Flavonoids of *Neotorularia aculeolata* Plant

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Abstract: *Neotorularia aculeolata* belongs to the family Cruciferae that has several uses in the Egyptian folk medicine for many years. Nothing could be traced about the chemical composition of the plant. Extraction, isolation and purification of the air-dried plant material using different chromatographic techniques (PC, TLC & CC) provided seven flavonoids. Identification of the isolated compounds using different chemical and physical techniques (UV, ^{1}H-NMR and C ^{13} NMR spectroscopy) allowed to characterize these compounds as kaempferol, kaempferol-7-O-rhamnoglucoside {Kaempferol-7-neohesperidoside}, quercetin, rutin, quercetin-3-O- β-D-glucoside-7-O-α-L-rhamnoside-3`-methylether, quercetin-3,7-di-O-α-L-rhamnoside-3`-methylether and myricetin.

Keywords: *Neotorularia aculeolata*, Cruciferae, kaempferol, quercetin, rutin, myricetin.

1. INTRODUCTION

Cruciferae (Brassicaceae) is one of the largest families in the plant kingdom that is rich in medicinal plants. It comprises approximately 380 genera and about 3350 species in 10 poorly defined tribes [1]. The family is represented in Egypt by 53 genera and 107 species mostly annual, biennial or perennial herbs**.** *N. aculeolata* (Boiss.) Hedge & J. Leonard is one of these annual plants in this family. It grows at Sinai proper; it always grows at the entire Sinai Peninsula including the coastal Mediterranean strip and El–Tih Desert east of Suez Canal, rock crevices and hillsides [2]. It is widely spread at Abo Egaila – El Qusayema road (North Sinai) from where it was collected for this study.

Cruciferous plants have been used since ages and are grown as vegetables, sources of oils and as condiments. They are known for their stimulant, diuretic, thermogenic, depurative, rubefacient, galactogogue, emmenagogue, tonic, aphrodisiac, ophthalmic activities and are used for scurvy, peptic ulcers, hepatopathy, splenomegaly, dyspepsia, diarrhea, dysentery, lumbago, syphilis, leucorrhoea, seminal weakness, asthma, cough, hiccough, tenesmus, hemorrhoids as well as anticancer activity especially as androgen receptor antagonist in human prostate cancer [3, 4]. Cruciferous plants are inducers of microsomal cytochrome P450 enzyme [5].

It was reported that kaempferol, quercetin and isorhamnetin glycosides, in addition to myricetin, luteolin and apigenin and their derivatives were isolated [6-8].

The presence of phenolic acids as: caffeic, ferulic, *P*–coumaric and vanillic acids beside the presence of the previous flavonoids and rutin were also isolated from *Brassica alba, B. oleraceae, B. campestris* and other cruciferous species [9-14].

2. RESULTS AND DISCUSIONS

2.1. Isolated Flavonoids

Seven flavonoids were isolated, purified by CC, PC and TLC and identified through R_f-values, UV spectra in methanol with different shift reagents (Table **1**) and ^{1}H & ^{13}C-NMR. These compounds were coded as A_1-A_7.

2.1.1. Compound A_1

This compound was obtained as yellow crystals, soluble in methanol, R_f-values of 0.85 in BAW and 0.4 in acetic acid 15%. It showed two major absorption bands in MeOH**;** band I at 367nm and band II at 268nm**,** which indicated a flavonol nucleus with free hydroxyl group at the C-3 [15, 16]. Addition of sodium methoxide resulted in a bathochromic shift in band I (+49 nm)**,** which proved the presence of a free OH**-** group at C-4**.** A bathochromic shift in band I (+53nm) with aluminum chloride**,** which was not affected by the addition of hydrochloric acid**,** indicating the presence of free hydroxyl group at C**-**3 and C**-**5**.** A bathochromic shift in band II (+7 nm) with sodium acetate indicated the presence of free hydroxyl group at C**-**7**.** Addition of H_3BO_3 gave no shift**,** which proved the absence of any catecholic hydroxyl group**.** From the UV analysis, compound A_1 is probably kaempferol. The identity of

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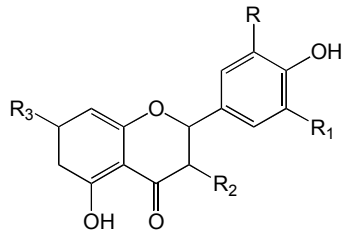
Table 1: UV Spectral Data of the Isolated Compounds

UV Data						
AcONa/ H_3BO_3	**AcONa**	**$AlCl_3$/ HCl**	**$AlCl_3$**	**MeONa**	**MeOH**	
274, 296 (sh), 320 (sh), 372	275, 302 (sh), 385	266, 305 (sh), 350, 420	266, 305 (sh), 350, 420	280, 318 (sh), 416	253 (sh), 268, 324 (sh), 367	A_1
260, 325, (sh), 370	266, 323, 385, 418 (sh),	244 (sh), 258, 266, 300 (sh), 350, 422	259 (sh), 266, 299 (sh), 353, 424	245, 267, 335 (sh), 425	253, 266, 323, 354	A_2
264, 292 (sh), 384	274, 320 (sh), 428	268, 300, (sh), 362 (sh), 428	272, 328, 445	262, 332, 440	267, 306 (sh), 370	A_3
220, 298, 387	271, 325, 393	271, 300, 340 (sh), 402	275, 303, (sh), 433	272, 327, 410	259, 266 (sh), 299 (sh), 350	A_4
262, 360	260, 360	270, 310, 350, 410	270, 310, 350, 410	270, 398	275, 355	A_5
256, 310, 360, 400	256, 310, 360, 400	256, 360	256, 360	270, 410	256, 350	A_6
258, 304 (sh), 382	269, 335	266, 275 (sh), 308 (sh), 366, 428	271, 316 (sh), 450	262 (sh), 285 (sh), 322, 423	254, 272, (sh),, 374	A_7



Comp.	Name	R	R_1	R_2	R_3
A1	Kaempferol	H	H	OH	OH
A2	Kaempferol-7-O-neohesperidoside	H	H	OH	Oglrh
A3	Quercitin	OH	H	OH	OH
A4	Rutin	OH	H	Oglrh	OH
A5	Quercitin-3-O-β-D-gluc-7-α-L-rham-3`-methylether	OMe	H	Ogl	Orh
A6	Quercitin-3,7di-O-α-L-rham-3`-methylether	OMe	H	Orh	Orh
A7	Myrecitin	OH	OH	OH	OH

Figure 1: Structures of compounds from *Neotorularia aculeolata*.

compound A_1 was further confirmed as kaempferol by ^{1}H-NMR spectrum in DMSO-d6, which showed signals at δ (ppm) 8.0 (2H, d, J= 8 Hz, H-2` and H-6`), 6.9 (2H, d, J= 8Hz, H-3` and H-5`), 6.4 (1H, d, J= 1.5 Hz, H-8), and 6.2 (1H, d, J= 1.5 Hz, H-6). Thus, from the above data and current literature this compound A_1 is identified as kaempferol [17].

2.1.2. Compound A_2

This compound was obtained as dull yellow crystals, soluble in methanol, R_f-values 0.3 in BAW and 0.35 in acetic acid 15%. It showed two major absorption bands in MeOH; the absorption maximal in methanol, band I (354nm) indicated that it was a flavonol with a 3-OH free. The addition of NaOMe caused a bathochromic shift in both band I and II, a fact which proved the presence of a free OH at 4` position. After addition of $AlCl_3$, a bathochromic shift proved the presence of a free OH at 5 positions. However, on addition of HCl, no change was observed indicating the absence of any catecholic hydroxyl groups. Meanwhile the addition of NaOAc caused no shift in band II thus suggesting the occupation of 7-position. Addition of H_3BO_3 caused no shift, this suggested the absence of any catecholic hydroxyl groups.

The ^{1}H-NMR spectral data of compound A2 showed the signals characteristic for kaempferol with additional signal for the sugar moieties. Two signals for the two anomeric sugar protons at δ 5.4 (1H, d, J=2.5Hz, H-1``

glucose) and δ 5.2 (1H, d, J=2.5Hz, H-1``` rhamnose). The remaining sugar proton as m at 3.2-3.9**,** signal at 1.2 (3H, d, J=6Hz, CH_3 rhamnose).

^{13}C-NMR spectrum data of compound A_2 showed a ketonic carbon at 176.1 at C-4 and the most acidic carbon at C-7 at 162.4 followed by C-4` at 159.4 and C-3 at 135.9. Two anomeric sugar carbons at 98.4 and 100.5 for C-1`` and C-1```, respectively indicating the disaccharide nature of compound A2. One methyl carbon of rhamnose was shown at 20.9. In A_2, C-2``` of rhamnose appeared at 70.5. Thus from the obtained R_f-values, UV, ^{1}H-NMR and ^{13}C-NMR spectral data of compound A_2, showed that it is identified as kaempferol-7-O-glucose (1→2)-rhamnose (Kaempferol-7-O-Neohesperidoside).

2.1.3. Compound A_3

This compound was obtained as yellow crystals, soluble in methanol, R_f-values 0.73 in BAW and 0.29 in acetic acid. Compound A_3 showed two major absorption bands in MeOH**;** band I at 370nm and band II at 267nm**,** which indicated a flavonol nucleus with free hydroxyl group at the 3 position [15, 16]. Addition of sodium methoxide resulted in a bathochromic shift in band I (+70nm)**,** which proved the presence of a free OH**-**group at 4$^{\backslash}$**-**position**.** A bathochromic shift in band I (+75nm) with aluminum chloride**,** indicated the presence of free hydroxyl group at C**-**3 and C**-**5**.** The hypthochromic shift of $AlCl_3$ spectrum in band I (-17 nm) after the addition of HCl indicated the presence of orthodihydroxy group in B-ring (3`, 4` position). A bathochromic shift in band I (+14 nm) with sodium acetate indicating the presence of free hydroxyl group at C**-**7**,** which was detected by H_3BO_3 addition, indicates the presence of orthodihydroxy group (3`, 4` position). Thus, from the UV analysis and R_f-values, compound A_3 may be identified as quercetin. The compound A_3 was further confirmed as quercetin by ^{1}H-NMR spectrum in DMSO-d6, which showed signals at δ (ppm) 7.7 (1H, d, J = 8.5 Hz, H-2`), δ 7.5 (1H, dd, J= 2.5, H-6`) and δ 6.8 (1H, d, J = 8.5, H= 5`), indicated the presence of aromatic ring with two substitutions, in *m, p*-substitution {δ 6.5 (1H, d, J = 1.5 H-6) and δ 6.2 (1H, d, J= 1.5, H-8)}. Thus, from the above mentioned data, compound A_3 is Quercetin [17].

2.1.4. Compound A_4

This compound was obtained as yellow crystals, R_f-values of 0.49 in BAW and 0.54 in acetic acid respectively. The absorption maxima in methanol, band I at 350 nm, indicates that it is a flavonol with 3-OH substitution. The remaining UV spectral data were found to be similar to that of quercetin type compound.

^{1}H-NMR spectrum of the compound A_4 in DMSO-d6, showed signals at δ (ppm) 7.6 (1H, d, J = 2.5 Hz, H-2`), δ 7.5 (1H, dd, J = 8.5, 2.5 H-6`), δ 6.8 (1H, d, J = 8.5, H= 5`), δ 6.4 (1H, d, = 1.5 H-8), δ 6.2 (1H, d, J = 1.5, H-6) and for sugar moiety δ (ppm): 5.3 (1H, d, J= 8Hz, H-1`` glucose), 4.5 (1H, d, J= 2.5Hz, H-1``` rhamnose), 3.4 (m, remaining sugar protons) and 0.8 (3H, d, J= 6Hz, CH_3 rhamnose). ^{13}C-NMR of A_4 gave the following peaks in DMSO-d6: δ (ppm): 146.9 (C-2), 135.5 (C-3), 175.8 (C-4), 160.7 (C-5), 98.2 (C-6), 163.9 (C-7), 93.3 (C-8), 156.2 (C-9), 103.1 (C-10), 122.1 (C-1′), 115.3 (C-2′), 145.0 (C-3′), 147.6 (C-4′), 115.6 (C-5′), 120.0 (C-6′), and for sugar moiety, 101.5 (C-1″), 74.3 (C-2″),75.9 (C-3″), 70.2 (C-4″), 76.2 (C-5″), 67.4 (C-6″), 101.2 (C-1‴), 70.8 (C-2‴), 71.0 (C-3‴), 72.2 (C-4‴), 69.1 (C-5‴) and 18.1 (C-6‴). Complete acid hydrolysis yielded glucose and rhamnose in the aqueous phase and quercetin in the organic phase in (a), (e) and (f) using specific spray reagents. From the above data and by comparison with published data, compound A_4 is identified as Rutin (quercetin-3-O-α-L-rhamnoside (1→6)-β-D-glucoside) [17].

2.1.5. Compound A_5

This compound was obtained as yellow crystals, R_f-values of 0.51 in BAW and 0.7 in acetic acid respectively. The absorption maxima in methanol, band I at 355 nm, indicates that it is a flavonol with 3-OH substitution. The remaining UV spectral data were found to be similar to that of quercetin type compound. ^{1}H-NMR spectrum of the compound A_5 in DMSO-d6, showed signals at δ (ppm) 7.95 (1H, d, J= 8.5 Hz, H-2′), 7.65 (1H, dd, J= 8.5 Hz, H-6′), 6.94 (1H, d, J= 8.5Hz, H-5′), 6.75 (1H, d, J= 2.5Hz, H-8), 6.45(1H, d, J=2.5 Hz, H-6), 5.6 (1H, d, J= 2.5Hz, H-1″ rhamnose), 5.4 (1H, d, J=2.5 Hz, H -1‴ glucose), 3.92 (s, OCH3) and 1.2 (3H, d, J=6Hz, OCH3 rhamnose). The isolated compound A_5 when subjected to partial acid hydrolysis afforded quercetine-3′-methoxide and the sugars were glucose and rhamnose. On the other hand, a known amount of the compound A_5 was subjected to complete acid hydrolysis using 2N HCl. It was observed that compound A_5 resisted acid hydrolysis, which coincided with C-glycoside flavonoid. From the data above and by comparison with published data**,** compound A_5 is identified as Quercetin-3-O-β-D-glucoside -7-O-α-L-rhamnoside-3`-methyether [17].

2.1.6. Compound A_6

This compound was obtained as yellow crystals, R_f-values 0.52 in BAW and 0.69 in acetic acid. The absorption maxima in methanol, band I at 350 nm, indicated that it was a flavonol with 3-OH substitution. The remaining UV spectral data was found to be similar to that of quercetin type compound. ^{1}H-NMR spectrum of the compound A_6 in DMSO-d6, showed signals at δ (ppm) 7.6 (1H, d, J= 8.5 Hz, H-2′), 7.4 (1H, dd, J= 8.5 Hz, H-6′), 6.9 (1H, d, J= 8.5Hz, H-5′), 6.7 (1H, d, J= 2.5Hz, H-8), 6.4 (1H, d, J=2.5 Hz, H-6), 5.6 (1H, d, J= 2.5Hz, H-1″ rhamnose), 5.5 (1H, d, J=2.5 Hz, H -1‴ rhamnose), 3.9 (s, OCH_3), 1.2 (3H, d, J=6Hz, OCH_3 rhamnose) and 0.8 (3H, d, J=6Hz, CH_3 rhamnose). The isolated compound A_6 when subjected to partial acid hydrolysis afforded quercetine-3′-methylether and the sugar was rhamnose. On other hand a known weight of the compound A_6 was subjected to complete acid hydrolysis using 2N HCl, which gave the aglycone quercetine-3′-methoxide and the sugar was rhamnose. From above data and by comparison with the published data, compound A_6 is identified as Quercetin-3,7-di-O-α-L-rhamnoside-3`-methylether [17].

2.1.7. Compound A_7

This compound was obtained as yellow crystals, R_f-values of 0.31 in BAW and 0.16 in acetic acid respectively. It showed two major absorption bands in MeOH**;** band I at 374 nm and band II at 254 nm**,** which indicated a flavonol nucleus with free hydroxyl group at the 3 position [15, 16]. Addition of sodium methoxide resulted in a bathochromic shift in band I (+49nm)**,** which proved the presence of a free OH**-**group at C-4`position**.** A bathochromic shift in band I (+76nm) with aluminum chloride**,** indicated the presence of a free hydroxyl group at C**-**3 and C**-5.** The hypthochromic shift of $AlCl_3$ spectrum in band I (-22 nm), after the addition of HCl, indicated the presence of orthodihydroxy group in B-ring (3`and 4` position). A bathochromic shift in band II (+15 nm) with sodium acetate indicated the presence of a free hydroxyl group at C**-7,** which was detected by H_3BO_3 addition, that indicated the presence of orthodihydroxy group (3`and 4` position). Thus, from the UV analysis and R_f-values compound A_7 may be identified as myricetin.^{1}H-NMR spectrum of the compound A_7 in DMSO-d6, showed signals at δ (ppm) 6.25 (1H, d, J= 2.5 Hz, H-6), 6.33 (1H, d, J= 2.5 Hz, H-8), 7.32 (s, H-2′ and H-6). From the above data and by comparison with published data**,** Compound A_7 is identified as Myricetin [17].

3. SUMMERY AND CONCLUSION

In the present study, the defatted desalted methanolic extract of the air-dried whole plant of *Neotorularia aculeolata* was purified by CC, PC and TLC ,to afford seven flavonoids {A_1 to A_7} these compounds are identified as:- kaempferol, kaempferol-7-O-rhamnoglucoside {Kaempferol-7-Neohesperido-side}, Quercetin, Rutin, Quercetin-3-O- β-D-glucoside-7-O-α-L-rhamnoside-3`-methylether , Quercetin-3,7-di-O-α-L-rhamnoside-3`-methylether and Myricetin.

To our knowledge this represents the first report for the isolation of these compounds from *N. aculeolata.*

4. EXPERIMENTAL

4.1. Material, Methods and Techniques

4.1.1. Plant Materials

Neotorularia aculeolata (Boiss.) plant was collected from North Sinai (Abo Egaila–El Qusayema road) in June 2006 and identified by Prof. Dr. Nahed El-Hadidi, Botany Department, Faculty of Science, Cairo University and by comparison with herbarium specimens at the Desert Research Center {DRC}. A voucher specimen was deposited in the Pharmacognosy lab, Future University, Egypt. The plant material was ground to fine powder and kept in an amber light proof container.

4.1.2. Extraction and Purification of Flavonoids:

The whole plant {2 kg} was defatted with petroleum ether (B.p. 40 -60°C): ether (1:1 v/v). The defatted powder was percolated with methyl alcohol (80%) till exhaustion to obtain a brownish alcoholic extract. This extract was concentrated by evaporation under reduced pressure at not more than 45°C to yield a sticky brownish residue (90g) which was suspended in hot distilled water, filtered and desalted.

4.1.3. Chromatographic Investigation:

4.1.3.1. PC

The concentrated aqueous extract was applied on Whatmman No.1 paper chromatography using descending technique with suitable solvent systems **[15].**

4.1.3.2. TLC

Precoated TLC plates silica gel 60SF_{254} (E- Merck) 20 x 20 cm were used for investigation of the

flavonoids using suitable solvent systems and precoated preparative TLC silica gel F_{254}

- Solvent systems used for PC and TLC:

  a) n-butanol-acetic acid- water (BAW) (4:1:5) organic phase.

  b) Acetic acid 15%.

  c) Ethyl acetate-methanol-water (30:5:4)and (30:2.5:2)one phase, for TLC only.

  d) Phenol-water(80:20)

- Spray Reagent for PC and TLC

Aluminum chloride reagent: 1 % aluminum chloride reagent solution in ethanol [18].

4.1.3.3. Isolation and Purification of The Flavonoidal Compounds

The desalted alcoholic extract was submitted to column chromatography and gradient elution was performed, using firstly chloroform with increasing concentration of ethyl acetate and methanol, respectively. Fractions of 50 ml were collected, then subjected to TLC using system (c) and similar fractions were pooled together. The pooled fractions were subjected to preparative TLC using system (a), the bands corresponding to the flavonoidal compounds were visualized under UV, eluted with methanol and water {1:1}. The eluted bands were purified on a Sephadex LH-20 column using methanol and water as eluting system where seven compounds could be isolated.

4.1.3.4. Identification of Flavonoids:

4.1.3.4.1. Spectral data

Ultraviolet (UV) with shift reagents and nuclear magnetic resonance (^{l}H-NMR and ^{13}C-NMR).

4.1.3.4.2. Acid Hydrolysis

- Partial and Complete Acid Hydrolysis

5 mg of each compound were dissolved in 5 ml methanol and an equal volume of 0.1N and 2N HCl aqueous solution {each separately} was added for mild and complete acid hydrolysis, respectively. The mixture was refluxed on a boiling water bath for 3 hours. The methanol was evaporated and the aglycone was extracted with ether. The aqueous layer containing the sugar part was evaporated to dryness and the residue was dissolved in 10% isopropanol solution.

- Identification of the Aglycone:

The ether extract of each compound containing the aglycone moiety was applied on paper chromatogram along side with authentic flavonoid aglycones using the solvent systems (a) and (b), visualized under UV, exposed to ammonia vapor and sprayed with AlCl3.

- Identification of the Sugars*:*

The isopropanol solution of each compound containing the sugar moiety was applied on PC along side with authentic sugars using the solvent systems (a) and (d). After development, they were visualized by spraying with aniline hydrogen phthalate reagent and heating at 110°C for 10 min..

5. AUTHENTIC REFERENCE

5.1. Flavonoid

Kaempferol ,Quercetin & myricetin were available at the medicinal and aromatic plants department, Phytochemistry unit, Desert Research Center Egypt**.**

5.2. Sugars

Glucose and rhamnose, (Sigma).

5.3. Apparatus

Shimadzu UV 240 (P/N 204 – 28000) instrument was used for recording UV spectra and measuring the absorbance under UV range.

A JEOL Ex – 270 NMR spectrometer apparatus (270 MH_z for ^{1}H –NMR and 67.5 MH_z for ^{13}C – NMR) was used for identification of the proton and / or carbon in methanol, DMSO, CCl_4 and $CDCl_3$ using trimethylsilyl ether (TMS) as an internal standard. Chemical shift values were recorded in δ ppm.

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