Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2015; 4(3): 01-08
Received: 04-06-2015
Accepted: 06-07-2015

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Highly methoxylated flavonoids and the anti-eczematous activity of *Rhamnus disperma* roots

Ehab A Ragab

Abstract
Chromatographic investigation of *Rhamnus disperma* roots was resulted in the isolation of two highly methoxylated flavonoids; myricetin 3,6,7,3`,4`,5`-*O*-hexamethyl ether (**1**) and myricetin 3,6,7,4`,5`-*O*-pentamethyl ether (**2**) together with quercetin 3-*O*-methyl ether (**3**) and quercetin (**4**). The structures of these compounds were assigned on the basis of spectroscopic data. Occurrence of compounds **1** and **2** in Genus *Rhamnus* are reported here for the first time. The alcoholic extract of *Rhamnus disperma* roots showed significant anti-eczematic activity against induced eczema in mice.

Keywords: *Rhamnus disperma*, highly methoxylated flavonoids, anti-eczematic activity.

1. Introduction
Family Rhamnaceae occur in all parts of the world in which climatic conditions allow the growth of woody plants [1]. One of the most important genus in the Rhamnaceae family is *Rhamnus*, it includes about 110 species of deciduous or ever green trees or shrubs, native in temperate regions of northern hemisphere, but a few extending in the tropics, planted for ornament [2]. Plants belonging to genus *Rhamnus* have been used in folk medicine for treatment of constipation, hepatic diseases and eczema [1]. Anthraquinones, anthrones, flavonoid glycosides and naphthaline derivatives were reported from genus *Rhamnus* [3-6]. In the flora of Egypt, the genus *Rhamnus* is represented by two species; *Rhamnus disperma* Ehrenb. and *Rhamnus lycioides* L [7]. Only one chemical study of an EtOH-H_2O (4:1) extract from the fruits and aerial parts of *Rhamnus disperma* has been reported, and phenolic metabolites were observed [8]. The present paper describes the isolation and identification of four flavonoids (**1-4**) along with evaluation of the anti-eczematic activity of the alcoholic extract of *Rhamnus disperma* roots.

2 Experimental
2.1 General experimental procedures:
UV spectra were determined with a Shimadzu UV-1650PC spectrophotometer; IR spectra were carried out on a Nicolet 205 FT IR spectrometer connected to a Hewlett-Packard Color Pro. Plotter. The ^{1}H-, ^{13}C- and ^{13}C-APT NMR measurements were obtained with a Bruker Avance spectrometer operating at 600 MHz (for ^{1}H) and 150 MHz (for ^{13}C), a Bruker BioSpin AG operating at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C) and a Bruker Avance III NMR spectrometer operating at 400 MHz (for ^{1}H) and 100 MHz (for ^{13}C) in DMSO-d_6 solution, and chemical shifts were expressed in δ (ppm) with reference to TMS, and coupling constant (*J*) in Hertz. EIMS was carried on Scan EIMS-TIC, VG-ZAB-HF, X-mass (158.64, 800.00) mass spectrometer (VG Analytical, Inc.). Si gel (Si gel 60, Merck), was used for open column chromatography. Solid phase extraction was performed on SPE-C_{18} cartridges (Strata columns). TLC was carried out on precoated silica gel 60 F254 (Merck) plates. Developed chromatograms were visualized by spraying with 1% vanillin-H_2SO_4, followed by heating at 100 for 5 min and by ammonia solution.

2.2 Plant material
Rhamnus disperma Ehrenb. roots were collected from Saint Kathrin Protectorate, South Sinai, Egypt in April 2013, and were identified by Dr. Ibraheem Elgarf, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Egypt. A voucher specimen has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

2.3 Extraction and isolation

Air-dried powdered roots (700gm) of *Rhamnus disperma* were subjected to exhaustive extraction with ethanol. The combined ethanolic extracts were concentrated under vacuum to dryness (145gm) and extracted twice with petroleum ether (pp. 40-60°C) for the removal of fatty substances. The defatted crude extract was dissolved in distilled water and partitioned successively with ethyl acetate (20gm) and *n*-butanol (40gm). The ethyl acetate-soluble fraction was chromatographed on Si gel CC and eluted with *n*-hexane-EtOAc (90:10→0:100). The resulting fractions were combined according to their composition to seven fractions (fr. A-G). Fraction A (960mg) was rechromatographed on Si gel CC and eluted with 100% chloroform to give four sub fractions of A-1 to A-4. The sub fractions A-2 (170mg) and A-4 (85mg) were separately subjected to Sephadex LH-20 eluted with MeOH to afford compound (**1**) (30mg) and compound (**2**) (38mg), respectively. Fraction E (800mg) was rechromatographed on Si gel CC and eluted with 100% EtOAc to give two sub fractions of E-1 (390mg) and E-2 (85mg). Sub fraction E-2 was further subjected to Sephadex LH-20 eluted with MeOH to afford compound (**3**) (11mg) and compound (**4**) (5mg), respectively.

Myricetin 3,6,7,3`,4`,5`-*O*-hexamethyl ether (**1**): Pale yellow amorphous powder [MeOH]; UV λ_{max} (MeOH) nm: 266sh, 274, 280sh, 336, λ_{max} (MeONa) nm: 274sh, 278, 291sh, 331, λ_{max} ($AlCl_3$) nm: 266sh, 274, 276sh, 310sh, 365, λ_{max} ($AlCl_3$/HCl) nm: 266sh, 274, 276sh, 305sh, 356, λ_{max} (AcONa) nm: 266sh, 274, 280sh, 336, λ_{max} (AcONa/boric acid) nm: 266sh, 274, 280sh, 336; IR υ_{max} (KBr) cm^{-1}: 3385, 2925, 1650, 1612, 1465, 1193; ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) see table 1; EIMS *m/z* 418 $[M]^+$.

Myricetin 3,6,7,4`,5`-*O*-pentamethyl ether (**2**): Yellow crystals [MeOH]; UV λ_{max} (MeOH) nm: 255sh, 272, 336, λ_{max} (MeONa) nm: 258sh, 266, 331, 376sh, λ_{max} ($AlCl_3$) nm: 259sh, 279, 299sh, 365, 406sh, λ_{max} ($AlCl_3$/HCl) nm: 255, 284, 304sh, 356, 401sh, λ_{max} (AcONa) nm: 255, 272, 336, λ_{max} (AcONa/boric acid) nm: 255, 272, 336; IR υ_{max} (KBr) cm^{-1}: 3387, 2923, 1652, 1611, 1462, 1191; ^{1}H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) see table 1; EIMS *m/z* 404 $[M]^+$.

Quercetin 3-*O*-methyl ether (**3**)**:** Yellow crystals [MeOH]; UV λ_{max} (MeOH) nm: 257, 267sh, 293sh, 357, λ_{max} (MeONa) nm: 274, 330, 406, λ_{max} ($AlCl_3$) nm: 276, 305sh, 335, 441, λ_{max} ($AlCl_3$/HCl) nm: 268, 276sh, 300sh, 360, 401, λ_{max} (AcONa) nm: 274, 321, 385, λ_{max} (AcONa/boric acid) nm: 261, 298sh, 380; IR υ_{max} (KBr) cm^{-1}: 3400, 1646, 1610, 1595. ^{1}H NMR (DMSO-d_6, 600 MHz) δ 12.69 (1H, brs, 5-OH), 7.53 (1H, d, *J*= 2.4 Hz, H-2`), 7.42 (1H, dd, *J*= 8.4, 1.8 Hz, H-6`), 6.89 (1H, d, *J*= 8.4 Hz, H-5`), 6.39 (1H, d, *J*= 1.2 Hz, H-8), 6.17 (1H, d, *J*= 1.2 Hz, H-6), 3.76 (3H, s, 3-$OCH_3$); $^{13}$C NMR (DMSO-$d_6$, 150 MHz) δ 177.83 (C-4), 164.29 (C-7), 161.21 (C-5), 156.30 (C-9), 155.54 (C-2), 148.71 (C-4`), 145.22 (C-3`), 137.59 (C-3), 120.72 (C-1`), 120.54 (C-6`), 115.72 (C-2`), 115.33 (C-5`), 104.05 (C-10), 98.55 (C-6), 93.58 (C-8), 59.63 (3-OCH_3); EIMS *m/z* 316 $[M]^+$.

Quercetin (**4**): Yellow crystals [MeOH]; UV λ_{max} (MeOH) nm: 254, 266sh, 300sh, 369, λ_{max} (MeONa) nm: 248sh, 278, 317, 415, λ_{max} ($AlCl_3$) nm: 274, 304sh, 332, 437, λ_{max} ($AlCl_3$/HCl) nm: 266, 301sh, 360, 402, λ_{max} (AcONa) nm: 256sh, 273, 330, 395, λ_{max} (AcONa/boric acid) nm: 264, 303sh, 388; IR υ_{max} (KBr) cm^{-1}: 3430, 1643, 1620, 1585, 1515. ^{1}H NMR (DMSO-d_6, 500 MHz) δ 12.48 (1H, brs, 5-OH), 10.90-9.42 (4H, brs, OHs), 7.67 (1H, s, H-2`), 7.54 (1H, d, *J*= 8.3 Hz, H-6`), 6.88 (1H, d, *J*= 8.4 Hz, H-5`), 6.40 (1H, s, H-8), 6.18 (1H, s, H-6); EIMS *m/z* 302 $[M]^+$.

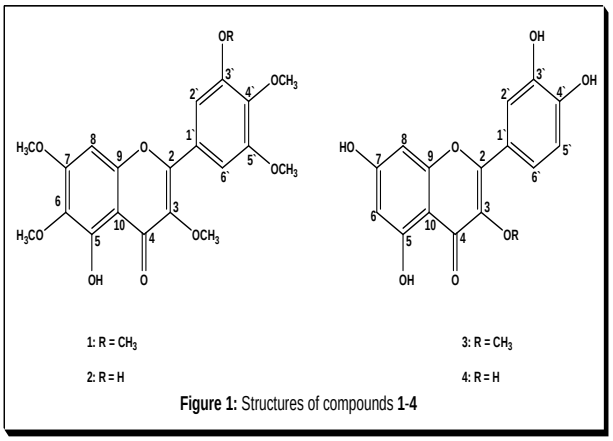


Figure 1: Structures of compounds **1-4**

2.4 Anti-eczematic activity

Healthy white male albino mice weighing between 25-30g were selected for evaluation of the anti-eczematic activity. All animals were housed 6 per cage and kept at the laboratory animal home of the Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt under standard environmental conditions of room temperature at 25 ± 2 °C with 12hr light/dark cycle and had free access to standard diet and water. All animal procedures were performed in accordance with the Ethics Committee of the Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt, and followed the recommendations for the proper care and use of laboratory animals (NIH publication No. 85-23, revised 1985).

All animals were first sensitized by painting the dorsal skin with 100 µL of 2% dinitrochlorobenzene (DNCB) in acetone followed by 100 µL of 0.2% DNCB solution after 4 days to induce eczema [9, 10]. The selected animals were divided into four groups of ten animals each. Group I served as a test sample which treated twice daily with the 1.0% w/w alcoholic extract ointment in Vaseline for 15 consecutive days. Group II served as a positive control treated twice daily with 0.1% w/w betamethazone ointment. Group III was kept as a negative control giving only Vaseline (White soft paraffin). Group IV was kept as an untreated group.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Student's *t*-test. A value of $p < 0.05$ was used to indicate statistically significant differences.

3. Results and discussion

The defatted alcoholic extract of *Rhamnus disperma* roots was suspended in water and successively extracted with ethyl acetate and *n*-butanol. The ethyl acetate-soluble fraction was subjected to repeated CC to afford four flavonoids (**1-4**) (Fig. 1). Occurrence of compounds **1** and **2** in Genus *Rhamnus* are

reported here for the first time while compounds **3** and **4** were isolated previously from the aerial parts of the same plant. Compounds **1** and **2** were isolated previously from the aerial parts of some *Cleome* species [11], but the ^{13}C-NMR data is presently reported for the first time.

Compound (**1**) was obtained as pale yellow amorphous powder and its molecular formula was assigned to be $C_{21}H_{22}O_9$ as indicated from the molecular ion peak observed at *m/z* 418 $[M]^+$ in its EIMS spectrum. Characteristic UV absorption maxima at 274 and 336 nm together confirmed that compound **1** is a flavonoid [12]. The IR spectrum showed absorptions for OH (3385 cm^{-1}), methoxyl (2925 and 1193 cm^{-1}), α, β-unsaturated carbonyl (1650 cm^{-1}) and aromatic (1612 and 1465 cm^{-1}) moieties. The ^{1}H NMR spectrum of **1** displayed a hydrogen-bonded hydroxyl group signal at δ 12.52 (1H, brs, OH-5) and an aromatic singlet at δ 6.95 corresponding to H-8 proton of ring-A indicating that the ring-A was substituted at 6-position. The presence of an aromatic singlet at δ 7.40 integrating for two protons corresponding to H-2` and H-6` protons indicating a 3`,4`,5`-trisubstituted ring-B. The $^1$H NMR spectrum also showed six methoxy groups at δ 3.78 (3H, s, OMe-3), 3.75 (3H, s, OMe-6), 3.94 (3H, s, OMe-7), 3.89 (6H, s, OMe-3`, 5`) and 3.85 (3H, s, OMe-4`). ^{13}C-APT NMR spectral analysis further confirmed the structure of **1**. It displayed 21 carbon signals (Table 1), corresponding to a flavonol derivative with six methoxy groups. Therefore, and by direct comparison with corresponding spectroscopic data published previously [11, 13], compound **1** was identified as Myricetin 3,6,7,3`,4`,5`-*O*-hexamethyl ether.

Compound (**2**) was obtained as yellow crystals and was assigned the molecular formula $C_{20}H_{20}O_9$ by EIMS as indicated from the molecular ion peak observed at *m/z* 404 $[M]^+$. This established that compound **2** has 14 mass units less than that of **1**. The UV and IR absorption bands were very similar to those of **1**. The ^{1}H and ^{13}C-APT NMR spectra of **2** were similar to those of **1** except for those of the ring B, they lacked the signals due to one of the methoxyl groups. The loss of the symmetry of C-2`/C-6` and C-3`/C-5` as indicated from the chemical shift values of ring B carbons (Table 1) and the resulted two broad singlets at δ 7.29 (H-2`) and 7.20 (H-6`) required the absence of the methoxyl group at C-3` position. In addition to the upfield shift of C-3` (δ 151.03) in **2** as compared to the methoxylated C-3` (δ 153.27) in **1** confirmed that the methoxyl group was lost from the C-3` position. Accordingly, compound **2** was identified as myricetin 3,6,7,4`,5`-*O*-pentamethyl ether and is in close agreement with the reported literature [11, 13].

Compound (**3**) was assigned the molecular formula $C_{16}H_{12}O_7$ by EIMS. The ^{1}H and ^{13}C-NMR spectroscopic data of **3** indicated this compound to be a flavonol derivative with one methoxyl group. In the ^{1}H NMR of **3** the presence of an ABX system at 7.53 (1H, d, *J*= 2.4 Hz, H-2`), 7.42 (1H, dd, *J*= 8.4, 1.8 Hz, H-6`), 6.89 (1H, d, *J*= 8.4 Hz, H-5`) together with the two doublets at δ 6.39 and 6.17 with a coupling constant of 1.2 Hz, typical of two *meta*-coupled protons corresponding to H-8 and H-6, respectively, indicated a quercetin skeleton. In addition to one methoxyl signal at δ 3.76 (3H, s, 3-OCH_3) confirmed a quercetin mono methyl ether. The ^{13}C NMR spectrum displayed 16 signals from which one signal is corresponding to the metoxyl group at δ 59.63 (3-OCH_3). The methoxyl group was located at C-3 as indicated from the downfield shift value of C-2 (δ 155.54) as compared to that of quercetin. Thus, compound **3** was identified as quercetin 3-*O*-methyl ether and in good agreement with the reported literatures [14, 15].

Similarly, compound (**4**) is a flavonol with a molecular formula $C_{15}H_{10}O_7$ as indicated from its EIMS. The ^{1}H NMR spectral data of **4** was assigned on the bases of comparison with those of **3** which revealed close correspondence in every aspect except that the signal of the methoxyl group was missing for **4**. The structure of **4** is, therefore, that of quercetin and in good agreement with the reported literatures [14, 15].

Table 1: ^{1}H and ^{13}C-APT NMR data of compounds **1** and **2** (DMSO-*d*$_6$, 400 MHz)

Position	1		2	
	^{1}H	**^{13}C-APT**	**^{1}H**	**^{13}C-APT**
2	-	155.98, C	-	155.70, C
3	-	139.06, C	-	138.92, C
4	-	178.85, C	-	178.80, C
5	-	152.08, C	-	152.08, C
6	-	132.16, C	-	132.07, C
7	-	159.31, C	-	159.21, C
8	6.95, s	92.06, CH	6.82, s	91.77, CH
9	-	152.31, C	-	152.22, C
10	-	106.19, C	-	106.11, C
1`	-	125.90, C	-	125.36, C
2`	7.40, s	106.44, CH	7.29, brs	110.42, CH
3`	-	153.27, C	-	151.03, C
4`	-	140.55, C	-	139.40, C
5`	-	153.27, C	-	153.47, C
6`	7.40, s	106.44, CH	7.20, brs	104.20, CH
5-OH	12.52, brs	-	12.52, brs	-
5`-OH	-	-	9.61	-
3-OMe	3.78, s	60.53, CH_3	3.78, s	60.45, CH_3
6-OMe	3.75, s	60.70, CH_3	3.73, s	60.49, CH_3
7-OMe	3.94, s	60.40, CH_3	3.91, s	60.29, CH_3
3`-OMe	3.89, s	56.61, CH_3	3.87, s	56.37, CH_3
4`-OMe	3.85, s	57.01, CH_3	3.83, s	56.88, CH_3
5`-OMe	3.89, s	56.61, CH_3	-	-

3.1 Anti-eczematic activity

In the present study, we have shown that application of 1.0% w/w of alcoholic extract of *Rhamnus disperma* roots alleviating the DNCB-induced eczema in mice as compared with a negative control. Table 2 demonstrates that, the induced eczema in mice treated with the alcoholic extract was significantly cured with 80% activity in a period ranged between 3-11 days as compared with that treated with 0.1% w/w of betamethazone ointment which gives 70% activity in a period ranged between 5-10 days. It could be concluded that the anti-eczematic activity is due to the constituents of the plant. Among them, flavonoids which increase wound healing due to anti-inflammatory and antioxidant activities, especially the methoxylated flavonoids which have a higher anti-inflammatory activity [16-18].

Table 2: Anti-eczematic activity of the alcoholic extract of *Rhamnus disperma* roots

Days of treatment	Number of treated mice in each group/days			
	Group I: Alc. Extract	Group II: Positive control	Group III: Negative control	Group IV: Untreated
1 st	-	-	-	-
2 nd	-	-	-	-
3 rd	3	-	-	-
4 th	1	-	-	-
5 th	1	3	-	-
6 th	-	-	-	-
7 th	-	2	-	-
8 th	-	1	-	-
9 th	2	-	-	-
10 th	-	1	-	-
11 th	1	-	1	-
12 th	-	-	2	-
13 th	-	-	-	-
14 th	-	-	-	-
15 th	-	-	-	1
N*	8	7	3	1
% of activity	80%	70%	30%	1%

* N= total number of treated mice in each group.

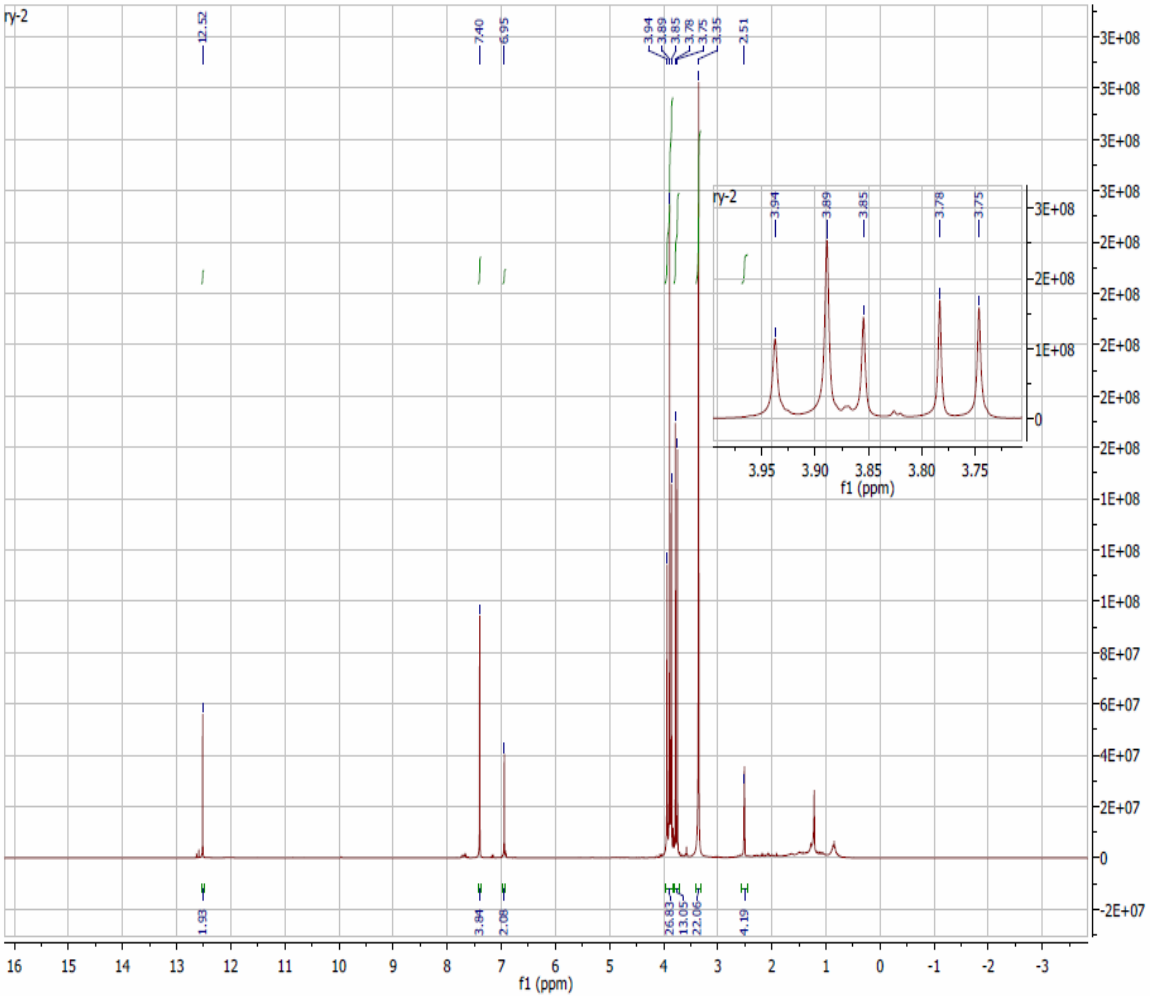
4. Conclusion

In conclusion, the present study has demonstrates that, highly methoxylated flavonoids were isolated for the first time from genus *Rhamnus* and from the roots of *Rhamnus disperma*. The present study also suggests that topical application of 1.0% w/w of alcoholic extract of *Rhamnus disperma* roots could be a potential therapeutic agent for eczema. Further investigations will be carried out to evaluate the potential anti-eczematic activity of the different fractions and isolated components.

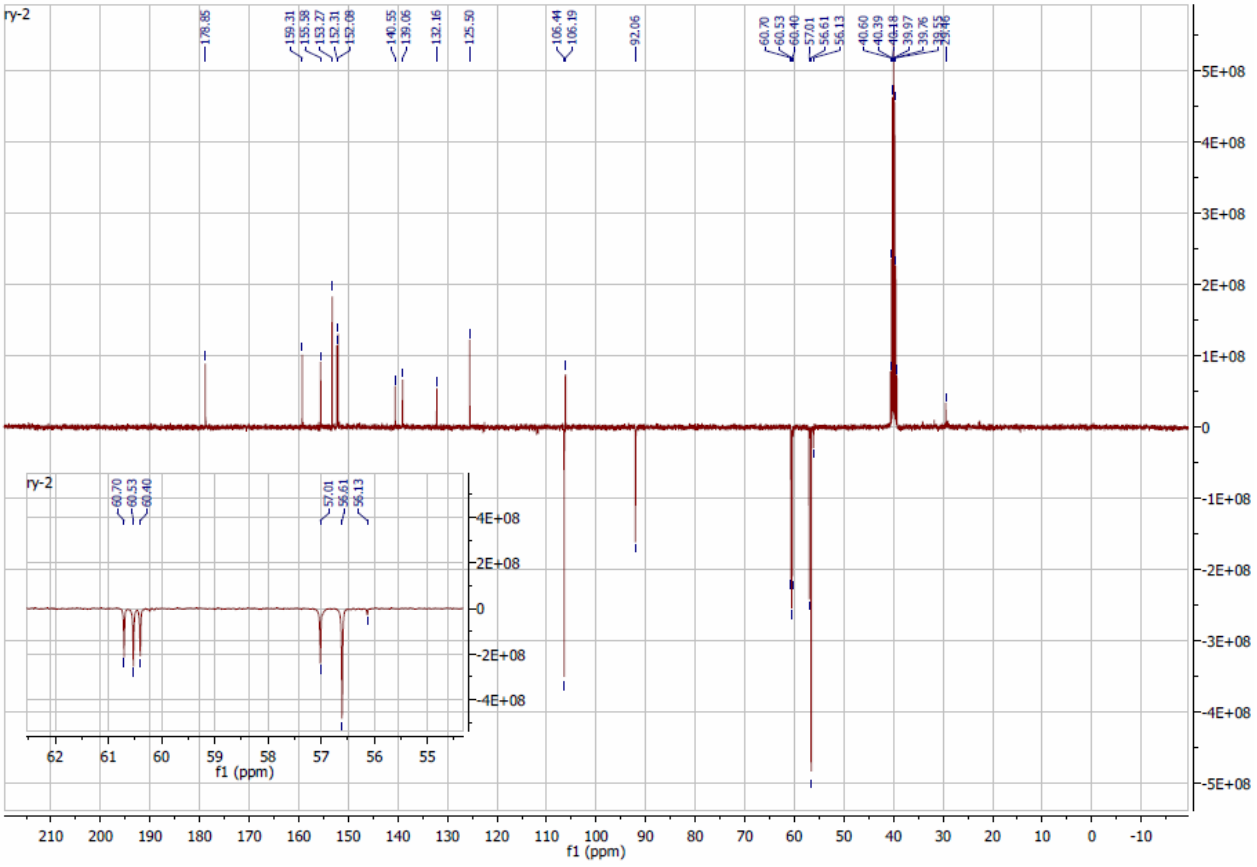
5. Acknowledgment

Thanks are due to Ahmed elsadeq, environmental researcher at South Sinai Protectorate, Saint Kathrin Protectorate, for supplying the plant material. Many thanks are due to Dr. Ahmed Mansour, Assoc. Prof. of Pharmacology, Faculty of Pharmacy, Pharmacology Department, Al-Azhar University, Cairo, Egypt, for supporting the anti-eczematic activity.

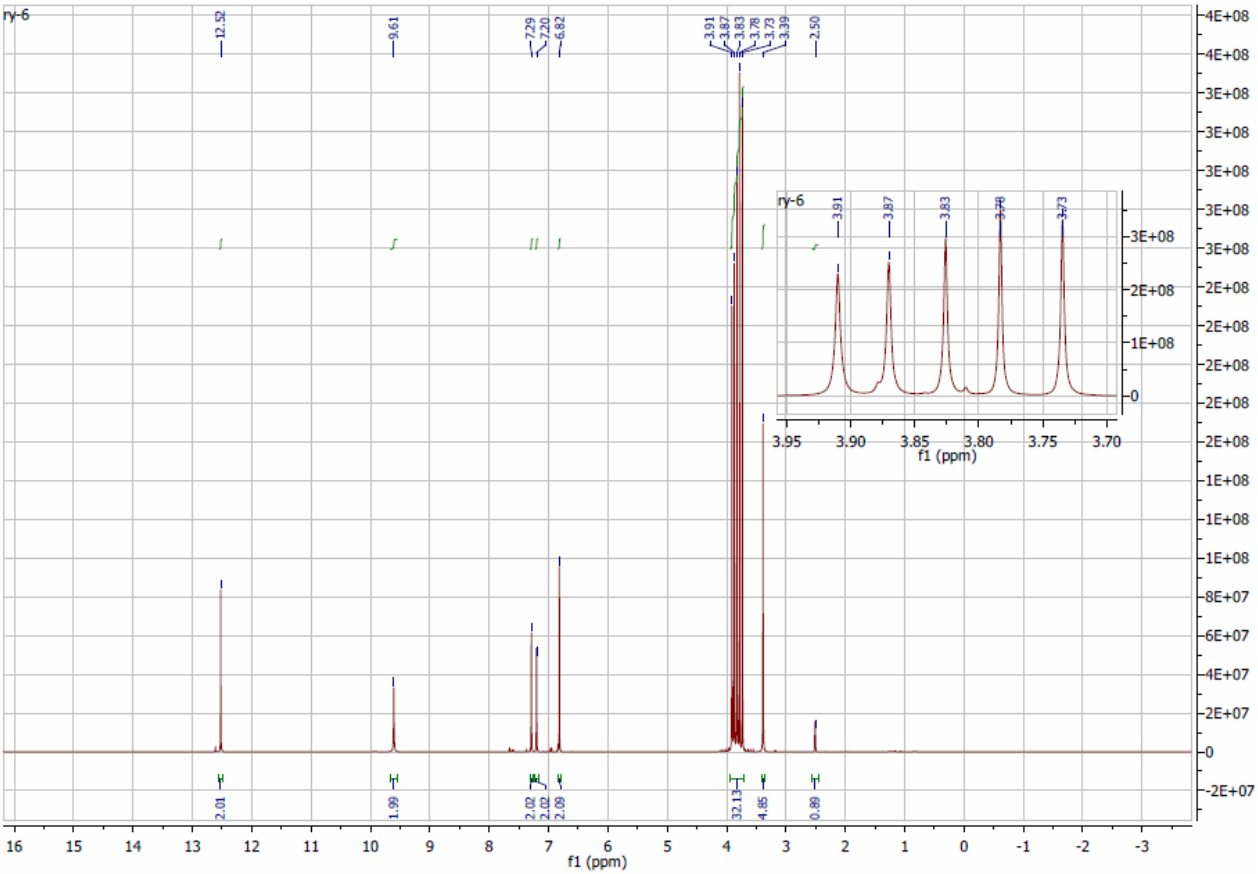
Supplementary material



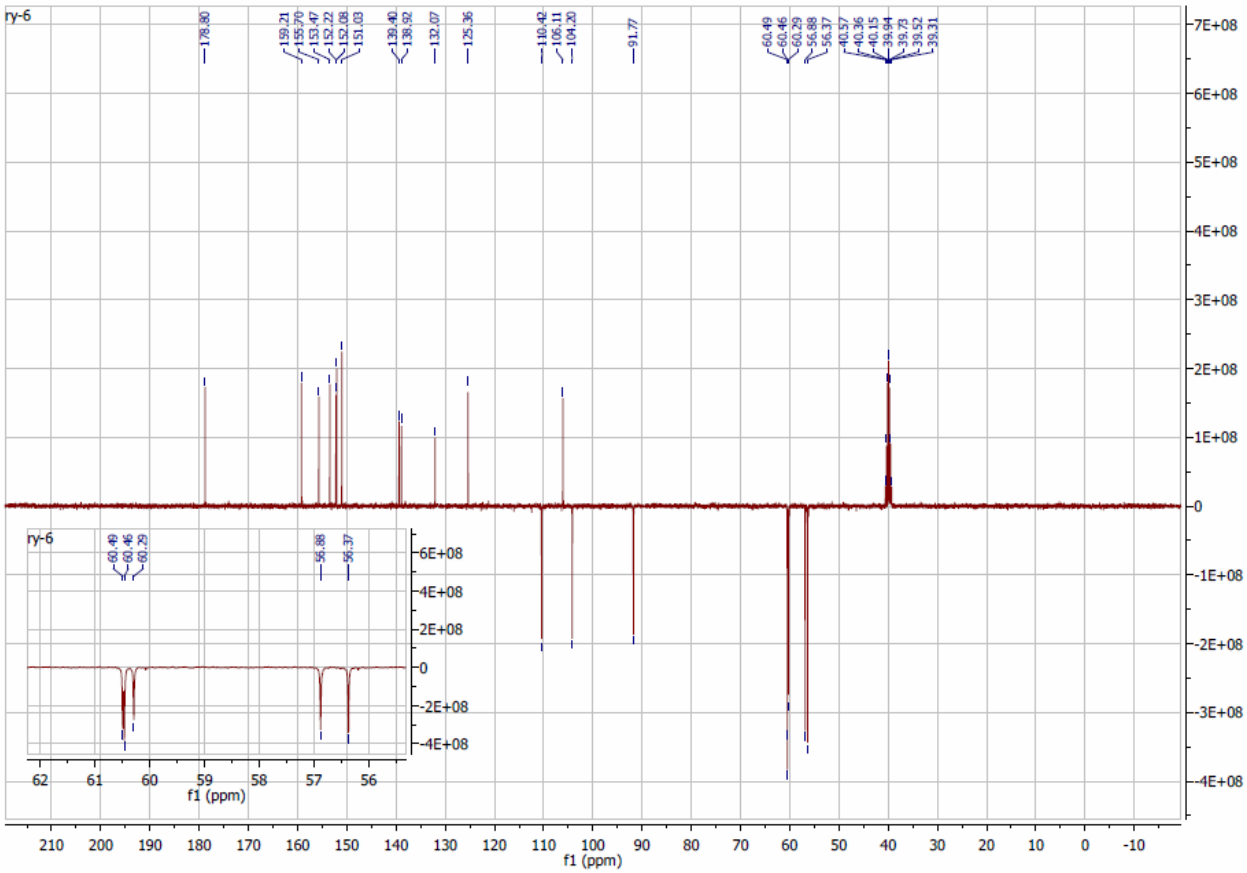
^{1}H NMR of compound (**1**)



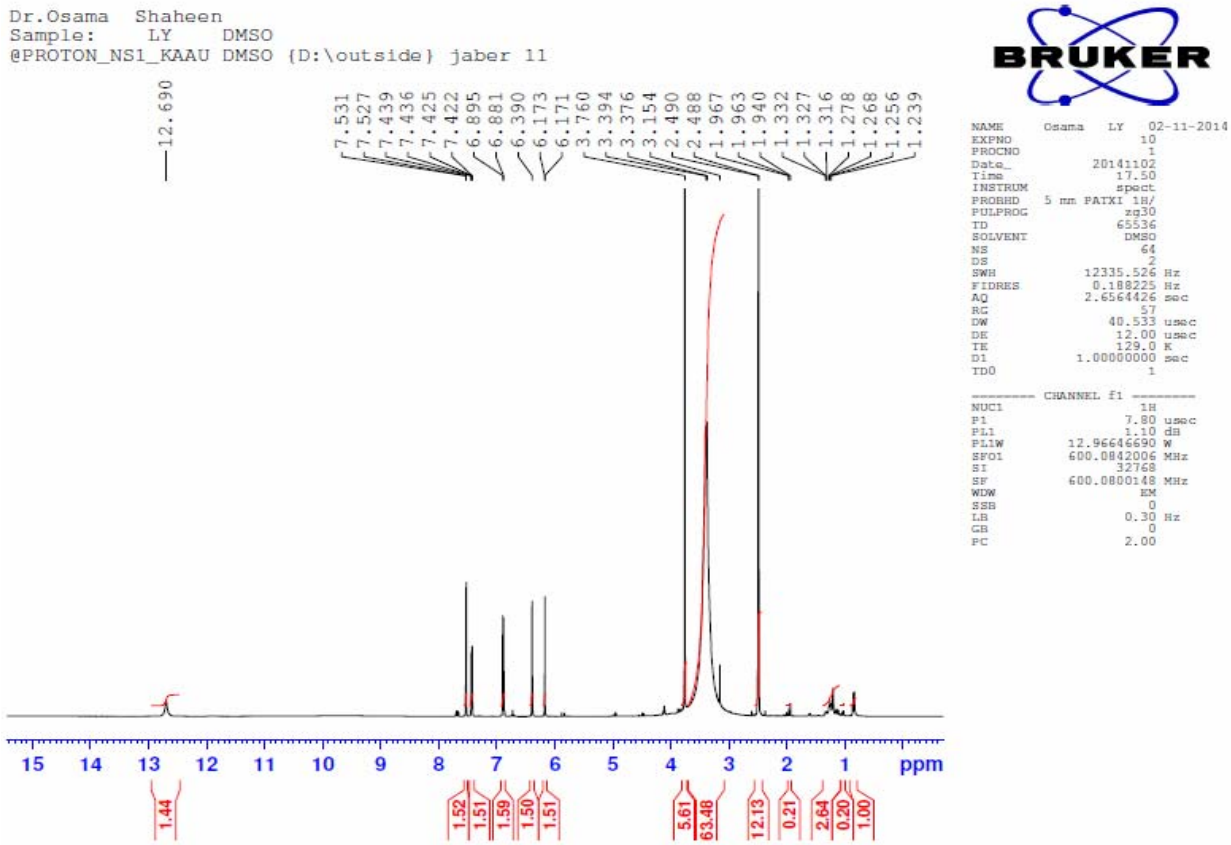
^{13}C-APT NMR of compound (**1**)



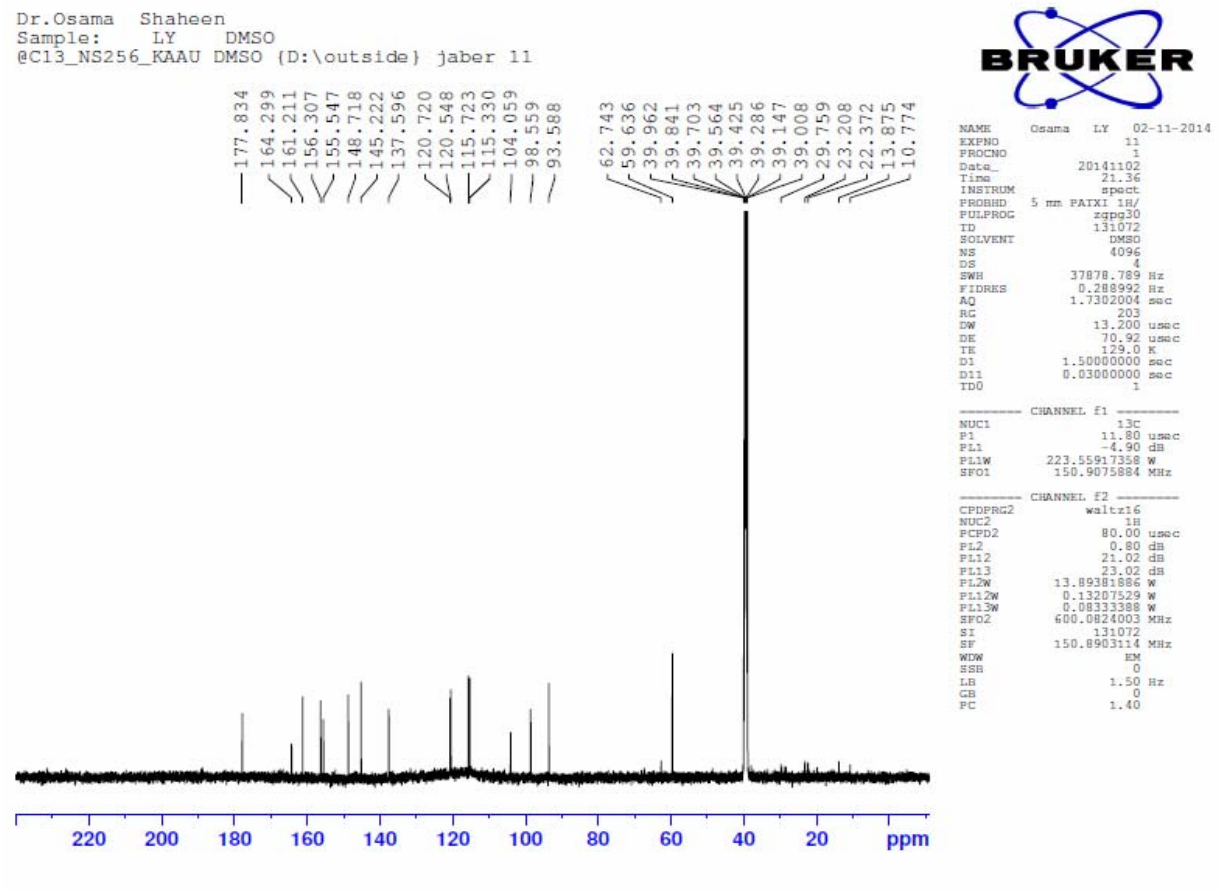
^{1}H NMR of compound (**2**)



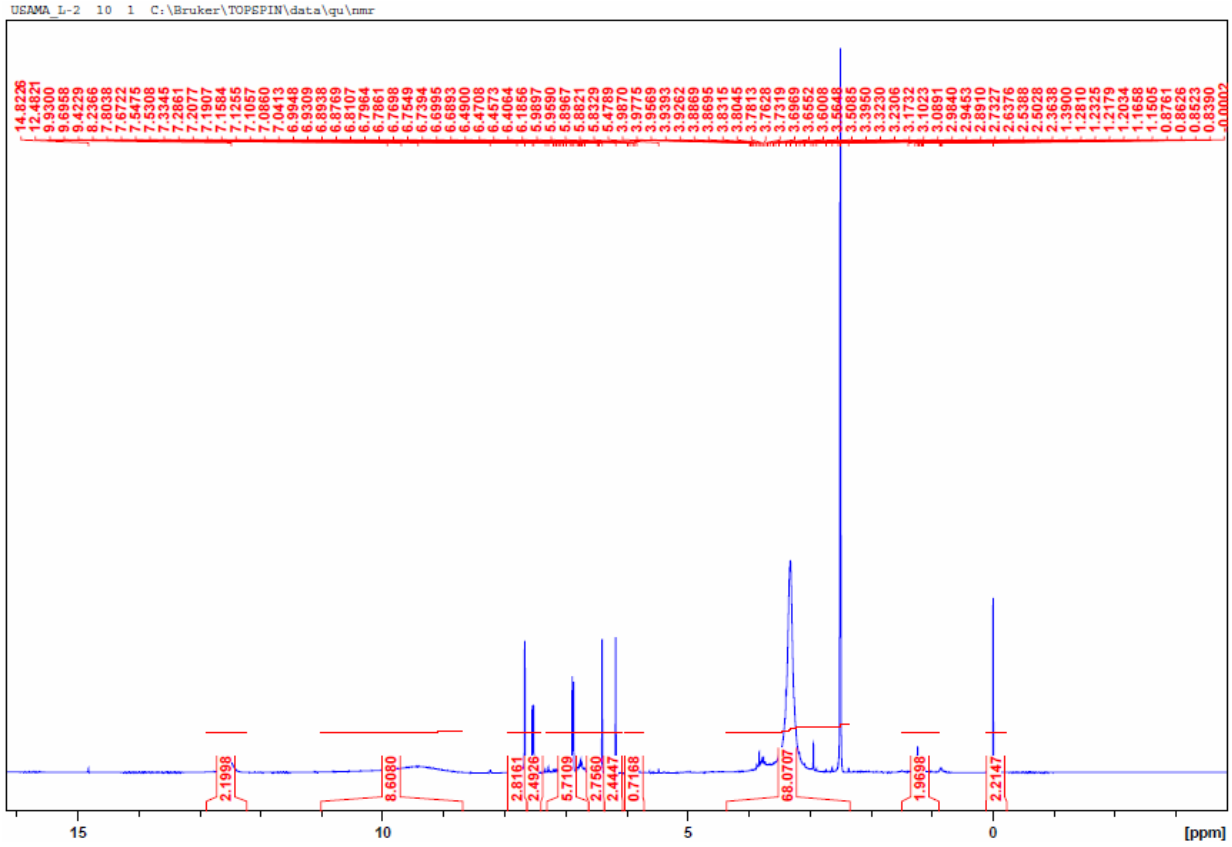
^{13}C-APT NMR of compound (**2**)



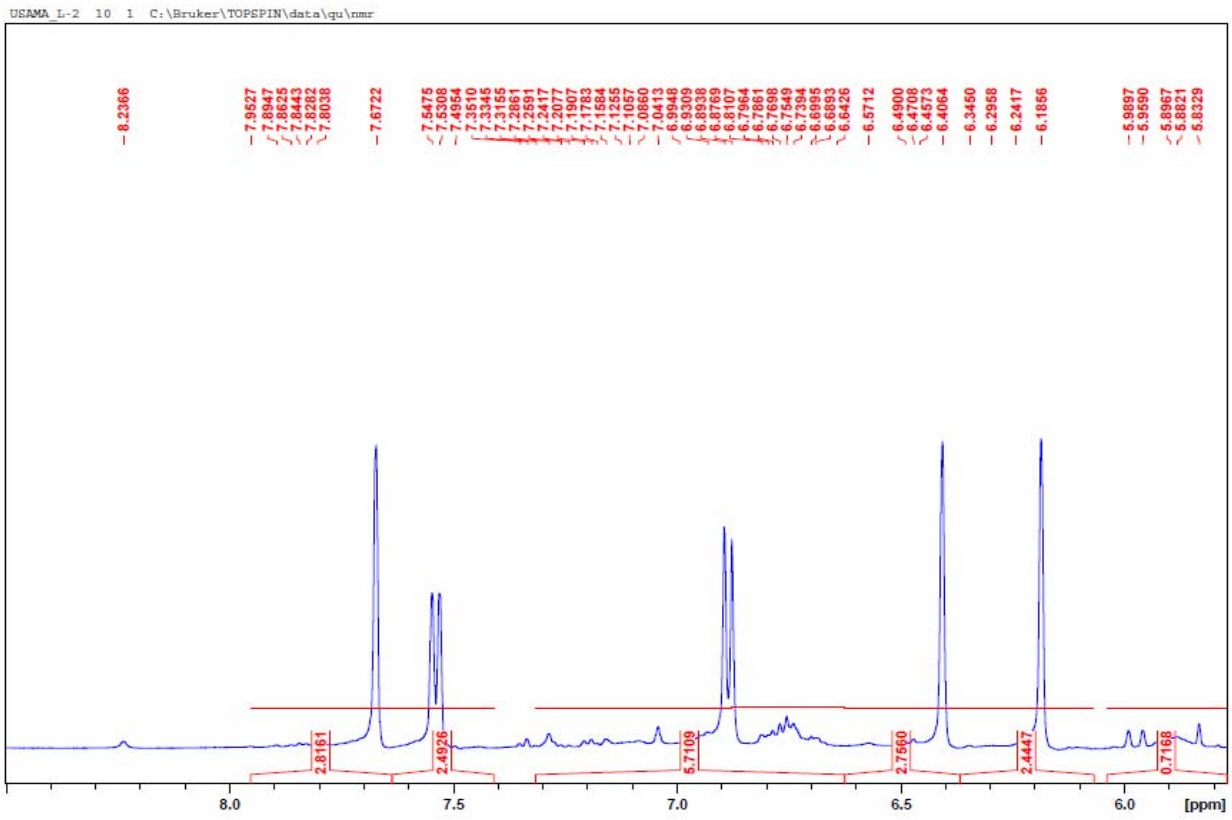
^{1}H NMR of compound (**3**)



^{13}C-NMR of compound (**3**)



^{1}H NMR of compound (**4**)



[1]H NMR of compound (**4**) (expanded aromatic region)

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