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Isoflavones and Fatty Acids from *Crotalaria aegyptiacea*

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The methanolic extract of *Crotalaria aegyptiaca* was concentrated and then extracted with chloroform. The ethyl acetate extract from chloroform residue was subjected to chromatographic techniques that revealed the presence of genistein, genistin and 5,7,4′-trihydroxy-6,8-diprenyl isoflavone. The fatty acids detected are capric acid, lauric acid, arachedic acid and myrisitic acid. The major hydrocarbon was proved to be squalene (36.24 %), while the major sterol was cholesterol (3.20 %).

Key Words: *Crotalaria aegyptiacea*, Isoflavone, Genistein, Genistin, 5,7,4′-Trihydroxy-6,8-diprenyl isoflavone, Capric acid, Lauric acid, Arachedic acid, Myrisitic acid, Cholesterol.

INTRODUCTION

The genus *Crotalaria* comprises about 550 species, distributed mainly in the temperate or tropical regions[1]. The genus *Crotalaria* represented in Egypt by 5 species[2]. *Crotalaria aegyptiacea* is shruby dwarf plant commonly found in Egyptian desert at kilo 101 Suez route. The plant is growing during the period of February to March[3]. *Crotalaria aegypteaca* was reported to be having antioxidant[4], antiinflammatory[5] and analgesic activity[6].

Preliminary phytochemical investigation of *Crotalaria aegyptiaca* revealed the presence of carbohydrates and/or glycosides, flavonoids, alkaloids and/or nitrogenous bases, saponins and sterols and/or triterpenes.

EXPERIMENTAL

^{1}H NMR (300 MHz, CD_3OD). ^{13}CNMR (125 MHz) spectra were recorded on a Bruker 300 MHz, TLC: percolated silica gel type 60 (Merck). Gas chromatograph was performed on Hewlett Packard Model 5890 and GC-14A Shimadzu.

Crotalaria aegyptiaca was collected from Suez Rout, Egypt. During the February 2002, it was identified by Dr. Mohamed Elmessery, Faculty of Science, Al-Azhar University, Cairo, Egypt. The collected sample of *Crotalaria aegyptiaca* was air-dried and kept in tightly closed containers.

Extraction and isolation: The air dried powder of *C. aegyptiaca* (2 Kg) was defatted with petroleum ether 2.5 L. (60-80 °C), then the defatted plant material exhaustively extracted with 70 % methanol. The methanolic extract was evaporated to dryness under reduced pressure. The residue was suspended in water and extracted with chloroform till exhaustion.

Chloroformic extract was evaporated to dryness under reduced pressure. The residue was suspended in water and then extracted with ethyl acetate till exhaustion, which was evaporated to dryness.

The ethyl acetate residue was fractionated on silica gel column with chloroform and then chloroform:methanol (gradient elution). The collected fraction (200 mL each) were monitored by TLC silica gel using solvent system, composed of chloroform:methanol (90:10), chloroform:methanol (85:15) and chloroform:methanol:water (75:23:2). The collected fractions were also examined on paper chromatography with solvent systems 15 % acetic acid and B.A.W. (*n*-butanol:acetic acid:water. 4:1:5).

The fractions 1 to 25 eluted from the column were collected and evaporated under vacuum and the residue was subjected to silica gel column and eluted by *n*-hexane-acetone (2:1).

Purification by repeated column chromatography and preparative thin layer chromatography using silica gel and solvent system *n*-hexane:acetone (2:1) result in isolation of compound **3** at R_f 0.39.

Fractions from 26 to 80 were collected and evaporated to dryness. Purification of the residue was carried out by repeated column chromatography and preparative thin layer chromatography using silica gel and solvent system *n*-hexane:ethylacetate (3:2). The compounds **1** and **2** were isolated at R_f values 0.30 and 0.63, respectively.

Acid hydrolysis of compound **1**, yielded the same compound. Acid hydrolysis of compound **2** revealed the presence of compound **1** in the chloroformic extract and glucose in the aqueous layer which was detected by comparison against standard monosaccharide's using paper chromatography with solvent system B.A.W. (*n*-butanol:acetic acid:water. 4:1:5) and sprayed with aniline hydrogen phthalate reagent[7].

Compound 1: The compound **1** was obtained as yellow crystals. The UV spectra indicated that compound **1** has λ_{max} of band II at 261 nm which is indicative to its isoflavone nature (259-268 nm). The compound exhibited dull blue colour on TLC silica gel plates[8,9] at UV-356 nm.

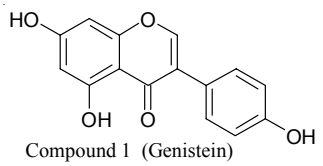
^{1}H NMR (CD_3OD) at 300 MHz δ: 8.04 (s, H-2), 6.21 (d, *J* = 2.1 Hz, H-6), 6.33 (d, *J* = 2.1 Hz, H-8), 7.36 (d, *J* = 8.5 Hz, H-2`),6.86 (d, *J* = 8.5 Hz, H-3`), 6.84 (d, *J* = 8.5 Hz, H-5`), 7.38 (d, *J* = 8.5 Hz, H-6`). ^{13}C NMR (CD_3OD, 100 MHz) 152.5 (C-2) 122.9 (C-3), 186.8 (C-4), 161.2 (C-5), 100.3 (C-6), 163.4 (C-7), 94.9 (C-8), 155.1(C-9/4`), 105 (C-10), 122 (C-1`), 131.41 (C-2`/6`), 116.3 (C-3`/5`).

Compound 2: It was obtained as dark yellow amorphous powder. The UV spectra indicated that compound **2** has λ_{max} of band II at 261 nm which is indicative to its isoflavone nature (259-268 nm). The compound exhibited dull blue colour on TLC silica gel plates[8,9] at UV-356 nm.

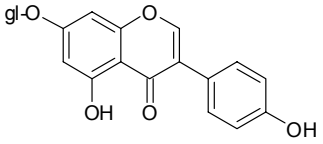
^{1}H NMR (CD_3OD) at 300 MHz, δ 5.08 (d, *J* = 16 Hz, H-1 for glucose), 8.04 (H-2), 6.46 (H-6), 6.63 (H-8), 7.36 (H-2`), 6.86 (H-3`), 6.84 (H-5`), 6.38 (H-6`).

Compound 3: It was obtained as pale yellow amorphous powder. The UV spectra indicated that compound **3** has λ_{max} of band II at 261 nm which is indicative to its isoflavone nature (259-268 nm). The compound exhibited dull blue colour on TLC silica gel plates[8,9] at UV-356 nm.

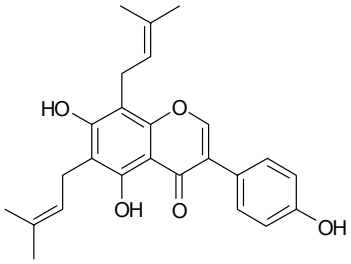
^{1}H NMR (CD_3OD) at 300 MHz, δ 8.18 (1H, s, H-2), 7.63 (2H, d, *J* = 8 Hz, H-2`/H-6`), 7.13 (2H, d, *J* = 8 Hz, H-3`/H-5`), 5.40 (2H, m, 2 protons vinyl), 3.80 (4H, m, 4 protons methylene), 1.89 (4 CH_3, s, 4 methyl groups). ^{13}C NMR at 100 MHz, 144.1 (C-1), 116.4 (C-2), 184.5 (C-3), 104.2 (C-4), 161.7 (C-5), 106.8 (C-6), 169.3 (C-7), 106.8 (C-8), 161.7 (C-9), 129.5 (C-1`/2`/5`), 114 (C-3`/4`), 135.5 (C-5`), 14.2 (C-1``), 116 (C-2``), 133.6 (C-3``), 21.1 (C-4``), 25 (C-5``), 11.9 (C-1```), 116 (C-2```), 133.6 (C-3```), 21.1 (C-4```), 25 (C-5```).



Compound 1 (Genistein)



Compound 2 (Genistin)



Compound 3: 5,7,4′-Trihydroxy-6,8-diprenyl isoflavone (6,8-diprenylgenistein)

Preparation of unsaponifiable matter and fatty acid: The air-dried powder of *Crotalaria aegyptiaca* was exhaustively extracted with petroleum ether (60-80 ºC). The petroleum ether was evaporated to dryness under vacuum. The extract was saponified by 10 % alcoholic potassium hydroxide under reflux[10,11]. After distillation of ethanol and dilution with water, the

unsaponifiable fraction was extracted with ether and washed with water. The residue left after evaporation of ether was weighed and kept for further study. The aqueous mother liquor was acidified with 10 % hydrochloric acid and the liberated fatty acids were extracted with ether and washed with water. The residue left after evaporation of ether was weighed and kept for further study (total fatty acids).

The yields of lipoidal matter as well as the percentage of unsaponifiable matter (USM) and total fatty acids (TFA) are listed in table.

Gas liquid chromatography of unsaponifiable matter: Tentative identification of hydrocarbons and sterols content of unsaponifiable matter fraction of *Crotalaria aegyptiaca* was carried out by GLC comparison of their retention times and co-injection with the available reference compounds. Quantification was based on peak area integration.

Table-1 shows the relative retention times of separated compounds as well as their percentage.

TABLE-1
GLC ANALYSIS OF HYDROCARBONS AND STEROLS IN UNSAPONIFIABLE MATTER FRACTION OF *Crotalaria aegyptiaca*

Identified compounds	RRT*	Concentration (%)
n-Pentadecane (C-15)	0.453	6.572
n-Eicosane (C-20)	0.608	1.236
n-Heneicosane (C-21)	0.648	4.622
n-Hexacosane (C-25)	0.899	0.356
n-Octacosane (C-28)	0.948	2.958
Squalene (C-30)	1.000	36.238
Cholesterol	1.177	3.200

RRT* = Relative retention time of squalene = 1, with retention time = 22.183.

Gas liquid chromatography of fatty acids: The fatty acids fraction of *Crotalaria aegyptiaca* was subjected to methylation and its methyl esters were analyzed adopting GLC conditions.

Identification of fatty acid methyl esters were tentatively identified by comparison of their retention times listed in Table-2.

RESULTS AND DISCUSSION

Dried whole plant of *C. aegyptiaca* (2 Kg) was extracted with 70 % methanol. The ethyl acetate extract from chloroform residue was subjected to silica gel column chromatography. By increasing portion of methanol in chloroform, three compounds were obtained.

TABLE-2
GLC ANALYSIS OF FATTY ACID METHYL ESTERS
FRACTION OF *Crotalaria aegyptiaca*

Identified compounds	RRT*	Concentration (%)
Capric acid ($C_{10:0}$)	0.502	1.095
Lauric acid ($C_{12:0}$)	0.586	9.156
Tridecylic acid ($C_{13:0}$)	0.632	0.822
Myrestic acid ($C_{14:0}$)	0.694	3.220
Pentacylic acid ($C_{15:0}$)	0.755	3.201
Stearic acid ($C_{18:0}$)	1.000	0.587
Arachedic acid ($C_{20:0}$)	1.300	17.770
Identified fatty acid (%)	–	35.851
Unidentified fatty acid (%)	–	64.149

RRT* = Relative retention time of stearic acid =1, with retention time = 19.617.

The isolated compounds (**1-3**) are subjected to acidic hydrolysis. The aqueous layer obtained after acidic hydrolysis were examined on TLC against authentic monosaccharides, only compound **2** showed a spot matched on TLC with that of authentic glucose and the chloroform extract in case of compound **1** and **3** containing the same compound before hydrolysis. So compound **1** and **3** are free aglycone.

The ethylacetate fraction obtained from acidic hydrolysis of compound **2** was compared with compound **1** and **3**. It was observed that the ethylacetate content is matched with compound **1**.

Compound 1: The bathochromic shift with $AlCl_3$ and the stable complex with $AlCl_3$/HCl shift reagent accompanied by bathochromic shift with $AlCl_3$ revealed the presence of free OH group at position 5 and the bathochromic shift in band II with NaOAc shift reagent indicate the presence of free OH group at position 7. UV spectra indicated that compound is an isoflavone with free hydroxyl groups at 5, 7 and 4` positions. Acid hydrolysis for compound **1** indicated free agylcone without any sugar moieties.

^{1}H NMR and ^{13}C NMR spectra revealed the presence of a singlet at δ 8.04 integrated for one proton at position 2 which is characteristic for isoflavone. From the spectrum, two doublet at δ 6.21 and δ 6.33 with meta coupling (d, J = 2.1 Hz) integrated for one proton at position 6 and 8 respectively, with free hydroxyl groups at 5 and 7. Protons at 2`, 6`and 3`, 5` were detected from spectrum at δ 7.38 and δ 6.86, respectively. From the previous data and from the acid hydrolysis for compound **1** which prove the free aglycone nature of compound **1** without any sugar moieties and by comparing ^{1}H NMR and ^{13}C NMR data with previously published data of related compounds[12]. Compound **1** was identified as genistein.

Compound 2: The bathochromic shift with $AlCl_3$ and the stable complex with $AlCl_3$/HCl shift reagent accompanied by bathochromic shift with $AlCl_3$ revealed the presence of free OH group at position 5. The absence of free hydroxyl group at 7-position was observed in lack of shift of band II in presence of NaOAc shift reagent. UV spectra indicated that compound **2** is an isoflavone with free hydroxyl groups at 5 and 7 positions. ^{1}H NMR spectra revealed the presence of a singlet at δ 8.04 integrated for one proton which is characteristic for free proton at position 2 of isoflavone nucleus.

From the spectrum, δ 6.46 and δ 6.63 with meta coupling (d, J = 2.5 Hz) integrated for one proton at position 6 and 8 with free hydroxyl group at position 5, protons at 2`, 6`and 3`, 5` were detected from spectrum at δ 7.36 and δ 6.86, respectively.

The anomeric proton for glucose appeared as doublet at δ 5.08 (d, J = 16 Hz) indicating the β configuration of the glucose unit and the other protons of glucose appear between δ 3.4 to 4.2.

Acid hydrolysis of compound **2** revealed the presence of genistein in the chloroformic extract and glucose in the aqueous layer which was detected by comparison with authentic samples on paper chromatography with solvent system BAW (*n*-butanol:acetic acid:water. 4:1:5) and sprayed with aniline hydrogen phthalate reagent[7]. Compound **2** was identified as genistin.

Compound 3: The bathochromic shift with $AlCl_3$ and the stable complex with $AlCl_3$/HCl shift reagent accompanied by bathochromic shift with $AlCl_3$ revealed the presence of free OH group at position 5 and the bathochromic shift in band II with NaOAc shift reagent indicate the presence of free OH group at position 7. UV spectra indicated that compound **3** is an isoflavone with free hydroxyl groups at 5, 7 and 4` positions and diprenyl groups at 6 and 8 positions.

From ^{1}H NMR the spectrum, protons 2`, 6` and 3`, 5` were detected from spectrum at δ 7.63 and δ 7.13 respectively. Four methyl groups were appeared at δ 1.89 and four methylene protons appear at δ 3.80. Vinyl protons were appeared at δ 5.40.

From the previous data and from the acid hydrolysis for compound **3** which prove the free aglycone nature of compound **3** without any sugar moieties and by comparing ^{1}H NMR and ^{13}C NMR data with previously published data of 6,8-diprenylgenistein[13,14] express that they are identical.

Compound **3** was identified as: 5,7,4`-trihydroxy-6,8-diprenyl isoflavone. (6,8- diprenylgenistein). It is worthy to mention that compounds **1**, **2** and **3**, were isolated from *Crotalaria aegyptiaca* for the first time. The percentage of total lipoidal matter is (3 %) in *Crotalaria aegyptiaca* and the percentage of USM and TFA which were found to be (95.2 %) and (1.5 %), respectively in the lipoidal matter of *Crotalaria aegyptiaca*.

The percentage of hydrocarbon and sterols were found to be (91.09 %) and (3.20 %) respectively in the unsaponifiable matter. The major hydrocarbon was proved to be squalene (36.24 %), while the major sterol was cholesterol (3.20 %).

Regarding the fatty acid, it was observed that the % of unsaturated fatty acids was much lower than that of saturated fatty acids which were found to be 3.24 and 82.07 %, respectively in the TFA of *Crotalaria aegyptiaca*.

Myristic acid (C-14) was the main saturated fatty acid being (46.089 %) followed by arachedic (C-20) acid being (17.77 %) in the TFA of *C. aegyptiaca* and finally eicosapentaenoic acid (C20:5) was the main unsaturated fatty acid being (2.876 %) in the TFA of *C. aegyptiaca*. It was found that capric acid, lauric acid and arachedic acid not identified before in *Crotalaria aegyptiaca*.

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