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GALLOTANNIN AND FLAVONOID GLYCOSIDES FROM THE STEM BARK OF *ACER NEGUNDO* (L.)

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تم فى هذا البحث تحضير خلاصة الكحول الميثيلى لقلف ساق نبات الأسر نيجوندو إل ثم تجزئتها بين مذيبات الهكسان ، خلات الايثيل والبيوتانول وبعد ذلك تم فحص وفصل مكونات خلاصة خلات الايثيل والبيوتانول كل على حده باسنخدام كروماتوجرافيا العمود ، طرق الفصل والتنقية المختلفة. وقد تمت دراسة الصفات الطبيعية والكيميائية للمركبات المفصولة باستخدام مطياف الأشعة فوق البنفسجية ودون الحمراء ومطياف الكتلة والرنين النووى المغناطيسى لأنوية الهيدروجين والكربون وأمكن التعرف على حامض الجاليك (١)، ١و٢ ثنائى جالويل ٠أ بيتا دى جلوكوبيرانوزيد (٢) ، كامبيفيرول ٣ ٠أ بيتا دى جلوكوبيرانوزيد (٣) ، كوارستين ٣ ٠أ بيتا دى جلوكوبيرانوزيد (٤) ، جالات الميثيل (٥) ، جالات الايثيل (٦) من خلاصة خلات الايثيل بينما أمكن التعرف على أيزورامنتين ٣ ٠ا روتينوزيد (٧) ، كوارستين ٣ ٠ا روتينوزيد (٨) من خلاصة البيوتانول.

*Gallic acid (**1**), 1,2-di-O-galloyl-β-D-glucopyranoside (**2**), kaempferol-3-O-β-D-glucopyranoside (**3**), quercetin-3-O-β-D-glucopyranoside (**4**) methyl gallate (**5**), ethyl gallate (**6**), isorhamnetin-3-O-rutinoside (**7**) and quercetin-3-O-rutinoside (**8**) were isolated for the first time from the methanolic extract of the stem bark of Acer negundo (L.). Identification of these compounds has been established by physical, chemical and spectral evidence (UV, IR, FAB-MS, ^{1}H- and ^{13}C-NMR).*

INTRODUCTION

Acer negundo (L.), Boxelder, Ash-leaved Maple[1] (Maple Family,[2] Aceraceae) is a tree that may reach about 1.5-2.0 meter in height. The leaves are opposite, pinnately compound, 3 to 5 (7 to 9) leaflets, ovate to oblong-lanceolate, coarsely serrate or terminal one lobed and their colour is bright green above, glabrous and lighter green beneath.[1] The flowers are dioecious, yellowish green. The fruit is samara, matures in September to October.[1] The genus *Acer* is cultivated for ornamental purposes.[2] The bark is prescribed as an astringent, for hepatic disorders and as eye wash.[3,4]

Several constituents have been isolated from plants of genus *Acer* such as anthocyanins, diacylated anthocyanins, galloylglucoside, galloylcyanidin glycosides,[5,6] phenolic hydrolysable tannins (1-O-galloyl-α-L-rhamnose, 1-O-galloyl-β-D-glucose),[7] diaryl-heptanoid acerogenin A-E, their glycosides as well as B-orcinol derivatives.[4,8,9] Flavonoids myricetin, luteolin glucosides, kaempferol, quercetin, acylated flavonols and biflavonoids were isolated from the leaves of different *Acer* species.[10-14]

In this study, we report the isolation and identification of gallotannin and flavonoid glycosides from the stem bark of the titled plant.

EXPERIMENTAL

General experimental procedures

1- Melting points are uncorrected and were determined by Stuart Scientific melting point SMP1 instrument (England).
2- UV spectra are measured in methanol using an Uvidec-320 (Jasco, Tokyo, Japan) spectrophotometer with matched 1 cm quartz cells.
3- ^{1}H- and ^{13}C-NMR spectra were measured in DMSO-d_6 and CD_3OD at 500 MHz by Bruker Avance spectrometer, at 400 MHz by JEOL TNM-LA400, FT NMR system, at 300 MHz by XL-300 Varian (Germany) and JEOL JNM-EX300 spectrometers (Japan) using TMS as internal standard.

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4- FAB-MS was measured by JEOL, JMS 600 H (Japan).
5- UV-lamp (254, 366 nm) VI, 6 LC Marine Lavalee-Cedex France.
6- Column chromatography was performed with silica gel (E. Merck, Germany), Develosil Lop ODS (30-50 μ, Nomura chemicals) and sephadex LH-20 (Pharmacia Biotech., AB, Upsala, Sweden).
7- TLC was performed with precoated silica gel 60 F_{254}, and RP-18 F_{254} (E. Merck, Germany).
8- Authentic samples were obtained from Department of Pharmacognosy, Faculty of Pharmacy, Assiut University.
9- Visualization:
Spots were visualized under UV and by spraying with 2.0% ferric chloride solution.
10- Solvent systems:

Solvent systems used for silica gel TLC screening:

I- Chloroform - methanol (90:10)
II- Chloroform - methanol - water (65:30:5)
III- Chloroform - methanol - water (75:22:3)
IV- n-Butanol - acetone - formic acid - water (60:17:8:15)

Solvent systems used for RP-18 TLC:

I- Water - methanol (40:10)
II- Water - methanol (30:20)

Plant material

The stem bark of *Acer negundo* (L.) was collected from the Faculty of Agriculture, Assiut University in May 2001 and identified by Prof. Dr. Gamal Taha, Department of Horticulture, Faculty of Agriculture, Assiut University.

Extraction and isolation

The air-dried powdered stem bark (1.5 Kg) of *Acer negundo* (L.) was extracted with methanol (5 liters) at room temperature by maceration and concentrated under vaccum. The concentrated extract (130 g) was diluted with distilled water and then subjected to solvent fractionation with n-hexane (4x500 ml), ethyl acetate (5x500 ml) and n-butanol (3x500 ml). The obtained fractions were separately concentrated under vaccum to solvent free residue (30, 60 and 30 g respectively) and examined for different constituents by silica gel TLC (systems I and II).

Isolation

A- Ethyl acetate fraction

About 10 g of ethyl acetate soluble fraction was chromatographed on silica gel column (300 g, 5x120 cm). Elution was started with chloroform followed by chloroform-methanol gradient. Fractions of 250 ml were collected, they are monitored by TLC and similar fractions were combined together. Fractions eluted with chloroform-methanol (80:20) were concentrated under vaccum (4.0 g), rechromatographed on ODS column (400 g, 5x120 cm) and eluted with methanol-water (10:20) to afford fractions I, II, III, it was then eluted with methanol-water (10:10) to afford fraction IV. Fraction I was rechromatographed on ODS column, it was eluted with methanol-water (10:40) to afford compound **1**. Repeated purification of fraction II on sephadex LH-20 and then on ODS column, eluted with methanol- water (10:20 and 10:10) yielded compound **2**. Fraction III was rechromatographed on sephadex LH-20 eluted with methanol to give compounds **3** and **4**. By repeated purification of fraction VI on sephadex LH-20 and then on ODS column, eluted with methanol-water (10:10), it yielded compounds **5** and **6**.

B- n-Butanol fraction

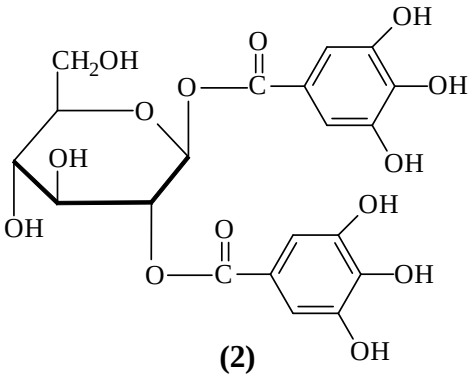
About 5 g of n-butanol soluble fraction was chromatographed on silica gel column (150 g, 5x120 cm). Elution started with ethyl acetate followed by ethyl acetate-methanol gradient. Fractions of 200 ml were collected and screened by TLC. Fractions eluted with ethyl acetate-methanol (80:20) were rechromatographed on sephadex LH-20 column with methanol to afford compounds **7** and **8** which were then purified by preparative silica gel plates 60 F_{254} and solvent system II.

Acid hydrolysis

Five-mg portion of each of the isolated glycosides was dissolved in 5 ml methanol to which 5 ml of 5% hydrochloric acid is added. The mixture was refluxed for 3 hours on a boiling water-bath, then cooled. The aglycone was extracted with chloroform, purified and subjected to TLC. The produced sugars were identified on silica gel TLC with solvent system IV.

Gallic acid (1): White crystalline needles [methanol], m.p 250-252°, (200 mg), R_f = 0.25 (system III), IR, ν, cm^{-1} (KBr disc), 3400, 1694, 1615.

1,2-Di-O-galloyl-β-D-glucopyranoside (2): Colourless needles [methanol], (50 mg), m.p 168-170°, R_f = 0.19 (system III), FAB-MS: at *m/z*: 485 for $C_{20}H_{20}O_{14}$. ^{1}H-NMR spectrum (300 MHz, CD_3OD): δ 3.09-4.40 (m, sugar protons), 6.04 (1H, d, J= 7.50 Hz, H-1′), 5.25 (1H, t, J= 7.50 Hz, H-2′), 7.12 (4H, s, galloyl-H), ^{13}C-NMR spectrum (75 MHz, CD_3OD): Table 1.



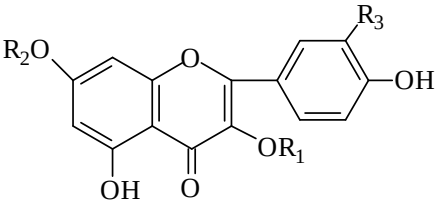
(2)

Table 1: ^{13}C-NMR data of compounds (**2** and **6**).

Group	2	6
Galloyl CO	168.66 170.05	168.10
Galloyl C-1	120.00 120.15	121.50
Galloyl C-2,6	109.85 110.10	109.70
Galloyl C-3,5	143.05 143.05	146.10
Galloyl C-4	139.80 139.59	139.30
CH_2	-------	61.31
CH_3	-------	14.30
Sugar moiety		
1`	92.30	
2`	74.24	
3`	75.44	
4`	70.17	
5`	77.89	
6`	61.94	

Spectra were measured by 75 MHz (2), 125 MHz (6) in CD_3OD, relative to TMS).

Kaempferol-3-O-β-D-glucopyranoside (3): Yellow amorphous powder (300 mg), R_f = 0.71 (system II), UV (λ_{max} nm) MeOH: 265, 300sh, 350, NaOMe: 273, 325sh, 404, $AlCl_3$: 275, 302sh, 347, 400, $AlCl_3$/HCl: 275, 348, 403, NaOAc: 273, 371, NaOAc/H_3BO_3: 265, 351. ^{1}H-NMR spectrum (300 MHz, DMSO-d_6): δ 3.09-3.66 (m, sugar protons), 5.46 (1H, d, J= 6.9 Hz, H-1″), 6.20 (1H, d, J= 1.80 Hz, H-6), 6.42 (1H, d, J= 1.80 Hz, H-8), 6.88 (2H, d, J= 9.00 Hz, H-3′,5′), 8.04 (2H, d, J= 9.00 Hz, H-2′, 6′), ^{13}C-NMR spectrum (75 MHz, DMSO-d_6): Table 2.



Compound	R_1	R_2	R_3
3	glucose	H	H
4	glucose	H	OH
7	glucose-rhamnos	H	OMe
8	glucose-rhamnose	H	OH

Quercetin-3-O-β-D-glucopyranoside (4): Yellow amorphous powder (350 mg), R_f = 0.55 (system II), UV (λ_{max} nm) MeOH: 257, 269sh, 362, NaOMe: 272, 324sh, 410, $AlCl_3$: 273, 305, 437, $AlCl_3$/HCl: 273, 348, 405, NaOAc: 274, 324, 375, NaOAc/H_3BO_3: 265, 298, 377. ^{1}H-NMR spectrum (300 MHz, DMSO-d_6): δ 3.09-3.63 (m, sugar protons), 5.46 (1H, d, J= 7.2 Hz, H-1″), 6.18 (1H, d, J= 1.80 Hz, H-6), 6.39 (1H, d, J= 1.80 Hz, H-8), 6.83 (1H, d, J= 9.00 Hz, H-5′), 7.51 (1H, d, J= 2.40 Hz, H-2′), 7.65 (1H, dd, J= 2.40, 9.00 Hz, H-6′), 12.63 (1H, s, 5-OH), ^{13}C-NMR spectrum (75 MHz, DMSO-d_6): Table 2.

Methyl gallate (5): White amorphous powder (50 mg), R_f = 0.38 (system III). ^{1}H-NMR spectrum (500 MHz, CD_3OD): δ 7.20 (2H, s,H-2,6) and 3.85 (3H, s, OCH_3).

Ethyl gallate (6): White powder (100 mg), R_f = 0.44 (system III). ^{1}H-NMR spectrum (500 MHz, CD_3OD): δ 7.03 (2H, s, H-2,6), 4.25 (2H, q, $\underline{CH_2}CH_3$) and 1.32 (3H, t, $CH_2\underline{CH_3}$). ^{13}C-NMR spectrum (125 MHz, CD_3OD): Table 1.

Table 2: ^{13}C-NMR data of compounds (**3**, **4** and **8**).

C-Atoms	3*	4*	8**
1	-----	-----	-----
2	156.19	156.16	156.17
3	133.15	133.31	136.09
4	177.42	177.44	178.71
5	161.20	161.25	161.99
6	98.81	98.70	98.12
7	164.54	164.23	162.85
8	93.71	93.53	93.30
9	156.42	156.34	156.28
10	103.87	103.96	104.00
1`	120.90	121.15	121.35
2`	130.89	115.22	114.97
3`	115.12	144.84	144.77
4`	159.97	148.49	147.48
5`	115.12	116.21	116.97
6`	130.89	121.62	121.49
1``	100.87	100.85	100.80
2``	74.22	74.01	73.13
3``	76.41	76.51	76.20
4``	69.89	69.94	69.63
5``	77.51	77.60	75.32
6``	60.83	60.98	66.12
1```			100.31
2```			70.99
3```			71.10
4```			71.38
5```			67.87
6```			17.02

Spectra measured by 75* and 100** MHz, (DMSO-d_6), relative to TMS).

Isorhamnetin-3-O-rutinoside (7): Yellow amorphous powder (50 mg), R_f = 0.46 (system II), UV (λ_{max} nm) MeOH: 254, 309sh, 355, NaOMe: 265, 320sh, 405, $AlCl_3$: 264, 356sh, 405, $AlCl_3$/HCl: 264, 354, 402, NaOAc: 272, 295, 377, NaOAc/H_3BO_3: 254, 295, 358. ^{1}H-NMR spectrum (300 MHz, CD_3OD): δ 3.20-3.60 (m, sugar protons), 4.47 (1H, d, J= 2.00 Hz, H-1‴, anomeric proton of rhamnose), 5.00 (1H, d, J= 7.50 Hz, H-1″, anomeric proton of glucose), 1.13 (3H, d, J= 6.50 Hz, CH_3-rhamnose), 3.86 (3H, s, OCH_3), 5.95 (1H, d, J= 2.00 Hz, H-6), 6.09 (1H, d, J= 2.50 Hz, H-8), 6.67 (1H, d, J= 8.50 Hz, H-5′), 7.58 (1H, dd, J= 2.50, 8.50 Hz, H-6′), 7.88 (1H, d, J= 2.50 Hz, H-2′).

Quercetin-3-O-rutinoside (8): Yellow amorphous powder (50 mg), R_f = 0.30 (system II), UV (λ_{max} nm) MeOH: 259, 266sh, 299sh, 359, NaOMe: 272, 325, 410, $AlCl_3$: 275, 303sh, 430, $AlCl_3$/HCl: 271, 300, 364sh, 402, NaOAc: 271, 325, 390, NaOAc/H_3BO_3: 262, 298, 387. ^{1}H-NMR spectrum (400 MHz, DMSO-d_6): δ 3.06-3.70 (m, sugar protons), 5.32 (1H, d, J= 7.1 Hz, H-1″, anomeric proton of glucose), 4.37 (1H, d, J= 1.5 Hz, H-1‴, anomeric proton of rhamnose), 1.03 (3H, d, J= 6.32 Hz, CH_3-rhamnose), 6.17 (1H, d, J= 2.20 Hz, H-6), 6.37 (1H, d, J= 2.20 Hz, H-8), 6.82 (1H, d, J= 8.00 Hz, H-5′), 7.52 (1H, d, J= 2.20 Hz, H-2′), 7.67 (1H, dd, J= 2.20, 8.00 Hz, H-6′), 12.53 (1H, s, 5-OH), ^{13}C-NMR spectrum (100 MHz, DMSO-d_6): Table 2.

RESULTS AND DISCUSSION

Repeated ODS column chromatography and sephdex LH-20 of ethyl acetate soluble fraction of the methanolic extract of the stem bark of *Acer negundo* (L.) afforded compounds **1**, **2**, **5** and **6** which gave dark blue colour with ferric chloride reagent.

Compound **1** was identified as gallic acid by comparing its physico-chemical data such as m.p, IR and co-TLC with reference sample.

^{1}H-NMR spectra of compounds **5** and **6** displayed two singlet signals at δ 7.03 and 7.20, each for two aromatic protons (H-2,6). The spectrum of compound **5** showed a singlet signal at δ 3.85 assigned for a methoxyl group. The spectrum of compound **6** showed a signal at δ 4.25 assigned for methylene group neighbouring to oxygen and a triplet at δ 1.32 for an aliphatic methyl group which were confirmed by signals in ^{13}C-NMR at δ 61.3 and 14.3 respectively. Other ^{13}C-NMR data were in agreement with those reported for galloyl moiety.[15] From the above data, compound **5** was identified as methyl gallate and compound **6** as ethyl gallate and this is the first report for their isolation from the stem bark of *Acer negundo* (L.).

The FAB-MS of compound **2** showed $[M+1]^+$ at *m/z* 485 was consistent with the molecular formula of $C_{20}H_{20}O_{14}$. The ^{1}H-NMR spectrum of compound **2** displayed two galloyl groups at δ 7.12 (4 H, s) and an anomeric proton at δ 6.04 (1 H, d, J= 7.50 Hz). The

configuration of the glucose C-1` position was concluded to be β on the basis of the J value (7.50 Hz).[16] The downfield position of the anomeric proton at (δ 6.04) indicated that one of the two galloyl groups is located at C-1`. It showed also a triplet at relatively lowfield (δ 5.25) and this signal could be assigned for the C-2` proton from the fact that it was shown to be coupled with the anomeric proton. The ^{1}H- and ^{13}C-NMR spectral data were in accordance with the reported data for 1,2-di-O-galloyl-β-D-glucopyranoside[17,18] which was isolated for the first time from the stem bark of *Acer negundo* (L.).

The UV spectral data in methanol for compounds **3**, **4**, **7** and **8** indicated their C3-OH substituted flavonol nature.[19] ^{1}H-NMR spectrum of compound **3** displayed two doublets of meta-coupled protons of ring A at δ 6.20 and 6.42 (J= 1.80 Hz) for H-6 and H-8. It showed also two doublets at δ 6.88 and 8.04 (J= 9.00 Hz) for H-3`,5` and H-2`,6` respectively. These data suggested a kaempferol skeleton with an anomeric glucose proton with β-configuration[16] (J= 6.90 Hz) at δ 5.46. Acid hydrolysis of compound **3** yielded sugar part identified as glucose (system IV) and kaempferol aglycone. From the UV data with different ionising and complexing agents,[19] the sugar attachement was assigned to be at C-3. ^{13}C-NMR data showed good agreement with those reported for kaempferol-3-O-β-D-glucopyranoside.[20]

^{1}H-NMR spectrum of compound **7** showed three aromatic protons of ring B which was confirmed through an ABX-type coupling at δ 7.58 (1H, dd, J= 2.50, 8.50 Hz, H-6`), 6.67 (1H, d, J= 8.50 Hz, H-5`) and 7.88 (1H, d, J= 2.0 Hz, H-2`), in addition, two doublets with J= 2.50 Hz at δ 5.95 and 6.09 assigned for H-6 and H-8 respectively. The spectrum showed also a singlet signal at δ 3.86 assigned to a methoxyl group, a doublet signal at δ 1.13 (J= 6.5 Hz) for CH_3-rhamnose and two doublets at δ 4.47 (J= 2.00 Hz) and at 5.00 (J= 7.5 Hz) for the anomeric protons of rhamnose and glucose respectively. From studying the effect of ionizing and complexing agents, acid hydrolysis followed by co-TLC for each of the sugar part and the aglycone as well as comparing its ^{1}H-NMR data with those reported,[21] it could be concluded that compound **7** was identified as isorhamnetin-3-O-rutinoside which was isolated from the leaves of the studied plant.[14]

The ^{1}H-NMR spectra of compounds **4** and **8** showed two doublets with meta coupling at δ 6.18, 6.17 for H-6 and at δ 6.39, 6.37 for H-8 respectively. The spectra showed also three aromatic protons of ABX-type coupling, two doublets at δ 6.83, 6.82 for H-5′, at δ 7.51, 7.52 for H-2′ and a doublet of doublet at δ 7.65, 7.67 for H-6′. The presence of sugar was confirmed by the appearance of one anomeric proton at δ 5.46 (1H, d, J= 7.20 Hz) in **4** characteristic for glucose and two anomeric protons at δ 4.37 (1H, d, J= 1.50 Hz) and at 5.32 (1H, d, J= 7.10 Hz) characteristic for rhamnose and glucose in **8**. Rhamnose was also confirmed by the presence of methyl signal at 1.03 (3H, d, J= 6.32 Hz) and at δ 17.02 in ^{13}C-NMR, Table 2. Acid hydrolysis of each of compounds **4** and **8** followed by co-TLC with authentic samples revealed the presence of glucose in **4**, glucose and rhamnose in **8**. From the aforementioned studies as well as comparison with reported data,[20] it could be concluded that compounds 4 and 8 were quercetin-3-O-β-D-glucopyranoside and quercetin-3-O-rutinoside respectively which were isolated for the first time from the stem bark of the studied plant.

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