differences of means within the extracting solvents

capacity, measurement of reducing potential combined with the estimation of total phenolic and total flavonoid contents were compared and correlated with each other (Table 3). As far as the correlation among results of different antioxidant assays is concerned a very good relationship between TPC and TFC was observed (r = 0.916). Iqbal *et al.* (2012) also reported a good correlation in three different varieties of Mulberry leaves from Pakistan. A strong correlation between TPC and TFC indicates that both phenolic and flavonoids components of Mulberry leaves extracts have a strong redox potential and act as potent antioxidants. Positive correlation (r = 0.765) was also observed between TFC and percent inhibition of peroxidation data. Furthermore, higher correlation (r = 0.865) was observed between TPC and percent inhibition. This might be ascribed to the fact that leaves extracts of Mulberry showing greater amount of TPC and TFC also exhibited higher levels of percent inhibition of linoleic acid peroxidation. These results are in line with the findings of Anwar *et al.* (2013) who reported that extracts with higher TPC also showed strong activity against linoleic acid peroxidation. A good correlation between TPC and percent inhibition was also observed (r = 0.865). A strong correlation between TFC and reducing power (r = 0.900) was also noted. This strong correlation might be attributed to the presence of higher amounts of flavonoids and phenolics in the leaves of different mulberry species from different tested varieties; however, correlation between TPC and reducing power was moderate (r = 0.872). Furthermore, a positive correlation between DPPH and TPC was observed (r = 0.854) among the extracts of different Mulberry varieties, indicating that phenolic compounds are powerful scavenger of free radicals. However, a moderate correlation between DPPH and TFC (r = 0.786), meanwhile, a moderate correlation between percent inhibition and DPPH (r = 0.717) was also observed. This weak correlation found between the present results of percent inhibition with those of reducing power and DPPH assays might be linked to the fact that some antioxidant components show their antioxidant potential not only by acting as hydrogen donors, but also as oxygen scavengers. A moderate correlation between reducing power (r = 0.746) and percent inhibition was observed in the present investigation. This moderate correlation could be explained by the facts that there are some anti-oxidative compounds that not only exhibit their antioxidant activity by donating hydrogen but also by scavenging oxygen. Consequently extracts containing such antioxidant compounds might exhibit higher levels of % inhibition as compared to their reducing power and thus can display a weak correlation (Manzoor *et al*., 2012).

Antimicrobial activity: Antimicrobial activity of leaves extracts from different species of mulberry was evaluated against eight pathogenic bacteria and fungi. The antimicrobial activity of different solvent extracts of mulberry leaves varied

significantly ($p<0.05$) in relation to extraction solvents and species selected. Among the solvent extracts, the 80% methanol extract of leaves of *Morus nigra* exhibited the strongest antimicrobial activity followed by *Morus alba, Morus macroura* and *Morus laevigata* leave extracts with zones of inhibition ranging from 11.6-25.6 mm, 10.1-21.6 mm, 9.0-20.6 mm and 8.6-15.9 mm, respectively (Table 4, 5,6 and 7). The minimum inhibitory concentration (MIC) values for 80% methanol extracts of leaves from *Morus nigra* L., *Morus alba* , *Morus macroura* and *Morus laevigata* ranged from 19.0-66.2 mg mL^{-1}, 34.0-88.1 mg mL^{-1} and 40.0-98.9 mg mL^{-1}, respectively. The antimicrobial activity in terms of zones of inhibition and MIC data of 80% methanol extract of Mulberry leaves was comparable with the respective standard drugs Rifampicin and Fluconazole. The higher antimicrobial activity of 80% methanol extract of *Morus nigra* leaves might be partly due to the higher contents of phenolic acids and flavonoids in this extract. Flavonoids are known to retard the growth of microorganism through inhibiting their nucleic acid synthesis, cytoplasmic membrane function and energy metabolism (Thiem and Grosslinka, 2004; Cushnie and Lamb, 2005). Different species of Mulberry leaves exhibited antimicrobial activity to varying extents. These differences can be attributed

Table 5: Antimicrobial activity in terms of inhibition zones and minimum inhibitory concentration of *Morus alba* L. leaves extracts against the selected strains of bacteria and fungi using different solvents

	Leaves extracts					
Tested organisms	Absolute methanol	80% methanol	Absolute ethanol	80% ethanol	Rifampicin	Fluconazole
Diameter of inhibition zone (mm)						
E. coli	16.5±0.7^{c}	20.00±0.7^{b}	10.1±0.5^{a}	13.6±0.4^{bc}	22.5±0.9^{b}	-
B. subtilis	12.9±0.4^{c}	14.80±0.4^{bc}	11.5±0.4^{a}	12.2±0.5^{bc}	24.3±0.6^{b}	-
S. aureus	14.7±0.5^{bc}	15.90±0.6^{bc}	12.3±0.5^{a}	12.9±0.2^{bc}	26.2±0.7^{a}	-
P. multocida	14.5±0.3^{bc}	16.00±0.21^{bc}	10.8±0.8^{c}	12.4±0.5^{c}	18.7±0.8^{c}	
A. niger	13.5±0.2^{bc}	14.10±0.5^{b}	11.5±0.4^{c}	13.4±0.9^{bc}		28.5±0.6^{a}
A. flavus	14.9±0.2^{bc}	20.60±0.2^{b}	12.8±0.6^{bc}	14.1±0.7^{bc}		26.6±0.8^{a}
R. solani	15.8±0.7^{bc}	21.60±0.7^{b}	13.4±0.4^{bc}	15.2±0.5^{bc}		28.9±0.5^{a}
A. alternata	16.8±0.4^{bc}	22.70±0.8^{b}	12.7±0.6^{bc}	13.8±0.5^{b}		24.3±0.4^{b}
Minimum inhibitory concentration (mg mL^{-1})						
E. coli	45.4±1.2^{e}	35.60±0.5^{f}	72.0±0.9^{bc}	56.2±0.8^{d}	32.5±0.7^{d}	-
B. subtilis	36.2±1.4^{f}	31.60±1.2^{fg}	65.5±1.7^{c}	60.3±1.6^{cd}	25.2±0.2^{g}	-
S. aureus	32.0±0.9^{fg}	26.00±0.9^{g}	75.0±0.9^{b}	62.8±1.4^{cd}	29.3±0.8^{fg}	-
P. multocida	41.2±1.3^{ef}	28.50±0.7^{g}	45.5±0.8^{e}	29.2±1.1^{fg}	34.5±0.8^{f}	-
A. niger	51.0±0.9^{de}	32.30±0.8^{fg}	78.8±0.7^{ab}	65.3±1.7^{c}		35.5±0.9^{f}
A. flavus	65.2±1.5^{c}	41.60±0.9^{ef}	75.2±0.8^{b}	69.7±1.2^{bc}		37.8±0.5^{f}
R. solani	69.2±1.7^{bc}	63.20±1.4^{cd}	85.2±1.9^{a}	66.7±2.1^{c}		39.7±0.6^{ef}
A. alternata	65.2±0.9^{c}	62.03±1.7^{cd}	62.9±1.1^{cd}	52.5±0.5^{de}		37.2±0.5^{f}

Values are Mean±SD of three separate experiments. Different superscript letters within the same row are significant ($p<0.05$) differences of means within the extracting solvents

Table 6: Antimicrobial activity in terms of inhibition zones and minimum inhibitory concentration of *Morus macroura* Miq. leaves extracts against the selected strains of bacteria and fungi using different solvents.

	Leaves extracts					
Tested organisms	Absolute methanol	80% methanol	Absolute ethanol	80% ethanol	Rifampicin	Fluconazole
Diameter of inhibition zone (mm)						
E. coli	14.50±0.5^{bc}	18.8±0.71^{b}	9.0±0.5^{c}	12.6±0.5^{a}	22.5±0.9^{ab}	-
B. subtilis	10.90±0.4^{c}	12.6±0.3^{bc}	10.0±0.4^{c}	10.6±0.8^{bc}	24.3±0.6^{ab}	-
S. Aureus	12.90±0.9^{bc}	14.6±0.5^{bc}	11.7±0.3^{c}	12.9±0.5^{bc}	26.2±0.7^{ab}	-
P. multocida	14.80±0.2^{bc}	12.9±0.7^{bc}	9.0±0.7^{c}	10.6±0.8^{c}	18.7±0.8^{b}	
A. niger	13.00±0.3^{bc}	13.8±0.6^{bc}	10.6±0.5^{c}	11.9±0.5^{c}		28.5±0.6^{a}
A. flavus	12.90±0.4^{bc}	18.8±0.9^{b}	11.6±0.4^{c}	12.6±0.3^{bc}		26.6±0.8^{a}
R. solani	13.90±0.8^{ab}	18.7±0.3^{b}	11.9±0.9^{c}	13.0±0.7^{bc}		28.9±0.5^{a}
A. alternata	14.70±0.3^{bc}	20.6±0.7^{b}	12.6±0.4^{a}	13.8±0.5^{ab}		24.3±0.4^{b}
Minimum inhibitory concentration (mg mL^{-1})						
E. coli	52.90±0.03^{e}	44.4±0.2	75.6±1.5^{bc}	69.0±0.9^{c}	32.5±0.7^{fg}	-
B. subtilis	42.97±0.8^{f}	40.1±0.1^{f}	72.7±1.1^{bc}	65.9±0.9^{cd}	25.2±0.2^{h}	-
S. aureus	44.00±0.09^{ef}	34.0±0.2^{fg}	80.4±1.8^{b}	69.0±1.6^{c}	29.3±0.8^{g}	-
P. multocida	59.50±0.2^{d}	34.0±0.2^{fg}	48.2±0.6^{ef}	35.5±0.9^{fg}	34.5±0.8^{fg}	-
A. niger	67.00±0.4^{cd}	47.0±0.9^{ef}	88.1±0.8^{a}	70.8±1.7^{c}		35.5±0.9^{ef}
A. flavus	72.00±0.4^{bc}	55.0±0.9^{de}	77.3±1.8^{bc}	75.9±1.1^{bc}		37.8±0.5^{ef}
R. solani	83.90±0.6^{ab}	79.2±0.5^{b}	89.2±1.3^{a}	68.7±0.9^{c}		39.7±0.6^{f}
A. alternata	79.40±0.6^{b}	77.2±0.5^{bc}	68.7±1.7^{c}	56.3±1.8		37.2±0.5^{f}

Values are Mean±SD of three separate experiments. Different superscript letters within the same row are significant ($p<0.05$) differences of means within the extracting solvents

Table 7: Antimicrobial activity in terms of inhibition zones and minimum inhibitory concentration of *Morus laevigata* W. leaves extracts against the selected strains of bacteria and fungi using different solvents

	Leaves extracts					
Tested organisms	Absolute methanol	80% methanol	Absolute ethanol	80% ethanol	Rifampicin	Fluconazole
Diameter of inhibition zone (mm)						
E. coli	12.60±0.7^{bc}	14.5±0.7^{bc}	8.60±0.6^{c}	10.60±0.4^{c}	22.5±0.9^{b}	-
B. subtilis	11.40±0.5^{c}	13.6±0.6^{c}	9.50±0.9^{c}	10.30±0.8^{c}	24.3±0.6^{ab}	-
S. Aureus	11.50±0.6^{c}	14.3±0.6^{bc}	12.70±0.4^{bc}	11.10±0.9^{c}	26.2±0.7^{ab}	-
P. multocida	10.40±0.7^{c}	10.6±0.4^{c}	8.60±0.7^{c}	9.50±0.5^{c}	18.7±0.8^{b}	
A. niger	12.30±0.4^{c}	14.9±0.3^{bc}	10.80±0.4^{c}	11.50±0.8^{c}		28.5±0.6^{a}
A. flavus	12.30±0.6^{c}	12.6±0.8^{bc}	11.60±0.7^{c}	11.90±0.6^{c}		26.6±0.8^{ab}
R. solani	10.80±0.5^{c}	14.2±0.8^{bc}	10.50±0.8^{c}	11.90±0.4^{c}		28.9±0.5^{a}
A. alternata	11.20±0.4^{c}	15.9±0.9^{bc}	10.60±0.8^{c}	11.30±0.5^{c}		24.3±0.4^{ab}
Minimum inhibitory concentration (mg mL^{-1})						
E. coli	69.20±1.9^{d}	50.2±0.8^{f}	86.31±1.2^{bc}	79.00±1.3^{d}	32.5±0.7^{h}	-
B. subtilis	54.00±0.8^{ef}	55.4±1.3^{ef}	86.31±1.6^{bc}	77.00±1.5^{cd}	25.2±0.2^{i}	-
S. aureus	50.10±1.6^{f}	40.0±0.7^{g}	95.50±2.1^{ab}	74.00±1.9^{cd}	29.3±0.8^{h}	-
P. multocida	71.50±0.9^{d}	45.4±0.9^{fg}	53.15±1.3^{ef}	40.00±0.08^{g}	34.5±0.8^{gh}	-
A. niger	81.51±1.3^{c}	51.0±1.2^{f}	94.70±1.5^{ab}	82.02±1.0^{c}		35.5±0.9^{gh}
A. flavus	97.20±1.8^{ab}	61.3±1.4^{e}	82.00±1.4^{c}	83.50±1.2^{bc}		37.8±0.5^{gh}
R. solani	96.50±2.1^{ab}	89.6±1.8^{b}	98.90±1.9^{a}	70.50±1.7^{d}		39.7±0.6^{g}
A. alternata	82.90±1.5^{c}	80.6±1.5^{c}	71.30±0.8^{d}	64.60±0.8^{de}		37.2±0.5^{gh}

Values are Mean±SD of three separate experiments. Different superscript letters within the same row are significant ($p< 0.05$) differences of means within the extracting solvents

to the accumulation and distribution of variable contents of antimicrobial agents in different species of Mulberry. In our study the antimicrobial activity of *Morus alba* leaves extract was found to be stronger than reported in an earlier study on this plant (Thabti *et al*., 2014).

CONCLUSION

In the present research study, the antioxidant and antimicrobial activities of leaves of four locally available varieties of Mulbery from Pakistan were evaluated. Among the four species analyzed, *Morus nigra* leave extracts exhibited stronger antioxidant and antimicrobial activities. Besides, the antioxidant and antimicrobial activities of the tested extracts were noted to be varied considerably depending on the genetic makeup of the species as well as a function of extraction solvents. The present results support that the aqueous-alcoholic solvents are more efficient towards recovery of antioxidant and antimicrobial agents from mulberry leaves.

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