ISSN 1110-5089

Zagazig J. Pharm. Sci., December 1995,
Vol. 4, No. 2, pp. 44-48

FLAVONOIDS FROM THE LEAVES OF *HIBISCUS CANNABINUS* L.

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ABSTRACT

From the leaves of *Hibiscus cannabinus* L., three flavonoidal glycosides were isolated and fully identified using different physical and spectral tools viz; chromatographic techniques (TLC, PC, CC), UV, ^{1}H- and ^{3}C- NMR (1-D and 2-D) experiments, as well as EI- and FAB- mass spectroscopy. These allowed to get confirmation of the structure of these compounds to be kaempferol-7-O-α-L-rhamnoside, apigenin-7-O-β-D-glucoside and kaempferol-3,7 di-O-α-L-rhamnoside. The antibacterial tests of these compounds were also demonstrated.

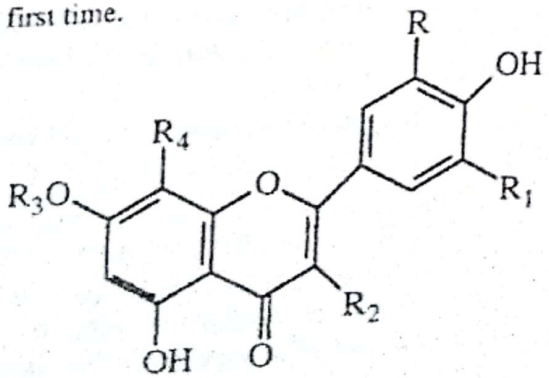
INTRODUCTION

The genus *Hibiscus* (Fam. Malvaceae) is widely distributed all over the world (200 species)[1]. *Hibiscus* cannabinus L. is an annual shrub which grows in the tropical and temperate regions[2]. The plants of this genus were reputed for their folkloric uses in treatment of chronic bronchitis and toothach[3], activation of gastric and hepatic secretions leading to rapid digestion and intestinal antiseptic effect as well as their sedative and diuretic actions[4].

The leaves were used as poultice on boils, cancerous swellings and abscesses, exhibit very fair antibacterial effect and can be used in treatment of gastric troubles[5].

Very little was recorded about the constituents of *H. cannabinus*, since three flavonoids viz.; cannabicetin (**1**), cannbicitrin (**2**) and hibicetin-3-rutinoside (**3**) were isolated from the flowers, but only one, kaempferol-3-rutinoside (**4**) was isolated from the leaves[6-8].

In this study, two kaempferol glycosides, a monoside (**5**) and a bioside (**7**), as well as an apigenin glucoside (**6**) were isolated and fully identified. From the available literature, it was found that these compounds (**5, 6 and 7**) were isolated from this plant for the first time.



	R	R_1	R_2	R_3	R_4
1	OH	OH	OH	H	H
2	OH	O-gl	OH	H	H
3	OH	OH	O-gl	H	OH
4	H	H	O-rh-gl	H	H
5	H	H	OH	rh	H
6	H	H	H	gl	H
7	H	H	O-rh	rh	H

gl = glucose, rh= rhamnose

EXPERIMENTAL

Plant material:

The air-dried leaves were collected from the flowering plants cultivated around the cotton fields near Mansoura, Dakahlia governorate, Egypt. The given plant material was identified by Dr. N. El-Hadidi, Professor of Taxonomy, Faculty of Science, Cairo University.

Authentic materials:

Kaempferol-7-glycoside (previously isolated and identified in Pharmacognosy Department, Faculty of Pharmacy, Mansoura Univ.), D-glucose (Lab. Merck, Germany) and L-rhamnose (DBH Chemnica Col., Poole, England).

Equipment:

Infra red spectrophotometer, Pye-Unicam sp-1000, Cambridge (England) using KBr pellets. Ultra violet spectro-photometer, Perkin Elmer 550 s, USA.

Nuclear magnetic resonance instrument was Varian XL-300, VXR series (Varian Associate, California, USA) operating at 300 MHz (^{1}H-NMR) and 75.5 MHz (^{13}C-NMR) using TMS as internal standard. APT, COSY and HETCOR spectra were performed using the Varian XL-300 instrument, DMSO-d_6 was used as NMR solvent. Mass spectra were determined on Finnigan MAT-90 instrument (Finnigan Instruments, California, USA) operating at 70 ev (EI-MS) or 7 kv using xenon gas and glycerol as matrix under pressure of 1 x 10^{-5} mm Hg (FAB-MS).

Extraction:

Six hundred grams of defatted (petroleum ether), air-dried, powdered leaves were extracted in a Soxhlet with ethyl alcohol 90% (800 ml). The concentrated alcoholic extract (100 ml) was partitioned successively with ether (5 x 100 ml) and ethyl acetate (8 x 100 ml). The solvent, of each extract, was distilled off.

Chromatographic investigation of flavonoids:

The preliminary chromatographic screening, of the two extracts, was carried out on silica gel TLC using $CHCl_3$ - CH_3OH (8:2, system I) and EtOAc-CH_3OH-H_2O (100: 16.5: 13.5, system II) as solvent mixtures and $AlCl_3$ as a spray reagent. This revealed that the ether extract has one major spot corresponding to one of the three spots detected in EtOAc extract.

Ten grams of the EtOAc extract was loaded upon silica gel column (1.5 x 120 cm, 200 gm). Elution was performed by gradient increase of the polarity of $CHCl_3$-CH_3OH solvent mixture. The fractions were monitored on silica gel TLC using solvent mixture (system I). The collected fractions (36-61) exhibited two flavonoid spots, while those (62-90) showed the third one. The latter was crystallized from CH_3OH to afford brownish yellow needle crystals (substance 7, 65 mg).

The mixture (fractions 36-61) was applied onto Whatmann filter paper No. 3 (210-20 x 40 cm) and developed with 15% HAc. Two yellow bands were localized under UV lamp and eluted with CH_3OH. The concentrated methanolic extract, of each band, was kept in refregerator to get amorphous yellow deposit (substances **5** and **6**).

The three isolates gave positive reaction for flavonoid glycosides ($AlCl_3$ and Molisch's tests).

Step-wise acid hydrolysis of the isolates and investigation of sugar moiety:

Ten milligrams, of each substance, were dissolved in 10 ml ethyl alcohol and mixed with 10 ml 4% alc. HCl. The procedure was adopted according to Mabry et al. (1970)[9].

The sugar moiety, of each substance, was investigated on cellulose TLC using n-BuOH-Pyridine-HAc-EtOAc-H_2O (50:20:10:25:20, two runs) as solvent system and aniline phthalate as spray reagent. L-Rhamnose was detected in substances 5 and 7, while substance 6 showed D-glucose.

Characterization of isolated substances:

Substance 5: It exhibited the following spectral data: UV (CH_3OH) λ_{max} (nm) 364, 323_{sh}, 265, 252_{sh}; +$NaOCH_3$ 430, 392sh, 269, 256sh; +NaOAc 361, 308ssh, 266sh; +NaOAc/H_3BO_3 362, 308ssh; 266sh; +$AlCl_3$ 426, 352, 269sh, 236 and +$AlCl_3$ /HCl 426, 352, 269sh, 236; EI-MS m/z (relative intensity, %) 432 (2), 414 (1), 328(1), 286(100), 257(4), 153(3), 143(5) and 93(5); FAB-MS m/z 431 (M^+-1), 367 and 285 (M^+-1-146).

Substance 6: It showed the following spectral data: UV (CH_3OH) λ_{max} (nm) 331, 304sh, 265, +$NaOCH_3$ 387, 320sh, 274; +NaOAc 356, 306sh, 268sh; +NaOAc/H_3BO_3 348, 310sh, 268sh; +$AlCl_3$ 292sh, 272; +$AlCl_3$/HCl 343, 292sh, 272; EI-MS m/z (rel. Int., %) 432 (3), 414 (2), 270 (100), 152 (31), 124(18), 118(13) and 84(14); FAB-MS m/z 433 (M^++1), 415, 271 (M^++1-162).

Substance 7: It exhibited the following spectral data: UV (CH_3OH) λ_{max} (nm) 325, 265, 235_{sh}, +$NaOCH_3$ 406, 269, 242_{sh}; + NaOAc 344, 335, 258_{sh}, +NaOAc/H_3BO_3 338, 318, 258_{sh}; +$AlCl_3$ 398, 348, 292_{sh}, 230; +$AlCl_3$ /HCl 398, 348, 292_{sh}, 230; FAB-MS m/z 579 (M^++1), 433 (M^++1-146), 287 (M^++1-146-146).

^{1}H-NMR and ^{13}C-NMR data of these substances are illustrated in table (1) and table (2).

Determination of the flavonoid content of the leaves:

1- Preparation of the standard curve:

According to Mabry et al. (1970)[9], the standard curve of kaempferol-7- glycoside was prepared using $AlCl_3$ (0.1 M) as colour reagent and the absorbance was measured at λ_{max} = 425 nm.

2- One gm of powdered leaves, was exhaustively defatted in a small Soxhlet with petroleum ether, then extracted with methanol. The concentrated methanolic extract was treated with $AlCl_3$ as before[9].

From the standard curve and by logical calculations, the flavonoid content in the leaves was found to be 3.36% (w/w).

Preliminary antibacterial testing:

The antibacterial activity of substances 5,6 and 7 was tested against certain strains of gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*) bacteria using the disc agar diffusion method[10]. The agar media were inoculated with the tested organisms, and the tested compounds were dissolved in

propylene glycol (5 mg/ml) and were aseptically transfered into sterile discs of Whatmann filter paper No. 3 (i.e. 40 µg/disc). Streptomycin antibiotic disc (BBL Microbiology Systems, Cockeysville, MD, USA) was used as a reference. The resulting inhibition zones were measured after 18 hours incubation (37°C).

Substance 5 exhibited little activity against *E. coli* (inhibition zone= 16 mm) and *S. aureus* (I.Z. = 14 mm) in comparison to streptomycin (I.Z. was 25 mm for *E. coli* and 23 mm for S. aureus). Subtances **6** and **7** exhibited no activity against both organisms.

DISCUSSION AND CONCLUSION

From the UV spectral data of the isolated compounds, it was concluded[9] that substance **5** is a flavonol (λ_{max} CH_3OH = 364 nm) with free OH groups at C-4' (high bathochromic shift with $NaOCH_3$ = 66 nm) and C-3 and C-5 (high bathochromic shift = 62 nm with $AlCl_3$ and not affected by HC1). The OH group at C-7 is substituted (no bathochromic shift with NaOAc) and the O-dihydroxy groups are absent (no significant bathochromic shift with NaOAc/$H3BO_3$).

Substance 6 showed UV absorption at 331 nm (band I, in CH_3OH) suggesting a flavone structure, having free OH groups at C-4' (significant bathochromic shift =56 nm with $NaOCH_3$) and C-5 (small bathochromic shift =12 nm with $AlCl_3$ not affected by HCl), but the OH group at C-7 is substituted (no clear bathochromic shift with NaOAc), and the O-dihydroxy groups are absent (no bathochromic shift with NaOAc/H_3BO_3).

Substance 7 may be a flavone (λ_{max} CH_3OH = 325 nm) exhibiting free OH groups at C-4' (high $NaOCH_3$ bathochromic shift = 81 nm) and C-5 (high $AlCl_3$ bathochromic shift = 73 nm, not affected by HCl). No o-dihydroxy groups are detected (absence of NaOAc/H_3BO_3 bathochromic shift) and the OH group at C-7 is substituted (no clear NaOAc bathochromic shift).

Accordingly, it was concluded that these isolates may be kaempferol-7- L-rhamnoside (substance 5), apigenin-7-D-glucoisde (substance6) and apigenin-7-L-rhamnoside (substance 7)[11].

But, according to the mass spectra and NMR (table 1 and 2) spectral data of these compounds, it was found that these data complied with the UV conclusions except substance 7 which was clearly assigned as kaempferol-3-7-di-O-L-rhamnoside.

For substance **5**, the presence of C-4 and C-3 signals at δ 176.2 and 136.2 respectively (table 2) as well as the absence of H-3 signal of ^{1}H-NMR confirmed its flavonol nature, while the presence of two 2H doublets at δ 6.50 (H-3', 5') and 8.10 (H-2'-6') as well as the two ^{13}C-NMR signals at δ 115.5 (C-3',5') and 129.7 (C-2',6'), confirmed that ring-B is occupied at position 4' only [12,13].

The presence of 1H doublet (J= 1.4 Hz) at δ 5.56 (H-1 of α-L-rhamnose), 3H doublet (J =6 Hz) at δ 1.15 (6-CH_3 of rhamnose) and the ^{13}C-NMR signals at δ 18.0 and 98.9 (C-6 and C-1 of rhamnose, resp.) indicated the rhamnose sugar unit which was confirmed through the FAB-MS ion peak at m/z 285 (M^+-1-146 of rhamnose)[12].

These assignments were ascertained through the COSY, HETCOR and APT NMR experiments and the TLC co-chromatography of the hydrolysate of substance **5**. Consequently, it was concluded that subtance **5** is kaempferol-7- O-α-L-rhamnoside.

For substance **6**, it was assigned as apigenin-7-O-β-D-glucoside according to the following criteria:

(a) its flavone nature was ascertained by the presence of 1H singlet at δ 6.84 (H-3) (table 1) and ^{13}C-NMR signals at δ 181.8 (C-4 of the flavones)[12] and 102.9 (C-3) (table 2).

(b) The glucose unit was assigned by FAB-MS ion peak at m/z 271 (M^++1-162 of glucose), 1H doublet (J= 7.6 Hz, H-1 of β-D-glucose) at δ 5.85, 2-H multiplet at δ 4.58 (CH_2-6 of glucose), ^{13}C-NMR signals at δ 100.3 and 60.7 (C-1 and C-6 of glucose, respectively)[12,13].

(c) Ring B exhibited only one OH group at C-r'. This was attributed to the presence of two 2H doublets at δ 6.97 (H-3', 5') and 7.93 (H-2', 6') (J= 8.7 Hz, o-coupling) and two ^{13}C-NMR signals at δ 116.0 (C-3',5') and 128.4 (C-2', 6').

These assignments were confirmed by the COSY and APT NMR experiments, TLC co-chromatography of the hydrolysate of substance 6, as well as the published data[12,13].

For substance **7**, it was clearly assigned as kaempferol-3, 7- di-O-α-L-rhamnoside depending upon the following findings:

(a) The flavonol nature was ascertained by the absence of the H-3 singlet in its 1H-NMR spectra and the C-4 signal appears at d 178.0 (ca. -4 nm upfield shift from flavones)[13].

(b) the presence of two α-L-rhamnose units was determined from the two anomeric proton-doublets at δ 5.32 (J = 1.2 Hz) and 5.5 7 (J= 1.1 Hz) and the two anomeric carbons at d 98.6 and 101.9, which was ascertained by the two FAB-MS ion peaks at

Table (1): ^{1}H-NMR spectral assignment of isolated substances 5, 6 and 7:

No. H atom	Substance 5			Substance 6			Substance 7		
	δ(ppm)	Multi-plicity*	*J* (Hz)	δ(ppm)	Multi-plicity*	*J* (Hz)	δ(ppm)	Multi-plicity*	*J* (Hz)
3	-	-	-	6.84	s	-	-	-	-
6	6.43	d	2.0	6.78	d	1.8	6.47	d	2.0
8	6.83	d	2.0	6.89	d	1.8	6.80	d	2.0
2'	8.10	d	8.7	7.93	d	8.4	7.81	d	8.0
3'	6.50	d	8.7	6.97	d	8.4	6.95	d	8.0
5'	6.50	d	8.7	6.97	d	8.4	6.95	d	8.0
6'	8.10	d	8.7	7.93	d	8.4	7.81	d	8.0
Sugar-7*									
1	5.56	d	1.4	5.85	d	7.6	5.57	d	1.1
2	3.86	d	1.0						
3						-			
4	3.39-3.84	m	-	4.35-4.50	m		3.11-3.68	m	-
5									
6	1.15	d	6.0	4.58	m	-	1.15	d	5.9
Sugar-3*									
1	-	-	-	-	-	-	5.32	d	1.2
2	-	-	-	-	-	-	3.87	d	1.7
3	-	-	-	-	-	-			
4	-	-	-	-	-	-	3.11-3.68	m	-
5	-	-	-	-	-	-			
6	-	-	-	-	-	-	0.82	d	5.3

** d = doublet, m = multiplet, s singlet; sugar-3 = sugar at C-3, sugar-7 = sugar at C-7. NMR solvent was DMSO-d6*

m/z 433 (M^{+}+1-146 of one rhamnose unit) and 287 (M^{+}+ 1-146-146 of two rhamnose units). This was also confirmed by the appearance of two 3 H doublets at δ 0.82 (*J* = 5.3 Hz) and 1.15 (*J* = 5.9 Hz) of CH_3-6 of rhamnose units, and the ^{13}C-NMR signals at δ 17.6 and 18.0 (C-6) of rhamnose units).

(c) The little up-field shift of the ^{13}C-NMR signals at C-3 (-1 ppm) and C-7 (o-2ppm) and the down-field shift of C-4 (+2 ppm), C-6 (+1 ppm) and C-8 (+1ppm) assigned the linkage of the two rhamnose units at C-3 and C-7 (12,13). (d) The presence of two 2H doublets at δ 6.95 (H-3',5') and 7.81 (H-2',6') having *J* = 8.0 Hz (o-coupling) as well as the two ^{13}C-NMR signals at 115.5 (C-3',5') and 130.8 (C-2',6') showed that ring B is hydroxylated only at C-4'.Thus, it was found that MS and NMR spectral experiments, rather than UV study only, were necessary to get clear assignment for substance 7.

The quantitative analysis of the flavonoid content (3.36% w/w), in addition to the fair antibacterial activity of substance 5 demonstrated the significance of the leaves of the plant under sutyd which contain mucilage[11] to be introduced in the formulations used for acute bronchitis and cough

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Table (2): 13C-NMR spectral assignment of substances **5, 6** and **7:**

No. C-atom	Chemical shift (δ, ppm)		
	Substance 5	Substance 6	Substance 7
2	147.6	164.3	156.2
3	136.2	102.9	134.6
4	176.2	181.8	178.0
5	160.4	161.5	161.0
6	98.5	99.5	99.5
7	161.5	162.9	161.8
8	94.4	95.0	94.7
9	155.8	156.8	157.9
10	104.8	105.4	105.9
1'	121.6	120.8	120.4
2'	129.7	128.4	130.8
3'	115.5	116.0	115.5
4'	159.5	161.1	160.3
5'	115.5	116.0	115.5
6'	129.7	128.4	130.8
Sugar-7*			
1	98.9	100.3	98.6
2	70.1	73.2	69.9
3	70.3	77.3	70.2
4	71.7	69.6	71.7
5	69.9	76.5	70.4
6	18.0	60.7	18.0
Sugar-3*			
1	-	-	101.9
2	-	-	70.2
3	-	-	70.3
4	-	-	71.2
5	-	-	70.7
6	-	-	17.6

**Sugar-3 and sugar-7 are sugar at C-3 and C-7, respectively.NMR solvent was DMSO-d6*

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فلافونات من اوراق نبات التيل

ممدوح منصور احمد

قسم العقاقير- كلية الصيدلة- جامعة المنصورة- المنصورة- مصر.

نبات التيل من النباتات المزروعة حول حقول القطن في مصر للحصول علي أليافه. وقد تم في هذا البحث فصل ثلاثة فلافونات من الأوراق وأمكن التعرف عليها من خلال تحاليل أطيافها المختلفة مثل طيف الأشعة فوق البنفسجية، والرنين النووى المغناطيسي في إتجاه واحد وفي إتجاهين للبروتون والكربون-١٣، وطيف الكتلة وكذلك الطرق الكروماتوجرافيه المختلفة. وقد تبين بوضوح أنها عبارة عن: كامفيرول-٧- رامنوزايد (مادة ٥)، أبيجينين -٧- جلوكوزايد (مادة ٦) و كامفيرول -٣ و ٧- ثنائي رامنوزايد (مادة ٧). وتم تحديد كمية الفلافونات الموجودة بالورقة (٣٦ ,٣٪) وبالفحص البكتريولوجي لهذه المواد كمضاد للبكتريا تبين أن مادة (أ) فقط هي التي لها تأثير قليل نوعاً ما.