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ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL PROFILE AND TRACE MINERALS OF BLACK MULBERRY (*MORUS NIGRA* L.) FRESH JUICE

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Abstract

In the present work, the fresh juice of black mulberry (*Morus nigra*) was tested for antimicrobial activity against various pathogenic microorganisms. Total antioxidant contents, total phenolic contents, total anthocyanins, trace minerals, total acid contents, total solids and ascorbic acid content were also evaluated. The results showed good antimicrobial activity both for Gram- positive and Gram-negative bacteria, with highest zones of inhibition for *Bacillus spizizenii* (19.68 mm, Gram-positive) and *Pseudomonas aeruginosa* (19.87 mm, Gram-negative). The black mulberry juice was rich in ascorbic acid (23.45 mg/100 g), had low overall acid content (1.60 %) and had 19% total soluble solids. The average total anthocyanins and total phenolic contents of black mulberry juice were 769 µg/g of cyanidin 3-glucoside equivalent (Cy 3-gly) per gram and 2050 µg of gallic acid equivalent (GAE) per gram of fresh juice. The average antioxidant activity (Trolox equivalent, TE) of fresh juice was 14.00 µmol/g according to a FRAP assay and 20.10µmol/g according to a DPPH assay. The fresh juice was also rich in a variety of trace minerals.

Introduction

Plants are excellent sources of food, chemicals and herbal medicines. Many important drugs have been directly or indirectly derived from them (Venkatesh & Chauhan, 2008). Mulberry belongs to the genus *Morus* of the family *Moraceae*. This genus has twenty-four species with one subspecies and has one hundred known varieties to date (Ercisli & Orhan, 2007; Hojjatpanah *et al.,* 2011). Plants are monoecious or dioecious trees that can grow up to 12 m in height. Geographically, the genus is found in temperate to subtropical regions of the northern hemisphere, and it has the ability to grow under a wide variety of soil, topographical and climatic conditions (Ercisli & Orhan, 2007). In term of food production, mulberry has three main species; white (*Mogus alba*), red (*Morus rubra*) and black (*Morus nigra*). The white mulberry (*Morus alba*) is recognized as the primary food source for silk worms and is widely cultivated in China (Venkatesh & Chauhan, 2008). Red mulberry (*Morus rubra*) is native to the United State of America and grows in forests; it is renowned as a rich source of flavones, namely rubraflavones (Rastogi & Mehrotra, 1990). The black mulberry is indigenous to Iran but was exported to Britain more than 500 years ago. Today it is cultivated in Southern Europe and Southwest Asia and is recognized as one of most important fruits in Mediterranean countries (Ercisli & Orhan, 2007; Hojjatpanah *et al.,* 2011). Black mulberry is mostly used for making processed foods such as pekmez, marmalades, juices, liquors, natural dyes, and frozen fruits for ice cream (Hojjatpanah *et al.*, 2011).

Black mulberry (*Morus nigra*) is a small to medium tree that reaches nine meters in height. The leaves are ovate-cordate, the flowers are mostly dioecious but sometimes monoecious. The fruits are syncarps, ovoid with purple to black, juicy, edible pulp (Venkatesh & Chauhan, 2008). The fruits of black mulberry are famous for their nutritional qualities, flavor and especially medicinal properties. The root bark of black mulberry is a vermifuge and purgative. The root bark extract contains deoxyjirimycin (DNJ). This alkaloid is considered to be effective against HIV (Venkatesh & Chauhan, 2008). The juice from roots of black mulberry has the ability to reduce blood sugar in diabetic patients and also has some effect on the pancreas and glycogenylsis (Ahmad *et al.,* 1985; Venkatesh & Chauhan, 2008). Similarly, root bark contains calcium malate, tannins, phytobaphenes, sugar, phytosterol, fatty acids and phosphoric acid (Venkatesh & Chauhan, 2008). The fruits of this plant have good impact on blood glucose level (Martin *et al.*, 2003) and can control blood cancer (Ahmad *et al.*, 1985). Additionally, black mulberry fruit contains high amounts of total phenolics, total flavonoids, and ascorbic acid (Hassimotto *et al.,* 2007; Nitra *et al.,* 2007). The fruit has a pleasant taste with a slightly acidic flavor and has an attractive dark red color (Koyuncu *et al.,* 2004; Özgen *et al.,* 2009b). The proximate analysis of black mulberry indicates that it contains 85.5% moisture, 0.7% protein, 0.4% fat, 12.2% carbohydrates, 0.8% fibre, and 0.4% mineral matter. It has nutritional values of 60 mg calcium, 20 mg phosphorus, 2.6 mg/100 g iron, 58 mg thiamine, 0.2 mg nicotinic acid, 92 mg riboflavin, and 10 mg /100 g ascorbic acid (Venkatesh & Chauhan, 2008).

Identification and quantification of anthocyanins, phenolics and antioxidant properties of different berries are now well defined (Sun *et al.,* 2002; Özgen *et al.,* 2006), but quantification and identification of these phytols from mulberry fruits is very limited (Özgen *et al.*, 2009b). Mulberry fruit juice is rich in anthocyanins. The major components identified include cyanidine-3-glucoside and cyanidine- 3-rutinoside (Sun *et al.,* 2002; Lee *et al.,* 2004). Cyanidin 3-*O*-,-D-glucopyranoside isolated from mulberry fruits inhibited the cerebral ischemic damage caused by oxygen glucose deprivation in PC12 cells (Kang *et al.*, 2006). The anthocyanins in black mulberry inhibited the copper-induced peroxidation of liposome and the co-oxidation of linoleic acid and *β*-

carotene (Hassimotto *et al.*, 2007). Naderi *et al*., (2004) found that extracts of *Morus nigra* fruits had a protective action against peroxidative damage to biomembranes and biomolecules. Morin, a flavonoid present in mulberry, significantly reduced the tissue level of cyclosporin, a potent immunosuppressive agent and considerably decreased nitric oxide production by the activated macrophages (Fang *et al.*, 2005). Black mulberry juice inhibited human cytochrome CYP3A activity in a pooled human liver microsome system (Kim *et al.*, 2006). The anti-stress activity of straight mulberry juice in mice was reported by Sakagami *et al.* (2006). Mulberry juice has scavenging properties against superoxide, hydroxyl and nitric acid, and these properties are confirmed by ESR spectroscopy. This scavenging activity is approximately 50% as effective as that of blueberry juice (Sakagami *et al.,* 2006). The anti-HIV activity of mulberry was more than four times than that of blueberries. The possible mechanism of this anti-HIV activity lies in lignified materials present in mulberry juice (Sakagami *et al.*, 2007).

Black mulberry has antimicrobial and anti-inflammatory properties as well (Butt *et al.*, 2008). The methanolic extract of *Morus* species has exudative, proliferative and anti-pyretic activities (Jawad *et al.,* 1988; Sener & Binjol, 1988). Root bark, which has a bitter acid taste, possessed cathartic and anthelmentic properties. Mulberry root is one of the constituents of a drug named "Glucosidase", which is used to treat high blood pressure. Root juice agglutinates the blood and is very useful in killing worms in digestive system (Shivkumar *et al.,* 1995; Venkatesh & Chauhan, 2008).

The present study was conducted to test the antimicrobial activity of black mulberry (*Morus nigra*) juice against a variety of pathogenic bacteria. The second objective was to compare antimicrobial activity with standard antibiotics, followed a phytochemical analysis of black mulberry juice.

Material and Methods

Collection and preparation of black mulberry (*Morus nigra*) fruit juice: Fresh black mulberry fruits were procured from the area surrounding Haraza University, Pakistan and verified by the Department of Botany. Approximately 500 g of fresh fruits were taken immediately to the laboratory for analysis. The fruits were mashed in a juicer (MK-8710, National, Japan). The fresh juice was filtered using filter paper and then subjected to physicochemical analysis. Three replications were run for each analysis. The following parameters were measured: total anthocyanins (TA), total phenolic content (TP), total antioxidant content (TAC), ascorbic acid (AsA), total acidity (TAY), soluble solid content (TSS), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe), and zinc (Zn). For determination of antimicrobial activity, 100 µl of fresh juice was used without any further processing to determine the actual antimicrobial activity of black mulberry.

Antimicrobial activity of fresh black mulberry fruit (*Morus nigra*) juice: The antimicrobial activity of fresh black mulberry fruit juice was evaluated *in vitro* against eight reference Gram-positive and Gram-negative bacterial strains: *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 49452, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus spizizenii* ATCC 6633, *Bacillus subtilis* ATCC 19659 and *Corynebacterium diphtheria* ATCC 39255. All reference strains were obtained from American Type Culture Collection (ATCC) and were maintained on Nutrient agar slants (Oxoid, UK) at 4°C.

Purity testing of each organism: Each organism was inoculated from a working culture of nutrient broth (Merck) on its respective selective medium for control as well as for purity testing. *Pseudomonas aeruginosa* was grown on Pseudomonas Cetrimide Agar (PCA; Oxoid, CM0579), *Salmonella typhimurium* on Xylose Lysine Deoxycholate Agar (XLD; Oxoid, CM0469), *Staphylococcus aureus* on Mannitol Salt Agar (MSA; Oxoid, CM0085), *Enterococcus faecalis* on Slanetz & Bartley (S&B; Oxoid, CM0377), *Escherichia coli* on Eosin Methylene Agar (EMB; Oxoid, CM0069), *Bacillus spizizenii* on Chromogenic Bacillus cereus Agar *(*CBA; Oxoid, SR0230), *Bacillus subtilis* on *Mannitol Egg Yolk Polymyxin* Agar (MYP; Oxoid**,** CM0929) and *Corynebacterium diphtheria* on Tinsdale Agar Base **(**TAB; Oxoid, CM0487). All plates were incubated at 37°C for 24 hrs.

Evaluation of antimicrobial activity: After incubation, one colony of each bacterium from its respective selective agar medium was inoculated into 5 ml nutrient broth and incubated for 4-6 hrs at 37°C. The inocula were standardized by matching turbidity with a McFarland standard (No. 1). The test culture was spread evenly on the surface of pre-sterilized plastic petri dish containing solidified Mueller Hinton Agar (MHA; Oxoid CM 0337) with a sterile cotton swab. Three wells were made in the MHA agar plate using a sterile cork borer of 6 mm. With a sterile micropipette tip, 100 µl of fresh black mulberry juice were poured into each well. The plates were incubated at 37°C for 24 hrs. After 24 hrs, the diameter of the resulting zone of inhibition was measured and the average values were recorded. Each antimicrobial assay was performed in triplicate. Standard discs (7 mm diameter) of ampicillin (AMP 25 µg), chloramphenicol (C 30 µg), ciprofloxacin (CIP 5 µg), tetracycline (TE 30 µg), erythromycin (E 15 µg), neomycin (N 30 µg) and gentamicin (CN 10µg), obtained from Oxoid Ltd, were used as positive controls for antimicrobial activity against different Gram-positive and Gram-negative bacteria.

Determination of ascorbic acid, total acidity and total soluble solids in black mulberry juice: The ascorbic acid content was determined by reflectometer strips (Merck RQflex, Darmstadt, Germany). Black mulberry juice samples were prepared according to Merck protocol for ascorbic acid. A mixture of 2 ml juice, 9 ml distilled water and 500 mg PVP (polyvinylpyrrolidine) was homogenized and filtered through filter paper. Ascorbic acid (AsA) was quantified as mg per 100 ml, following the protocol of Hietarahta *et al.,* (2010). The total acidity was determined according to the AOAC method (Anon.,

2003). Total soluble solids were determined with a digital refractometer (Kyoto Electronics, Japan) by using one drop of juice in digital refractometer at 25°C and were expressed as a percentage of fresh juice (volume basis).

Determination of total anthocyanins, total phenolic content and total antioxidants in black mulberry juice: The total anthocyanin (TA) content was quantified according to the bisulfate bleaching method described by Ribéreau-Gayon *et al.* (1965). The results were expressed as mg of cyanidin 3-glucoside equivalent per g of fresh juice. Total phenolic contents (TP) were estimated by using the Folin-Ciocalteu colorimetric method described by Ough and Amerine, (1988). TP was expressed as µg of gallic acid equivalent (GAE) per g of fresh juice. Total antioxidant contents (TAC) were estimated by using ferric reducing ability of plasma (FRAP) (Benzie & Strain, 1996) and the 2,2-diphenyl-1-picrylhydrazil (DPPH) assays to measure the free radical scavenging capacity (Brand-Williams *et al.*, 1995). Results were expressed in mol of Trolox equivalents per g of fresh juice for both FRAP and DPPH assays.

Determination of trace mineral elements: Potassium, calcium, magnesium, sodium, iron, manganese and zinc were determined after wet digestion using an HNO_3-$HClO_4$ acid mixture (4:1 by volume) with a PerkinElmer 360 Atomic Absorption Spectrophotometer (PerkinElmer, Waltham, MA, USA). Results for the trace minerals were expressed in mg per 100 g of fresh juice.

Results and Discussions

The antimicrobial activity of fresh juice of black mulberry is presented in Table 1. The zones of inhibition against various Gram-positive and Gram-negative bacteria were measured in mm. Zones of inhibitions were seen against all species, confirming the antimicrobial activity of black mulberry juice. The maximum zone of inhibition was against *Pseudomonas aeruginosa* (19.87 mm), followed by *Bacillus spizizenii* (19.68 mm) and *Bacillus subtilis* (18.46 mm). The minimum zone of inhibition was obtained against *Escherichia coli* (9.98 mm). Among the Gram- positive species, *Bacillus* species exhibited highest zones of inhibition (Table 1) while for Gram- negative bacteria, *Pseudomonas aeruginosa* had higher inhibition than *Salmonella typhimurium* or *Escherichia coli.* The stem, bark and stem wood of *Morus nigra* also showed antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus flavus*, *Streptococcus faecalis*, *Salmonella abony* and *Pseudomonas aeruginosa* (Mazimba *et al.,* 2011). Antibacterial activity was also reported from a methanol extract of stem bark of *Morus mesozygia* (Kuete *et al.,* 2009).

Black mulberry juice, in comparison with standard antibiotics like ampicillin, chloramphenicol, ciprofloxacin, tetracycline, erythromycin, neomycin and gentamicin, generally produced smaller zones of inhibition (Table 1); however, black mulberry juice has advantages over the tested antibiotics because microbes have not yet developed resistance against it and it contains higher amounts of phytochemicals (Figs. 1 and 2).

Table 1. Comparison of antimicrobial activity of black mulberry (*Morus nigra*) fresh juice to selected antibiotics.

Bacterial strains tested for antimicrobial activity	Mean zone of inhibition (mm)							
	Fresh Juice (100 µl)	CIP 5 µg	TE 30 µg	AMP 25 µg	C 30 µg	E 15 µg	N 30 µg	CN 10 µg
Gram-positive strains								
Bacillus spizizenii ATCC 6633	19.68 ± 0.70	34.19 ± 0.67	29.79 ± 0.65	38.72 ± 0.44	23.76 ± 0.58	32.23 ± 0.80	24.26 ± 0.75	26.16 ± 0.38
Bacillus subtilis ATCC 19659	18.46 ± 0.90	36.17 ± 0.71	29.93 ± 0.71	38.66 ± 0.63	25.40 ± 0.71	31.83 ± 0.84	23.06 ± 1.00	26.35 ± 0.54
Corynebacterium diphtheriae ATCC 39255	15.57 ± 0.55	35.98 ± 0.50	25.6 ± 0.34	23.47 ± 0.68	22.01 ± 0.95	0.00 ± 0.00	23.50 ± 0.69	26.95 ± 0.87
Enterococcus faecalis ATCC 49452	16.03 ± 0.65	26.71 ± 0.65	34.38 ± 0.94	35.37 ± 1.20	24.20 ± 0.62	20.26 ± 0.33	8.02 ± 0.30	8.88 ± 0.50
Staphylococcus aureus ATCC 6538	17.37 ± 0.75	29.87 ± 0.90	33.46 ± 0.95	39.98 ± 0.83	27.49 ± 0.68	28.90 ± 0.38	20.12 ± 0.50	21.68 ± 0.65
Gram-negative stains								
Escherichia coli ATCC 25922	9.98 ± 1.15	36.10 ± 0.40	24.70 ± 0.84	21.67 ± 0.64	25.68 ± 0.92	11.86 ± 0.45	20.79 ± 0.66	23.16 ± 0.74
Pseudomonas aeruginosa ATCC 27853	19.87 ± 0.60	30.10 ± 0.85	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	10.99 ± 0.61	20.03 ± 0.84
Salmonella typhimurium ATCC 14028	11.73 ± 0.80	35.25 ±0.90	21.73 ± 0.90	27.40 ± 0.50	26.55 ± 0.65	8.69 ± 1.20	22.42 ± 0.95	22.53 ± 0.66

CIP = ciprofloxacin, TE = tetracycline, AMP = ampicillin, C = chloramphenicol, E = erythromycin, N = neomycin and CN = gentamicin

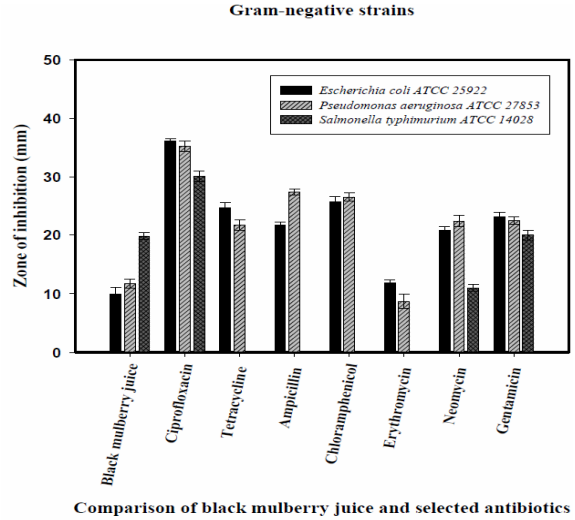

Fig. 1. Comparison of antimicrobial activity of fresh black mulberry juice with standard antibiotics (Gram-negative).

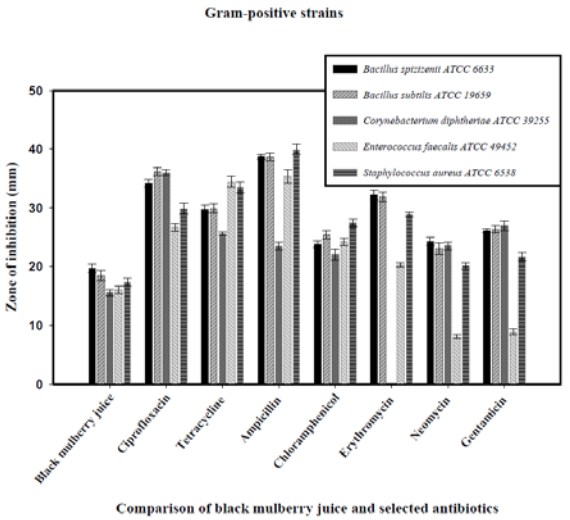

Fig. 2. Comparison of antimicrobial activity of fresh black mulberry juice with standard antibiotics (Gram-positive).

Ascorbic acid, total acidity and total soluble solids in black mulberry juice: The contents of ascorbic acid, total acidity and soluble solids are presented in Table 2. The ascorbic acid (AsA) in fresh black mulberry juice averaged 23.45 mg per 100 ml. This amount was in agreement with previous results obtained by Ercisli *et al.,* (2010) and Lale and Ozcagiran, (1996). Iqbal *et al.* (2010) reported 32.25 mg of ascorbic acid per 100 g of black mulberry fruit. *Morus* fruits are considered to contain a moderate amount of ascorbic acid. The total acidity present in fresh mulberry juice averaged 1.60%, which is a little higher then has been reported for red and white mulberries (Ercisli *et al.*, 2010) and for black mulberry (Iqbal *et al.*, 2010). Total soluble solids in black mulberry juices averaged 19.40%; this high content of soluble solids gave a thick appearance to black mulberry juice. Koyuncu *et al.* (2004) reported 13.11 to 16.23% total soluble solids in black mulberry genotypes, and similarly, Iqbal *et al.* (2010) reported 8.88% TSS in black mulberry fruits obtained from Khyber Pakhtunkhwa area, Pakistan. The total soluble solids in mulberry grown in different agro-ecological regions ranges from 15 to 31% (Lee *et al.,* 2004; Ercisli *et al.,* 2010; Iqbal *et al.*, 2010).

Table 2. Physico-chemicals and phytochemicals present in black mulberry (*Morus nigra*) fresh juice.

Physico-chemical composition	Fresh black mulberry(*Morus nigra*) juice
Ascorbic acid (ASA) mg/100 ml	23
Total acidity (%)	2
Total soluble solids (%)	19
Phytochemicals	
Total anthocyanins as (Cy 3-glu) µg/g	769
Total phenolics as (GAE) µg/g	2050
Total antioxidants	
FRAP as (TE) µmol/g	14
DPPH as (TE) µmol/g	20

TE: Trolox equivalent, GAE: gallic acid equivalent, FRAP: ferric reducing ability of plasma, DPPH: 2,2-diphenyl-1-picrylhydrazil

Total anthocyanins, total phenolic content and total antioxidants: The total anthocyanins (TA), total phenols (TP) and total antioxidants (TAC) are presented in Table 2. The TA of fresh mulberry juice averaged 769 µg/g in Cy 3-glu equivalent. Previous studies have shown that black mulberry fruit contained higher total anthocyanins than red and white mulberry fruits (Lee *et al.,* 2004; Özgen *et al.,* 2009b; Ercisli *et al.,* 2010). Ercisli *et al.* (2010) reported 723-787 µg/g Cy-3-glu equivalent of TA in different genotype of *Morus nigra* grown in Turkey. Similarly, TA contents in purple and black mulberries were 99 and 571 µg/g Cy 3-glu equivalent (Özgen *et al.*, 2009b). High performance liquid chromatography (HPLC) measurements indicated that TA in different cultivars of mulberries grown in China ranged from 22 to 3300 mg/l Cy-3-glu equivalent (Song *et al.*, 2009). The anthocyanin content (expressed in cyanidin 3-glucoside) of raw black mulberry juice was calculated to be 16 mg per 100 ml (Hojjatpanah *et al.*, 2011).

The total phenolic contents (TP) of fresh mulberry juice averaged 2050 µg/g as gallic acid equivalent (GAE). The results agree well with those of Ercisli *et al.* (2010), who reported TP in different genotypes of black mulberry as gallic acid equivalent ranging from 1836 to 2483 µg/g. The total phenolic contents in different mulberries ranged from 1500 to 2570 µg/g (Bae & Suh,

2007; Lin & Tang, 2007; Hojjatpanah *et al.,* 2011). The average phenolic content in black mulberry accessions from all over Turkey was 2737 µg/g GAE on a fresh weight basis (Özgen *et al.*, 2009a).

The antioxidant activity (FRAP and DPPH assays) of black mulberry juice is reported in Table 2. In the FRAP assay, the antioxidant activity (TAC) averaged 14 µmol/g Trolox equivalent (TE). In the DPPH assay it averaged 20 µmol/g TE. These result agree with those obtained by Ercisli *et al.* (2010). They reported TAC from different genotypes of black mulberry, ranging from 13-14 µmol/g TE in a FRAP assay and 19-21 µmol/g TE in a DPPH assay. The TAC in black mulberry ranges from 13-14 µmol/g TE in FRAP assays of different accessions from all over Turkey (Özgen *et al.*, 2009a). All these values suggest that black mulberries provide more antioxidants than vegetables like pepper, tomato and spinach, which have values ranging from 0.98 to 2.65 mmol/100 g (Halvorsen *et al.*, 2002).

Trace minerals: Table 3 presents the trace minerals present in black mulberry juice. Potassium was present in the highest amount and averaged 1300 mg/100 g of fresh juice. Other minerals like calcium, magnesium, sodium, iron, manganese, and zinc averaged 150, 130, 160, 40, 7 and 45 mg/100 g, respectively. All these contents were less than previously reported by Imran *et al.,* (2010) and much higher than those described by Ercisli and Orhan (2007). The mineral composition of mulberry fruits (*M. alba*, *M. rubra*, and *M. nigra*) grown in Turkey was: potassium (834–1668 mg/100 g), calcium (132–152 mg/100 g), magnesium (106–115 mg/100 g), sodium (59–61 mg/100 g,) iron (4.2–4.5 mg/100 g), and zinc (2.8–3.2 mg/100 g) (Ercisli & Orhan, 2007). The mineral composition of *Morus nigra* from northern areas of Pakistan included potassium (1731 mg/100 g), calcium (470 mg/100 g), sodium (272 mg/100 g), magnesium (240 mg/100 g), iron (77.6 mg/100 g) and zinc (59.2 mg/100 g) (Imran *et al.*, 2010). Trace minerals, especially iron, calcium and zinc, are very important for health. Black mulberry appears to have a balanced mineral profile in combination with high antioxidant and phenolic contents.

Table 3. Trace mineral composition of fresh juice of black mulberry (*Morus nigra*).

Trace minerals	Amount in black mulberry (*Morus nigra*) fresh juice (mg/100 g)
Potassium	1300
Calcium	150
Magnesium	130
Sodium	160
Iron	40
Manganese	7
Zinc	45

Conclusions

In this study, antimicrobial activity of fresh black mulberry juice was tested against various pathogenic bacteria and the results demonstrated antimicrobial activity against both Gram- positive and Gram-negative species. High antioxidant and phenolic contents aid in antimicrobial activity. Black mulberry juice is highly nutritious and is full of anthocyanins. It has high amounts of zinc and iron, which could help to improve the micronutrient status of pregnant women and children, because black mulberry fruit is very cheap and readily available. Black mulberry juice is a potential candidate for phyto-medicine and may be an effective antibiotic against certain bacteria.

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