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Flavonoid Glycosides from *Prunus armeniaca* and the Antibacterial Activity of a Crude Extract

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Investigations on the chemical constituents of the fruits of *Prunus armeniaca* have led to the isolation of two new flavonoid glycosides, 4',5,7-trihydroxy flavone-7-*O*-[β-D-mannopyranosyl (1'''→2'')]-β-D-allopyranoside (**1**) and 3,4',5,7-tetrahydroxy-3',5'-di-methoxy flavone 3-*O*-[α-L-rhamnopyranosyl (1'''→6'')]-β-D-galactopyranoside (**2**), from the butanolic fraction of the fruits. The butanolic extract exhibited antibacterial activity against both Gram positive and Gram negative bacteria. The structures of these compounds were elucidated through spectral studies, including 2D-NMR (COSY, NOESY, *J*-resolved), HMQC and HMBC experiments.

Key words: *Prunus armeniaca*, Fruits, Flavonoid glycosides

INTRODUCTION

The genus *Prunus* belongs to the family Rosaceae, and consists of about 175 species distributed all over the world. *P. armeniaca* has been reported to have many medicinal properties (Baquar, 1989; Chopra *et al.*, 1956). In Pakistan, the inner valleys of Baluchistan and Kashmir are famous for its cultivation (generally 2000m). Various flavonoids have been reported from *Prunus* species (Masao,1957; Nagarajan and Seshadri, 1964). Different parts of the plant are used for the treatment of many ailments, mainly against diseases of bacterial and fungal origins. In the Yunani system of medicine, it is used as an antidiarrohetic, emetic and anthelmintic in lever diseases, piles, earache and deafness, and as an expectorant remedy for dry throat, laryngitis, lung diseases and abscesses (Kritikar and Basu, 1988). It is also regarded as a bechic, depurative sedative for the respiratory centre, a tonic and antispasmodic, as well as a remedy for severe colds and bronchial asthma (Perry and Judith, 1980).

The dried fruits of the plant have been studied for their various chemical constituents and biological activities (Rashid *et al.*, 2005). Herein, the isolation and structural elucidation of two new flavonoid glycosides are reported. One of the flavonoid (**1**) was glycosylated with a disaccharide, consisting of D-allose and D-mannose. The combination of allose and mannose in the diglycosides from this source are reported for the first time and are unique compared to the other flavonoid glycosides previously reported from different *Prunus* species (Agrawal, 1989).

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MATERIALS AND METHODS

General experimental procedures

Melting points were recorded in glass capillary tubes, using a Buchi 535 melting point apparatus, and are reported uncorrected. IR and UV spectra were obtained on JASCO A-302, JASCO DIP-360 and schimatzo-610 respectively. ^{1}H and ^{13}C-NMR were run on Bruker AM-300 and AM- 400 FT NMR spectrometers. Mass spectra were recorded on Varian-MAT 112S and Finningan MAT-112 and 312 double focusing mass spectrometers. Negative FAB-MS was recorded on a Varian MAT-312 spectrometer. The HPLC consisted of a Schimadzu model LC-6A pump as the solvent delivery system, a Rheodyne sample injector with a 100 microliter loop, a RP-18 column and a Schimadzu model RD-6A refractive index detector connected to a recorder. Column chromatography and analytical TLC were performed using silica gel (Merck 7739, 7734, 70-230 mesh ASTM and PF 254+366), Flash chromatography (Silica gel 9383, Merc, 0.043-0.064 mm).

HPTLC was conducted on Silica gel GF_{254} and RP-18 F_{254} (Merc plates).

Plant material

Dried fruits were purchased in October 2004 from a fruit market in Karachi. A voucher specimen (Ch-2059-2004) was deposited in the herbarium of the Department of Botany, University of Karachi.

Extraction and isolation

Dried fruits (10 kg) were soaked in ethanol for 15 days (5 L × 3 batches) at room temperature. The dark brown colored extract was concentrated on a rotary evaporator with the resulting brown gummy material (375 g) then successively fractionated with n-hexane, chloroform, ethyl-acetate and saturated butanol.

The butanolic fraction of the ethanolic extract was concentrated, to a dark brown sticky mass (85 g). This concentrated fraction was subjected to Silica gel column chromatography using chloroform-methanol (5%, 10%, 15%, 20%, 30%, 40% and 1:1) and chloroform-methanol-water (65:35:1) as solvent systems. The fractions of similar composition were combined, with two main fractions obtained; **F** (50 mg) and **G** (40 mg). TLC of the two different fractions was conducted with BuOH:AcOH:H_2O (12:3:7, 12:3:5, 3:1:1) and EtOAc:$CHCl_3$:H_2O (17:3:1) as the mobile phases. Fraction **G** was obtained with chloroform-methanol (70:30), and indicated the presence of two compounds with distinct R_f, which gave, prominent colored spots with ferric chloride reagent. Further separation was achieved by HPLC employing an RP-18 column, eluted using an isocratic methanol-water system (65:35), followed by HPTLC and fractional crystallization to afford compounds **1** (15 mg) and **2** (17 mg).

4',5,7-Trihydroxy-flavone-7-*O*-[*β*-D-mannopyranosyl-(1'''→2'')]-*β*-D-allopyranoside (1)

Yellow powder, 15 mg, mp: 162C, UV (MeOH) λ_{max}(nm): 272, and 333 nm; IR (KBr) V_{max} (Cm^{-1}): 3700-3310 (OH), 2920 (CH-str), 1722 (α, β unsaturated carbonyl group), 1662, 1600 (C=C), 1180 (C-O), 1070 and 1032; HR-FABMS (negative) m/z: 593.5179 $[M-H]^+$ (Calcd. for $C_{27}H_{29}O_{15}$, 593.5171); ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 7.75 (1H, d, *J*=2.5 Hz, H-6'), 7.66 (1H, dd, *J*=8.2, 2.5 Hz, H-2') 7.28 (1H, d, *J*=8.4 Hz, H-3', H-5'), 7.26 (1H, d, *J*=2.5 Hz, H-8), 7.06 (1H, d, *J*=2.4 Hz, H-6), 6.99 (1H, s, H-3) 4.89 (1H, d, *J*=5.2 Hz, mannosyl H-1'''), 5.77 (1H, d, *J*=7.5 Hz, allosyl H-1''), 3.10-3.55 (12H, m, sugar proton). 3.55-3.64 (m, H-6'''); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 182.1 (C-4), 164.1 (C-2), 162.8 (C-7), 161.2 (C-9), 156.8 (C-5), 148.4 (C-4'), 121.5 (C-6'), 120.5 (C-1'), 116.3 (C-5', 3'), 110.9 (C-2'), 105.4 (C-10), 103.4 (C-3), 102.1 (C-1''), 99.7 (C-6'), 98.1 (C-1''), 95.4 (C-8), 79.7 (C-2'''), 77.0 (C-5'''), 74.4 (C-5''), 73.7 (C-3''), 71.8 (C-3''), 70.1 (C-2'''), 68.2 (C-4'''), 67.2 (C-4''), 61.8 (C-6'''), 60.6 (C-6'').

Acid hydrolysis of compound 1

Compound **1** (5 mg) was subjected to acid hydrolysis with 2% H_2SO_4 in methanol (5 mL), by refluxing for three hours, cooled and then extracted with ethyl-acetate. The ethyl acetate fraction and the aqueous fraction were obtained. Ethyl acetate and water fractions were worked up in the usual manner to afford a dihydroxy aglycone (Table I) yellow powder, m.p 165-166°C, the aqueous fraction containing two sugar moieties mannose and allose. Hydrolysis of compound **1** showed initial removal of one mannose unit followed by one of allose unit. D-mannose and D-allose were mainly identified by paper chromatography by comparison with authentic samples which were further confirmed by comparing the R_f values with standards using descending techniques, with BuOH: AcOH: H_2O 4:1:5 and 3:1:1 as the mobile phases, the spots were detected by spraying with aniline phthalate and heating at 110°C.

3,4',5,7-Tetrahydroxy-3',5'-dimethoxy flavone-3-*O*-[-*β*-L-rhamnopyranosyl (1→6)]-*β*-D-galactopyranoside (2)

Yellow powder, 17 mg, mp: 176C, UV (MeOH) λ_{max}(nm): 252 (band II) & 357 (band I); IR (KBr) V_{max} (cm^{-1}): 3620-3560 (OH), 1650 (C=O), 1605 (C=C), 1508, 1450 & 1150 (C-O); HR-FABMS (negative) m/z: 654.6321 $[M-H]^+$ (Calcd. for $C_{29}H_{33}O_{17}$; 654.5774); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 7.61 (2H, s, H-2' and H-6'), 6.45 (1H, d, *J*=2Hz H-8), 6.24 (1H, d, *J*=2 Hz H-6), 5.41 (d, *J*=7.8 Hz), 4.55 (1H, d, *J*=1.4 Hz) and 3.95 (6H, s, 3'-OMe & 5'-OMe); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 179.1 (C-4), 166.1 (C-7), 163.0 (C-5), 158.6 (C-9), 158.4 (C-2), 149.1 (C-5'), 149.0 (C-3'), 140.4 (C-4'), 135.2 (C-3), 121.7 (C-1'), 108.5 (C-2', 6'), 105.9 (C-10), 104.3 (C-1''), 102.1 (C-1'''), 100.3 (C-6), 95.0 (C-8), 75.5 (C-5''), 75.2 (C-3''), 73.5 (C-4'''), 73.3 (C-2''), 72.5 (C-3'''), 72.3 (C-2'''), 70.1 (C-4''), 69.9 (C-5'''), 67.5 (C-6''), 57.5 (C-OCH_3, 3' and 5'), 18.5 (C-6''').

Acid hydrolysis of compound 2

A solution of compound **2** (5 mg) in 5% HCl/methanol (5 mL) was refluxed for three hrs. The reaction mixture was extracted with ethyl acetate. The ethyl acetate fraction containing aglycone (Table I) yellow amorphous powder, m.p. 280-285°C and the aqueous fraction containing two sugar units (galactose and glucose) were concentrated to dryness. The sugars were identified by comparison with standards using descending paper chromatography, with BuOH: AcOH: H_2O 4:1:5 as the mobile phase, the spots were detected by spraying with aniline phthalate followed by heating at 110°C.

Antibacterial assay

The antibacterial activity of the butanolic extract of *Prunus armeniaca* was determined against 20 gram-positive bacteria, including 10 MRSA isolates, 2 non tuberculous Mycobacteria (NTM) and 13 gram-negative bacteria. All the environmental and clinical isolates were obtained from the Immunology and Infectious Disease Research Laboratory (IIDRL) Culture Collection Unit, Department of Microbiology, University of Karachi and were identified and characterized using conventional microbiological methods (Marie, 1985). A stock solution of the butanolic extract (10 mg/mL) was prepared by dissolving in 100% DMSO (Merk). The stock solution was filtered and sterilized by passing through a 0.22 μm pore size syringe filter (Nalgen).

The antibacterial activity of the extract was determined by evaluating the minimum inhibitory concentrations (MICs) using the Micro Broth Dilution method, as described in the National Committee for Clinical Laboratory Standards (NCCLS) (Wayne, 1998). Briefly, serial dilutions of the butanolic extract were prepared directly into Mueller Hinton broth (Oxoid-UK) in a round bottom 96-well Microtitre plate. The wells were seeded with cultures at 2×10^5 cfu/mL. The plates were incubated at 37°C for 24 h, except for the *M. fortuitum* and *M. smegmatis*, which were incubated for 2 and 3 days, respectively. The results were recorded in the form of MICs, defined as the minimum amounts of the extract and pure compound that inhibited the visible growth of the organisms.

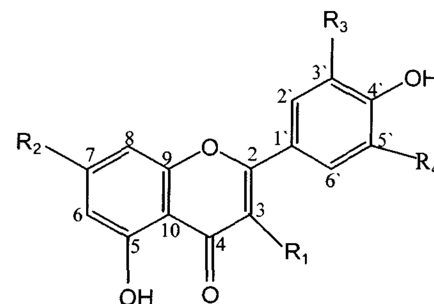
RESULTS AND DISCUSSION

Compound **1** was isolated as a light yellow powder from the butanol soluble fraction of the ethanolic fruit extract via repeated column chromatography, flash chromatography and finally by HPLC. The IR spectrum showed a strong absorption band between 3700-3450 cm^{-1}, indicating the presence of a hydroxyl group, 2920 (CH-stretching), α, β unsaturated carbonyl absorption at 1720 cm^{-1} (C-4 flavonoid), 1660, 1600 due to an olefinic double bond in an aromatic system. A broad carbonyl stretching band in the region of 1100-1600 cm^{-1} suggested the glycosidic nature of a flavonoid. The UV spectrum showed λ_{max} at 272 and 333 nm giving a very strong indication of the presence of a flavonoid (Vorin, 1983). The compound was assigned the molecular formula; $C_{27}H_{30}O_{15}$, with the help of a peak observed in the HR-FABMS (negative mode) at m/z 593.5179 $[M-H]^+$ (Calcd. for $C_{27}H_{29}O_{15}$ 593.5171).

The ^{1}H-NMR spectrum showed signals for phenyl ring B at δ 7.75, 7.66 and 7.28 corresponded to hydrogens on the C-6', 2', and 3',5' respectively. The aromatic protons of ring A appeared in the ^{1}H-NMR spectrum at δ 7.26 (d, 1H, *J*=2.5 Hz) and 7.06 (d, 1H, *J*=2.4 Hz), which could be due to the hydrogens on the C-8 and C-6 of a flavone derivative.

The compound was subjected to acid hydrolysis with 2% H_2SO_4, which afforded an aglycone with a fragment at *m/z* 270 [$C_{15}H_9O_5$, M-allose - mannose + H]$^+$, and other prominent fragments 152 [A_1-$C_7H_4O_4$]$^+$, 124 [A_1-CO]$^+$ and 118 [B_1-C_8H_6O]$^+$, as well as two saugar units D-mannose and D-allose were mainly identified by paper chromatography compared with authentic samples (Gupta and Bahar, 1985). All the ^{1}H- and ^{13}C-NMR data of the two sugars moieties were also confirmed by 2D-NMR spectroscopic techniques. The $[\alpha]_D^{20}$+15° and +14° values of allose and mannose respectively, confirmed their D-configuration due to their δ-shifts (Whistler and Wolform, 1962). The ^{1}H- and ^{13}C-NMR spectra also confirmed the presence of aglycone and two sugar residues. A signal at δ 162.9 in the ^{13}C-NMR spectrum was assigned to the C-7 on the basis of the long range ^{13}C-^{1}H correlation observed in HMBC experiments with the two protons signals at δ 7.26 (1H, d, *J*=2.5 Hz, H-8) and 7.06 (1H, d, *J*=2.4 Hz, H-6) confirming the site of glycosidation at the C-7 position of the aglycone. The C-7 signals showed three-band correlations with the anomeric proton of the allosyl unit at δ 5.77 (d, 1H, *J*=7.5 Hz). The anomeric proton of the mannosyl residue at δ 4.89 (1H, d, *J*=5.2 Hz) also showed long range correlations with the ^{13}C-NMR signal at δ 79.8, corresponding to a proton at δ 3.54 (H-2") in HMQC spectrum. The later signal showed a ^{1}H-^{1}H correlation, as observed in a Cosy experiment with the anomeric proton at δ 5.77 which was assigned as the H-1" of the allosyl moiety. Therefore glucosidation of allosyl at the C-2" position was evident. The b-configuration of two anomeric carbons was also confirmed from the coupling constant of the H-1" of allose (*J*=7.5 Hz) with that of the H-1"' of mannose (*J*=5.2 Hz) (Agrawal, 1989). 2D-NMR allowed the assignment of all ^{1}H- and ^{13}C-NMR signals of the allosyl and mannosyl moieties.

The ^{13}C-NMR spectrum, broad band and DEPT of the



Compound	R_1	R_2	R_3	R_4
1	H	O-All-Man	H	H
2	O-Gal-Rha	OH	OCH3	OCH3

Fig. 1. Compounds **1** and **2**

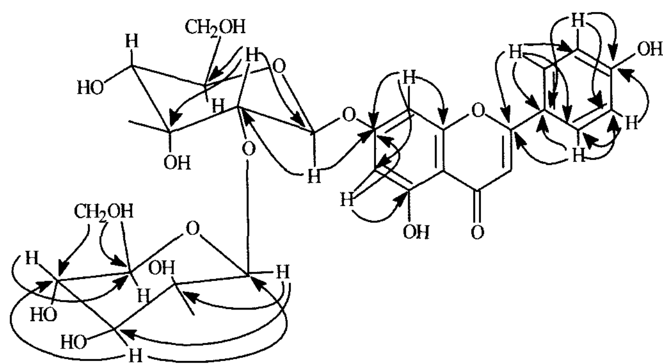


Fig. 2. Important HMBC interactions of **1**

above compound revealed the presence of two methylene, seventeen methine and eight quaternary carbon atoms. On the basis of above discussions and spectral data; compound **1** was assigned the structure 4',5,7-trihydroxy flavone-7-*O*-[*β*-D-mannopyranosyl-(1'''→2'')]-*β*-D-allopyranoside.

Compound **2** was obtained as yellow powder from the butanolic fraction through column and flash chromatography and finally purified by HPLC using an RP-18 column, with an isocratic methanol water system (65:35), to afford 17 mg of compound **2**. The IR spectrum of compound **2** showed absorption at 3620-3560 cm^{-1} indicating the presence of a hydroxyl group (OH), other prominent peaks were observed at 1650 (α, β unsaturated carbonyl group), 1605 (C=C aromatic), 1508 and 1454 cm^{-1}. The UV spectrum showed λ_{max} at 252 (band II) and 357 (band I) nm, strongly indicating the presence of a flavonoid system (Voirin, 1983). The compound was assigned the molecular formula $C_{29}H_{34}O_{17}$, from the HR-FABMS (negative mode) at m/z 654.6321 $[M-H]^+$ (Calcd. for $C_{29}H_{33}O_{17}$, 654.5774).

Complete acid hydrolysis of the compound afforded aglycone, galactose and rhamnose. These sugars were identified by comparison with authentic samples by TLC (in acetone, isopropanol and 0.2 M acetic acid, 3.5: 3.5: 2), detection was achieved with aniline phthalate. Aglycone showed fragments at m/z 347 [M-rhamnose and galactose + H]$^+$, 152 $[A_1\text{-}C_7H_4O_4]^+$, 194 $[B_1\text{-}C_{10}H_{10}O_4]^+$ and 124 $[A_1, C_7H_4O_4\text{-CO}]^+$, and identified from the data available in the literature as 5,7-dihydroxy flavonoid (Wagner and Mohnchari, 1976). The ^{1}H-NMR spectrum at 400 MHz in DMSO-d_6, indicated a methoxyl signal at δ 3.95 (6H, s, 3'-OMe and 5'-OMe) (Table I). Aromatic protons signals were observed at δ 6.24 (1H, d, H-6'), 6.45 (1H, d, H-8) and 7.61 (2H, s, H-2' and H-6'), which confirmed the 3-O-glycoside structure. The ^{1}H-NMR also supported the presence of one rhamnose and one galactose unit, with a rhamnose signal at δ 4.55 (1H, d, *J*=1.4 Hz, H-1''') and a galactose signal at δ 5.41 (1H, d, *J*=7.8 Hz, H-1''), indicating a β-linkage of the sugars, with a large coupling constant.

The ^{13}carbon-data of the compound also supported that the C-6'' (galactose) was down field shifted to δ 67.5 (Table I), which indicated that the glycosylation of the

Table I. ^{1}H- and ^{13}C-NMR spectral data of compounds **1** and **2**

Position	Compound 1		Compound 2	
	^{13}C	^{1}H (m, *J*)	^{13}C	^{1}H (m, *J*)
2	164.1		158.4	
3	103.4	6.99 (s)	135.2	
4	182.1		179.1	
5	156.8		163.0	
6	99.7	7.06 (d, 2.4)	100.3	6.24 (d, 2.0)
7	162.8		166.1	
8	95.4	7.26 (d, 2.5)	95.0	6.45 (d, 2.0)
9	161.2		158.6	
10	105.4		105.9	
1'	120.5		121.7	
2'	110.9	7.66 (dd, 8.2, 2.5)	108.4	7.61 (s)
3'	116.3		149.0	
4'	148.4		140.4	
5'	116.3	7.28 (d, 2.5)	149.1	
6'	121.5	7.75(d, 2.5)	108.5	7.61 (s)
1''	98.1	5.77 (d, 7.5)	104.3	5.41 (d, 7.8)
2''	79.8	3.54 (m)	73.3	3.78 (m)
3''	71.8	3.36 (m)	75.2	3.56 (dd,9.6,3.3)
4''	67.2	3.10 (m)	70.1	3.76 (br d, 2.5)
5''	74.4	3.18 (m)	75.5	3.68 (br t, 6.0)
6''	60.7	3.34, 3.56 (m)	67.5	3.76 (dd, 10.2, 3.6)
1'''	102.1	4.89 (dd, 5.2)	102.1	4.55 (d, 1.4)
2'''	70.1	3.16	72.3	3.56 (m)
3'''	73.7	3.24 (m)	72.5	3.48 (dd, 9.4, 3.3)
4'''	68.2	3.21 (m)	73.5	3.26 (t, 9.4)
5'''	77.0	3.16 (m)	69.9	3.52 (m)
6'''	61.8	3.55, 3.64 (m)	18.4	1.18 (d, 6.3)
OCH_3 3' and 5'			57.4	3.95, s (6H)

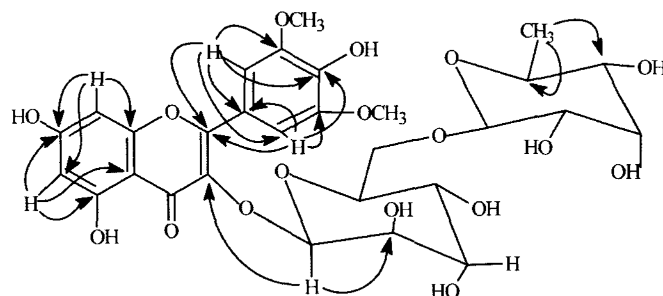


Fig. 3. Important HMBC interaction of **2**

galactose moiety with the rhamnosyl was via 6-hydroxyl. The HMBC spectrum also showed a cross peak between the C-6" of galactose and the H-1"' of rhamnose, and also confirmed a linkage point between the two sugar moieties. In addition, down field shielding of the C-2 also indicated that position-3 was substituted by the glucosyl chain, which was further confirmed by a cross peak between the H-1" of galactose and the C-3 of aglycone.

On the basis of above data and that available in the literature (Tang *et al.*, 2003), the compound was characterized as 3,4',5,7-tetrahydroxy-3',5'-dimethoxy flavon 3-*O*-[-α-L-rhamnopyranosyl-(1$\rightarrow$6)]-β-D- galactopyranoside.

The antibacterial activity of the butanolic extract was determined against gram positive, gram negative and non-tuberculous Mycobacteria (NTM). According to the results, the extract was found to possess significant antibacterial activity with MICs ranging between 31.25-500 µg/mL (Tables II and III).

Table II. Antibacterial Activity of the ButanolicExtract Against Gram Positive Bacteria

S No.	Gram-positive bacteria	Minimum Inhibitory Concentration (MICsµg/mL)
1	Staphylococcus aureus ATCC 6538	250
2	MRSA	78.125
3	Staphylococcus epidermidis	125
4	Streptococcus faecalis	250
5	Streptococcus pyogenes	250
6	Micrococcus luteus	31.25
7	*Bacillus subtilis* ATCC 6633	62.5
8	Corynebacterium diphtheriae	62.5
9	Mycobacterium fortuitum	250
10	Mycobacterium smegmatis	125

Table III. Antibacterial activity of the butanolic extract against gram negative bacteria

S No.	Gram-negative bacteria	Minimum Inhibitory Concentration (MICs µg/mL)
1	*Escherichia coli* ATCC 25922	125
2	ETEC	125
3	EPEC	500
4	Salmonella typhi	125
5	MDR *Salmonella typhi*	125
6	*Salmonella Paratyphi* A	125
7	*Salmonella Paratyphi B*	125
8	Shigella dysenteriae	125
9	Enterobacter aerogenes	250
10	Klebseilla pneumoniae	125
11	*Pseudomonas aeruginosa* ATCC 9027	125
12	Proteus mirabilis	125
13	Proteus vulgaris	125

The butanolic extract was found to be more effective at inhibiting the growth of Gram-positive bacteria (MIC values 31.25-250 µg/mL), with the highest activity exhibited against *Micrococcus luteus* (MIC 31.25 µg/mL) (Table II). The MICs of the extract were found to be 125 and 250 µg/mL against almost all Gram-negative bacteria, with the exception of the lowest activity against the Enteropathogenic *E. coli* (EPEC), which had an MIC value of 500 µg/mL (Table III).

In the case of MRSA, 10 isolates of clinical origin were screened, with the MICs of the extract given as the average of the collective MICs. According to the observation the average MICs of the butanolic extract was found to be 78.125 µg/mL (Table II).

The present study was connected with our previous work on the fruit extracts (ethanolic and ethyl acetate), essential oils and pure compounds isolated from *P. armeniaca* (Ahmed *et al.*, 2004 Rashid et al 2005). This study on the butanolic extract further strengthens the evidence for the presence of antibacterial constituents in apricots.

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