

Chemical Investigation of *Opuntia tuna* Mill Growing in Egypt

Wafaa A. Tawfik, Nahla S. Abdel-Azim, Abdel-Aaty A. Shahat, Nahed M. Hassan,
Shams I. Ismail and Faiza M. Hammouda

Phytochemistry Dept., National Research Centre, 12311, Dokki, Egypt

Abstract: Family Cactaceae is characteristic of the plant with prickly pear which has been considered as a source of fiber. The fruit of the genus *Opuntia* is of much greater value economically used to some extent as forage. It is also cultivated for its edible fruits. *Opuntia tuna* is wildy growing in Egypt and is also cultivated for their edible uses (Watt and Brandwijk, 1962). *Opuntia tuna* was subjected to chemical investigation in this work for the first time. We report here on the flavonoids of the flowers and the mucilage and the betalaines of the fruits. Isolation and purification processes were carried out by applying successive chromatographic techniques (column chromatography, TLC, PPC and HPLC). Identification of the isolated compounds was carried out by spectroscopic analysis (UV, MS, ¹H-NMR and ¹³C-NMR). Quantitation of betalaines was established by TLC densitometry. The isolated flavonoids were identified as isorhamnetin (I), isorhamnetin-3-O-glucoside (II) and isorhamnetin-3-O-rhamnoglucoside (Rizk and Nowaihi; 1989) (III). The mucilage study revealed the presence of L-rhamnose, L-arabinose, D-galactose, fructose D-glucuronic acid and D-galacturonic acid (Norma *et al*, 1987). Indicathanthin, betanin and isobetanin were identified (El-Moghezy *et al*, 1982).

Key words:

INTRODUCTION

Opuntia is one of the most important genera of the family Cactaceae which is known as prickly pear group of much great economic value and cultivated for its edible fruits and as forage (Watt and Brandwijk, 1962).

The genus *Opuntia* is represented in Egypt by two species, *Opuntia ficus indica* and *Opuntia tuna* Mill. *Opuntia tuna* is wildy growing in Egypt and is also cultivated for their edible uses.

Opuntia ficus indica has been a subject of interest to many investigators regarding to its chemical constituents and its nutritive value as edible fruits (Rizk and Nowaihi, 1989). The edible fruit is much eaten by both Europeans and Africans. The Pedi is not only eaten fresh but also as dry. The red extracted juice of the fruits is being used as a valuable source for natural food colorant particularly for coloring cheese and a paste made from their juice. It is also applied as a poultice of the leaf to various painful conditions, to ulcers and sores and to boils. They also used the sweetened decoction for whooping cough (El-Moghezy *et al*, 1982).

Opuntia ficus indica contains many active chemical constituents other than flavonoids, alkaloids and mucilages, such as triterpenes, amino acids and lipids (Vidal and Varela, 1968).

The present work deals with the chemical investigation of *Opuntia tuna* growing in Egypt for their flavonoids, mucilage and betalaines.

MATERIALS AND METHODS

General :

TLC was carried out on precoated G plates (Kieselgel G, type 60, Merck) developed with Ethyl acetate - Acetic acid - Formic acid - Water (30 : 0.8 : 1.2 : 8) (Abd Elshafeek *et al.*; 2000).

Ascending paper chromatography (PC) was applied using Whatmann 3MM chromatographic sheets and developed with 25% AcOH. Spots were detected by spraying with 1% alcoholic AlCl₃ and /or diphenyl boric acid - ethanolamine complex (Neu's spray reagent) (Markham, 1982). NMR spectra were recorded in CD₃OD on a Bruker DRX-400 spectrometer operating at 400.13 MHz for ¹H and at 100.61 MHz for ¹³C. Chemical shifts were presented in ppm downfield from TMS. Mass spectra were recorded on an Autospec-oa-ToF instrument.

Corresponding Author: Faiza M. Hammouda Phytochemistry Dept., National Research Centre, 12311, Dokki, Egypt
fmhammouda@hotmail.com

High performance liquid chromatography (HPLC) for carbohydrate analysis was carried out using an isocratic pump (model Le. 10AS, Shimadzu, Japan). Refractive index detector RID-6A, Shimadzu, Japan. Arheodyne injector (model 7161, Catati, California, USA), equipped with 20 μ l injector loop. An injector model C-R7A, Shimadzu, Japan. Phenomenx column USA, Kromasil 10 NH₂ (250 X 4.6 mm i.d.). Ser.:12885, 20 μ l injected. The sensitivity was set at 0.001AuFS.

Plant Material:

Opuntia tuna L. Mill was collected from Kafr-Elsheikh governorate, Egypt and was kindly identified by Prof. Dr. S. F. Khalifa, Botany Department, Faculty of Science, Ain Shams University. The yellow petals of the flowers were separated, air dried and powdered.

Extraction and Isolation:

About 50g of the dried flower petals of *O. tuna* were extracted with 70 % ethyl alcohol till exhaustion. The combined alcoholic extract was evaporated *in vacuo* at about 50°C till dryness; the residue was dissolved in 500 ml hot distilled water and left in the refrigerator overnight. The precipitated material was filtered and the clear filtrate (the aqueous mother liquor) was extracted with successive portions of chloroform followed by ethyl acetate and finally with butanol.

Each of the combined solvents was dried over anhydrous sodium sulfate and evaporated *in vacuo* at 50 °C. The chloroform, ethyl acetate and butanol free solvent residues amounted to 0.308 g, 2.6 g and 2.9 g; respectively.

About 0.3 g of the chloroform fraction was dissolved in about 5 ml methanol (95 %) and subjected to preparative paper chromatography using Whatmann 3MM developed with 25 % AcOH. Two main bands (I and II, R_f 0.27 & 0.61; respectively) were localized in UV light and eluted with methanol (100 %). The eluent of the first band was subjected to further purification using Sephadex LH-20 column (30 X 1.5 cm). Elution was afforded with methanol (100 %). The fractions containing compound (I) in a pure form were collected and evaporated *in vacuo* till dryness (0.012g) and its purity was checked by two dimensional paper chromatography using different solvent systems. This compound was found to be isorhamnetin (Markham, 1982) in comparison with its authentic.

About 0.5g of the ethyl acetate fraction was dissolved in about 10 ml methanol (95 %) and subjected to preparative paper chromatography using Whatmann 3MM and 25% acetic acid as a developing solvent. Two main bands having R_f 0.38 and 0.58; respectively were localized in UV light, cut as strips and eluted in descending chromatographic tank using methanol (90 %).

The eluant of the first band was subjected to further purification using Sephadex LH-20 column (30 X 1.5 cm). Elution was afforded with methanol (95 %). Fractions of 10 ml for each were collected. The fractionation was monitored by paper chromatography (Whatmann-3MM) developed by 15 % ACOH. Fractions were collected and re-purified using Sephadex LH-20 column (30 X 1.5 m) and methanol (95 %) as eluent. The fractions containing compound (II) in a pure form were collected and evaporated *in vacuo* till dryness to give 0.013 g and its purity was checked by two dimensional paper chromatography using different solvent systems.

The eluant of the second band was further purified using preparative paper chromatography (Whatmann 3MM) and 15 % AcOH as developing solvent system. The main compound (III) with R_f 0.69 was eluted as described before using methanol (85 %) and was further purified by passing through Sephadex LH-20 column and eluted with methanol (90 %). The fractions containing flavonoid compound (III) in a pure form were collected and evaporated *in vacuo* till dryness yielding 0.15g and its purity was checked by two dimensional paper chromatography using different solvent systems.

Isolation of Betalaine:

About 500g of *Opuntia tuna* Mill fruits were extracted with 80% ethyl alcohol for 30 mins in a blender several times until complete decolorization (Piatelli and Minale, 1964)

The homogenate was heated at 40° C for 5 min, filtered and adjusted to pH 4.5. The brown precipitate was removed by centrifugation to obtain a clear dark red-violet pigment which was concentrated under vacuum at 40 °C then extracted with hot ethanol. The combined ethanolic extract was stored at 4°C overnight then filtered. A red-violet precipitate was formed while the filtrate consisted of a light-yellowish orange material. The filtrate was evaporated to give 0.4 g of yellowish orange pigment (pigment I).

Further extraction of the remaining precipitate was carried out with acidified ethanol. The extract was neutralized with dilute NaOH and then the solvent was removed under vacuum at 40°C to give 0.5 g of red-violet pigment (pigment II).

Tlc-densitometry Analysis:

1% stock solutions of the samples were prepared. Quantitative amounts of each sample were applied on TLC plates, Silica Gel ready-made plate (Merck 60 F 254, 20 X 20 cm). Samples were applied in band wise using CAMAG Automatic TLC sampler III, distance from lower edge was 15mm, band length was 8mm, track distance was 20mm and 4 bands represented all samples were applied. The plate developed with CH₃COOH: MeOH (40:60).

Samples evaluation was done using CAMAG Automatic TLC Scanner with CATS evaluation Software at wave length scan 538 nm and scanning speed 20 mm/s.

Isolation of Mucilage:

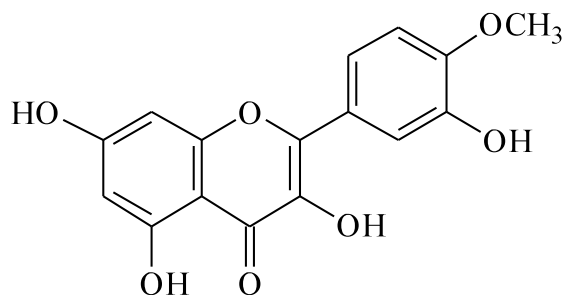
About 50g of the fresh fruits of *Opuntia tuna* (L.) Mill. was macerated in one liter of slightly acidified distilled water at pH 4, stirred for three hours at 25 °C, left overnight and filtered. The process was repeated twice and the combined filtrate was concentrated under vacuum to about 200 ml. The mucilage was precipitated from the aqueous extract by adding, slowly while stirring, four volumes of absolute ethanol. The precipitate was separated by centrifugation, then washed several times with absolute ethanol, followed by acetone and filtered to give 0.7g of mucilage dried powder. One hundred mg of the obtained mucilage was hydrolyzed with 0.5 M sulphuric acid in a sealed tube for 20 hours on boiling water bath and then filtered. The filtrate was freed from (SO₄²⁻) by precipitation with barium carbonate and filtered. The filtrate was evaporated under vacuum and the residue was dissolved in 10 % isopropyl alcohol and subjected to paper chromatography on Whatmann NO. 1 sheets, using n-butanol - acetic acid - water (4:1:5) as an eluting solvent. The chromatogram was visualized by spraying with aniline oxalate reagent and heating to 105 °C for 5 minutes (Solution A, 6.8 g oxalic acid were dissolved in 200 ml water, Solution B, 5 ml aniline were dissolved in 200 ml ethanol, Solution A and solution B were mixed and kept in brown stoppered bottle) (El-Moghezy *et al.*, 1982).

RESULTS AND DISCUSSION

Identification of Compound (I):

The UV absorption spectra of compound (I) in methanol showed band-I at 370 nm which indicates that this compound is a flavonol type structure (Nielsen *et al.*, 1993). A bathochromic shift (58 nm) in band-I with high intensity was noticed on addition of NaOMe indicating the presence of free OH group at C-4'. The AlCl₃ spectrum showed a bathochromic shift (47 nm) in band-I indicating the presence of free OH group at C-5. No hypsochromic shift was noticed in band-I when HCl was added which indicated that the compound has no orthodihydroxy system in ring-B. The bathochromic shift in band-II (13 nm) in NaOAc spectrum indicates the presence of free OH group at C-7. The absence of the ortho-dihydroxy system was proved from the NaOAc / H₃BO₃ spectrum where no bathochromic shift was noticed. The UV data proved that the compound is flavonol type structure containing three OH groups at C-5, C-7 and C-4' and there is no ortho dihydroxy system.

The positive electron spray (ES⁺) mass spectrum of compound (I) showed a molecular ion peak M⁺ at m/z 317 (100 %) which corresponding to molecular formula C₁₆H₁₂O₇ +1. Another important fragment ion peaks at m/z 302 (M⁺ - CH₃) and 287 (M⁺ - OCH₃) confirm the presence of a methoxy group in the compound. This fragmentation pattern was in accordance with that reported for isorhamnetin (Mabry and Markham, 1975). From the above chromatographic and spectroscopic data, compound (I) was found to be isorhamnetin (Markham, 1982) in comparison with its authentic.



Compound (I); isorhamnetin

Identification of Compound (II):

The chromatographic behavior of the compound in different solvent systems showed that it is flavonoidal glycoside in nature.

The UV absorption spectra of compound (II) showed band-I at 355 nm in methanol which indicates that it is of a flavonol type in nature. A bathochromic shift (58 nm) with high intensity of band-I was observed in NaOMe spectrum which indicated the presence of free OH group at C-4'. The presence of free OH group at C-5 was proved through the AlCl₃ spectrum where there was a bathochromic shift (47 nm) in band-I relative to the methanol spectrum. The absence of an ortho-dihydroxy system in ring-B was confirmed where no hypsochromic shift was noticed when HCl was added to the AlCl₃ spectrum. The presence of a bathochromic shift (14 nm) in band-II with NaOAc indicated the presence of free OH group at C-7.

The +ve ES-MS of compound (II) displayed a molecular ion peak M⁺ at m/z 479 which suggesting the molecular formula C₂₂H₂₂O₁₂ (+1). The fragment ion peak at m/z 317 indicated the presence of only one hexose moiety (M⁺ - 162 (hexose)). The other fragments at m/z 302 (M⁺ - (CH₃ - hexose)) and 257 (M⁺ - (OCH₃ + C=O hexose)) confirmed that the aglycone is isorhamnetin.

The ¹H-NMR spectrum of compound (II) showed signals at δ in ppm 8.03 (1H, d, H-2''), 7.85 (1H, dd, H-6), 6.9 (1H, d, H-5), 6.3 (1H, d, H-8), 6.1 (1H, d, H-6). The signal at δ 5.5 (1H, d, H-1'') was assigned for the anomeric proton of the sugar moiety of glucose. The other sugar protons were appeared at δ 3.4 ppm and the signal at δ 3.79 ppm was assigned for methoxy group protons (Table 1).

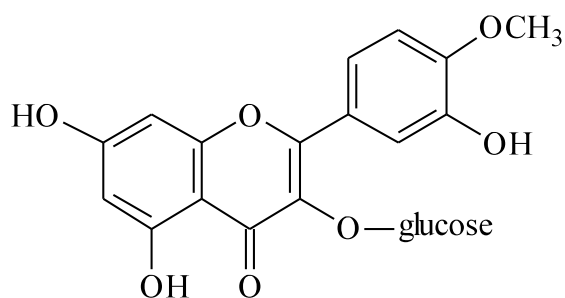
¹³C-NMR spectrum confirmed the presence of the most important carbon signals like C-4 displayed at δ 177.3 ppm which attributed to the carbonyl group (C=O) and the methoxy group carbon displayed at δ 56.3 ppm.

Acid Hydrolysis of Compound (II):

About 5mg of compound (II) were dissolved in 15ml acidified methanol and refluxed on a boiling water bath for three hours. The solution was concentrated *in vacuo*, diluted with distilled water and extracted with diethyl ether. The ethereal extract was washed with distilled water and evaporated *in vacuo* at 40 °C till dryness to give the aglycone. The isolated aglycone was purified by passing through Sephadex LH-20 column and its purity was checked by two dimensional paper chromatography using different solvent systems. The color under UV light as well as R_f values in different solvent systems were in agreement with those of an authentic isorhamnetin.

The EI mass spectrum of the isolated aglycone was found to be identical with that of compound (I) The aqueous acidic solution after separation of the aglycone was neutralized with barium carbonates and filtered through centered glass funnel. The clear filtrate was evaporated till dryness and the residue was dissolved in 10 % isopropanol and subjected to paper chromatography using ethyl acetate-pyridine-water (12: 5: 4) as a developing solvent.

The chromatogram was visualized by spraying with aniline phthalate and heated at 105°C for few minutes. Glucose was the only sugar detected in the hydrolysate. The above mentioned data resulted in the identification of compound (II) as isorhamnetin-3-O-glucoside.



Compound (II); isorhamnetin-3-O-glucoside

Identification of Flavonoid Compound (III):

The chromatographic behavior of compound (III) on paper chromatography with different solvent systems indicates that it is a flavonoidal diglycoside in nature.

The UV absorption spectra of compound (III) showed band-I at 356nm in methanol which indicated that compound (III) was a flavonol type structure. A bathochromic shift (40nm) of band-I was observed with NaOMe which indicated the presence of free OH group at C-4'. A bathochromic shift in band-I (45nm) with AlCl₃ spectrum indicated the presence of free OH group at C-5. No hypsochromic shift was noticed on addition of HCl indicating no orthodihydroxy system in ring-B. Also, a bathochromic shift (10nm) in band-II with NaOAc indicated the presence of free OH group at C-7.

The +ve ES mass spectrum of the compound (III) displayed a molecular ion peak M⁺ at m/z 625 which corresponding to the molecular formula C₂₈H₃₂O₁₆ +1.

The fragments at m/z 479 (M^+ - Deoxy hexose moiety (146) and 317 (M^+ - (Deoxy hexose + hexose)) confirmed the presence of two sugar moieties. Another two important fragments at m/z 302 (M^+ - (Deoxy hexose + hexose + CH_3)) and 285 (M^+ - (Deoxy hexose + hexose + OCH_3)) proved the presence of a methoxy group on the aglycone.

1H -NMR spectrum of compound (III) showed signals at δ in ppm 7.85 (1H, d, H-2'), 7.52 (1H, dd, $J=8.4$ Hz, 2Hz, H-6'), 6.92 (1H, d, $J=2$, H-5'), 6.44 (1H, d, $J=2$, H-8), 6.21 (1H, d, $J=2$ Hz, H-6). Two anomeric protons displayed at 5.5 and 4.5 ppm were attributed to glucose and rhamnose; respectively indicating the rhamnoside structure of compound (III). The methoxy group protons were appeared at 3.8 (3H, s, OCH_3) and the methyl group protons of the rhamnose moiety appeared at δ 0.98 ppm.

^{13}C -NMR spectrum of compound (III) showed the most important carbon signals like C-4 at 177.19 ppm, two anomeric carbons of the two sugar moieties at 101.03 and 100.7 ppm for C-1'' and C-1''' ;respectively, OCH_3 at 55.49 ppm and the C-6''' of the rhamnose moiety at 17.59 ppm. The other data were shown in (Table 2).

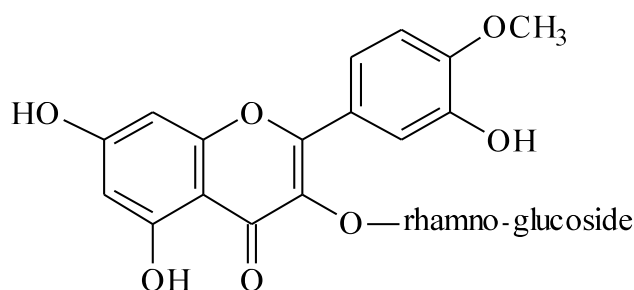
Table 2: 1H - & ^{13}C - NMR Assignments of Compound (III)

No	^{13}C -NMR	1H -NMR	No	^{13}C -NMR	1H -NMR
C-2	156.33	-	C-6'	122.12	7.52 (dd)
C-3	132.33	-	OCH_3	55.49	3.8 (s)
C-4	177.19	-	C-1''	101.03	5.5 (gluc.)
C-5	161.0	-	C-2''	74.1	3-4
C-6	98.0	6.21 (d)	C-3''	75.7	3-4
C-7	164.0	-	C-4''	70.7	3-4
C-8	93.69	6.44 (d)	C-5''	76.23	3-4
C-9	156.39	-	C-6''	66.01	3-4
C-10	103.8	-	C-1'''	100.7	4.5
C-1'	120.8	-	C-2'''	70.4	3-4
C-2'	113.09	7.85 (d)	C-3'''	69.9	3.4
C-3'	149.25	-	C-4'''	71.6	3-4
C-4'	146.7	-	C-5'''	68.1	3-4
C-5'	115.10	6.92 (d)	C-6'''	17.59	0.98 (CH_3)

Dept-90 and Dept-135 experiments proved the presence of two methylene groups attributed to the two sugar moieties.

Acid Hydrolysis:

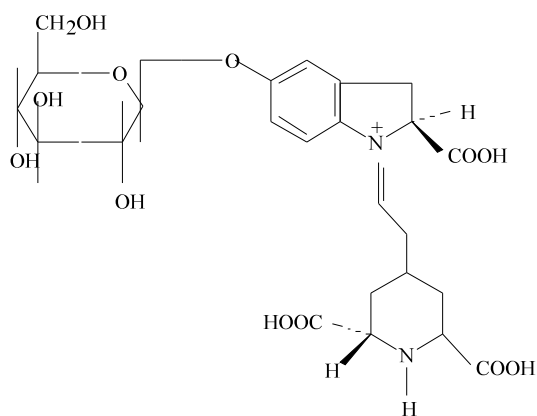
About 5mg of the compound (III) were subjected to acid hydrolysis. The obtained aglycone was further purified over Sephadex LH-20 column, eluted with methanol (100 %) and identified with (UV, MS) as isorhamnetin. The aqueous solution containing the sugar moiety was evaporated and dissolved in isopropanol and investigated by paper chromatography. Both rhamnose and glucose were detected in the hydrolysate. So, the chromatographic and spectroscopic data confirmed that compound (III) is isorhamnetin-3-O-rhamnoglucoiside.



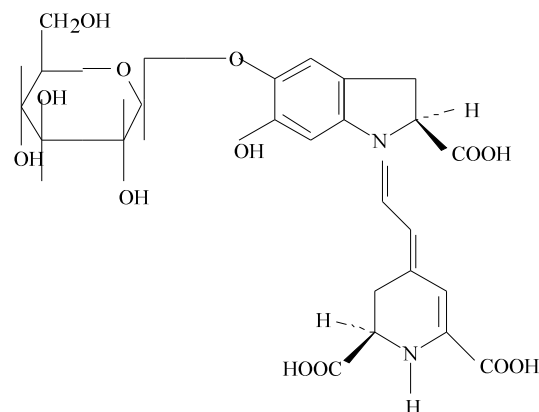
Compound (III); isorhamnetin-3-O-rhamnoglucoiside

Identification of Betalaine Pigments:

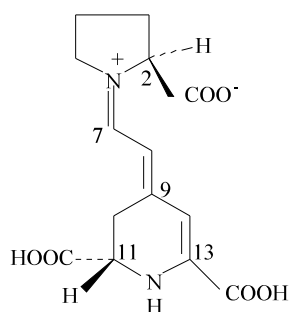
UV spectra of the betalaine pigments were measured in absolute spectroscopic methanol in the region of 250-600 nm. The UV absorption spectrum of pigment I exhibited an absorption band at 475 nm, while pigment II showed an absorption band at 534 nm. These data were in accordance with those reported for indicanthin and betanin and its isomer isobetainin (Forni *et al.*, 1992).



Betanin



Isobetanin



Indicaxanthin

The isolated pigments showed R_f values at 0.63 and 0.79 with maximum absorption at 475 and 534nm for pigments I and II; respectively. These results were in agreement with those reported (Forni *et al.*, 1992).

Identification of the Mucilage:

The brown spots appeared on the chromatogram were identical with authentic, L-Rhamnose (R_f 0.6), D-galacturonic acid (R_f 0.2), L-Arabinose (R_f 0.4) and D-galactose (R_f 0.3). Also, the hydrolysate was dissolved in water-acetonitrile (1: 3) and subjected to HPLC analysis. The major components of the hydrolysate were found to be glucuronic acid (91.962%), rhamnose (3.4821%), galactose (1.9403%). Fructose was also detected in minor amount (0.22%) in addition to four unidentified compounds.

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