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FLAVONOID GLYCOSIDES AND HYPOTENSIVE EFFECT OF *JUGLANS NIGRA* L. CULTIVATED IN EGYPT

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فى دراسة كيميائية لمكونات أوراق نبات الجوجلانس نيجرا ل. (الجوز الأسود) التابع للعائلة الجوزية والمنزرع فى مصر ، تم فصل ثلاثة مركبات لأول مرة من العائلة الجوجلانديسية وهم الكامبفيرول-٣-أ-جلوكوزيد ، الكوارستين-٣-أ-جلوكوزيد ، الكوارستين-٣-أ-٦-ايثيل جلوكيورونوزيد بالإضافة إلى الإستجماستيرول-٣-أ-جلوكوزيد. وقد تم التعرف على المركبات السابق ذكرها بواسطة الطرق المختلفة للتحليل الكيماوى والطيفى وكذلك بالمقارنة بعينات أصيلة. هذا وقد أوضحت الدراسة البيولوجية لهذا النبات أن الخلاصة الكحولية للأوراق لها تأثير خافض لضغط الدم.

From the methanolic extract of the leaves of Juglans nigra L. (Black Walnut) Family Juglandaceae (Walnut family), kaempferol-3-O-β-glucoside, quercetin-3-O-β-glucoside and quercetin-3-O-β-glucuronoide-6"-ethyl ester were isolated for the first time from the family Juglandaceae, in addition to stigmasterol-3-O-β-glucoside. Most of the structures were established on the basis of UV, MS and NMR (^1H, ^{13}C-NMR and DEPT) spectroscopic data. Moreover, the hypotensive and toxicological studies were done.

INTRODUCTION

Juglans nigra L. is a large tree chiefly from north temperate regions and mountains in the north tropics. It is grown mainly in Eastern United States, mountains regions of North Africa, East Asia, and cultivated in Egypt.[1,2] The plant is used in folk medicine as antihypertensive and antidiabetic, for eczema, syphilis, as astringent and anthelmintic.[3] Naphthoquinones, naphthanyl glycosides, flavonoid glycosides, regiolone, sitosterol, betulinic acid, polysaccharides, phenolic compounds catechins proanthocyanidins and fatty acids were isolated from different species of Juglans.[4-14]

EXPERIMENTAL

Plant material

The leaves of *J. nigra* L. were collecred in the period from October to December 1999, from the Assiut University Campus. Its identity was confirmed by Dr. Salah M. El-Nagar, Associate Professor of Taxonomy, Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt. A voucher specimen is deposited at the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Assiut, Egypt.

Equipment

Column chromatography was carried out on silica gel (63-100 μm, Merck) and Diaion HP-20 (Mitsubishi). Silica gel 60 F_{254} layers (Merck) were used for TLC. UV spectra were measured in methanol and different ionizing and complexing agents using Uvidec model 320 spectrophotometer with matched 1 cm quartz cells (Jasco, Tokyo, Japan), UV-lamp (254, 366 nm, VL 6LC, Marine Lavalee-codex, France). ^1H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6 (400-100 MHz respectively) using JEOL JNMLA400. While FABMS spectra were recorded in positive and negative ion mode (matrix, glycerol, NBA) by a JEOL, JMS600H

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model 500 spectrometer. Authentic samples were obtained from Department of Pharmacognosy, Faculty of Pharmacy, Assiut University. The following solvent systems were used for TLC screening:

I. Chloroform : Methanol 9:1 (solvent system to detect compound 1).
II. Chloroform : Methanol 8.5:1.5 (solvent system to detect compound 2).
III. Chloroform : Methanol 8:2 (solvent system to detect compound 3).
IV. Chloroform : Methanol : water 6:5:1 (solvent system to detect compound 4).
V. Ethyl acetate : hexane 8:2 (solvent system to detect the aglycone part).
VI. n-BuOH : acetone : formic acid : water 60:17:8:15 (solvent system to detect the sugar part).

Extraction and isolation

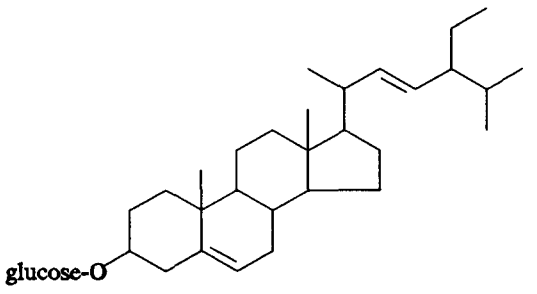
The air dried powdered aerial parts of *J. nigra* L. (2.5 kg) was extracted with 70% ethanol. The dried ethanol extract (230 g) was suspended in water and extracted with chloforom. The aqueous fraction (43 g) was applied to a column of Diaion HP-20 and eluted with H_2O, MeOH and acetone respectively. The MeOH eluate (30 g) was chromatographed on silica gel column using $CHCl_3$/MeOH gradient. Elution with $CHCl_3$: MeOH (9.5:0.5) afforded compound **1**, $CHCl_3$: MeOH (8.5:1.5) afforded compound **2**, $CHCl_3$: MeOH (8:2) afforded compound **3** and $CHCl_3$: MeOH (7:3) afforded compound **4**.

Acid hydrolysis

Each glycoside (25 mg) was separately hydrolysed with 1 M H_2SO_4 for 90 min, after completion, the solution was shaken with $CHCl_3$. Each recovered aglycone part was identified by direct comparison with authentic samples on TLC using solvent system V. The aqueous layer was neutralized with $KHCO_3$ and the sugar part was identified by TLC (using a solvent system VI) and comparison with the authentic sample.[15]

Identification of the isolated compounds

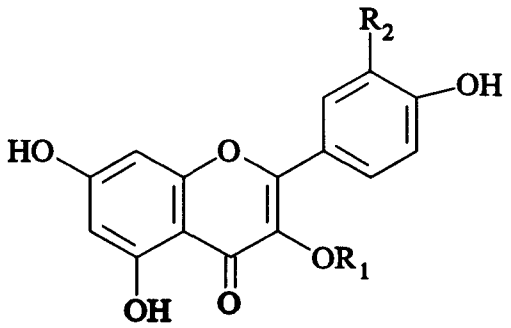
Compound 1: White fine needles, 400 mg, R_f 0.33 (solvent system I), m.p 232-234°. It was identified as stigmasterol-3-O-glucoside by direct authentication (m.p, mmp, co-chromatography using solvent system I and superimposalbe IR spectra).



Compound 1

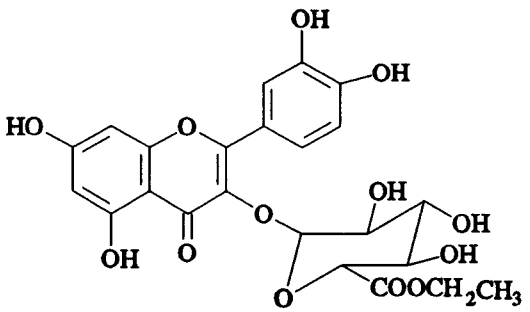
Compound 2: Yellow powder, 65 mg, R_f 0.41 (solvent system II), m.p 243-244°. UV λ_{max} nm (MeOH): 265, 296 (sh), 347; +NaOMe: 262, 329 (sh), 398; +NaOAc: 273, 311 (sh), 378; +NaOAc/H_3BO_3: 266, 299 (sh), 349; +$AlCl_3$: 272, 304 (sh), 395; +$AlCl_3$/HCl: 373, 302 (sh), 393. ^{1}H-NMR spectrum (DMSO-d_6) at δ: 5.46 (1H, d, J= 7.4 Hz, anomeric proton), 6.2 (1H, br.s, H-6), 6.4 (1H, br.s, H-8), 6.9 (2H, d, J= 7.1 Hz, H-3`,5`),8.03 (2H, d, J= 7.1 Hz, H-2`,6`),12.6 (1H, s, 5-OH). ^{13}C-NMR given in Table 1.

Compound 3: Yellow powder, 260 mg, R_f 0.42 (solvent system III), m.p 221-223°, UV λ_{max} nm (MeOH): 257, 356; +NaOMe: 268, 328 (sh), 409; +NaOAc: 268, 364; +NaOAc/H_3BO_3: 260, 377; +$AlCl_3$: 274, 332 (sh), 430; +$AlCl_3$/HCl: 268, 362 (sh), 378. ^{1}H-NMR spectrum (DMSO-d_6) at δ: 5.44 (1H, d, J= 6.84 Hz, H-1``), 6.19 (1H, br.s, H-6), 6.39 (1H, br.s, H-8), 6.77 (1H, d, J= 8.5 Hz, H-5`),7.40 (1H, d, J= 8.5 Hz, H-6`), 7.60 (1H, br.s, H-2`). ^{13}C-NMR given in Table 1.



Compound	R_1	R_2
2	glucose	H
3	glucose	OH

Compound 4: R_f 0.53 (system V), UV λ_{max} nm (MeOH): 257, 357; +NaOMe: 267, 330 (sh), 401; +NaOAc: 266, 370; +NaOAc/H_3BO_3: 262, 377; +$AlCl_3$: 272, 420; +$AlCl_3$/HCl: 271, 362. FABMS (negative ion mode), m/z $(M-H)^-$: 505.54 (2.2%), 476.34 (0.8%), 301.39 (2%). ^{1}H-NMR spectrum (400 MHz, DMSO-d_6) at δ: 1.1 (3H, t, J= 7.1 Hz, CH_3 of ethyl ester moiety), 5.2 (1H, d, J= 5.1 Hz, H-1``), 6.2 (1H, br.s, H-6), 6.4 (1H, br.s, H-8), 6.8 (1H, d, J= 7.1 Hz, H-5`), 7.4 (1H, d, J= 7.1 Hz, H-6`), 8.01 (1H, br.s, H-2`). ^{13}C-NMR given in Table 1.



Compound 4

Biological screening

Extraction and preparation of solution

300 g of *J. nigra* L. leaves was extracted with 95% ethanol. The extract was evaporated under reduced pressure till a thick syrupy mass of a constant weight and then divided into two equal parts. One part was diluted with water and shaken with chloroform till exhaustion, the aqueous layer was concentrated under reduced pressure to obtained the aqueous residue. 10 g of this residue was dissolved in 30 ml saline to give the required solution. The solution of other part (total extract) was prepared by dissolving 10 g in 50 ml saline.

Hypotensive activity[16]

Normotensive adult rabbits of either sex (2-2.5 kg) were anaesthetized by I.P. administration of urethane (1.6 g/kg). The trachea was exposed and cannulated to facilitate spontaneous artificial respiration throughout the experiment. The solutions were injected (5 ml) through a polyethylene cannula that inserted into the external juglar vein. The arterial blood pressure was recorded from the carotid artery via a polyethylene arterial cannula that is connected to a Universal Oscillograph. Animals were allowed to equilebrate for at least 15 min. before administration of any drug. The mean blood prssure was calculated as the mean value of the summation of both diastolic and systolic blood pressure. Changes in blood pressure were expressed as the percent of the control value, obtained immediately before the administration of test substance. The results are listed in Table 2.

Evaluation of toxicity[17]

About 56 albino mice with an average weight (20-30 g) were classified into 6 homogenous groups each composed of 6 animals with equal sex distribution. Each group was held on a separate cage. The animals received a gradual increasing dose of the tested dried ethanolic extract in saline solution by I.P. route. With each test a parallel control group was given the saline by I.P. route in the same volume as the tested group. All animals are ketp under a constant observation for 6 horus to observe any change in the general behaviour or other physiological activities. Mortalities in each group were recorded 24 hr after extract administration. The results are listed in Table 3.

Table 1: ^{13}C-NMR data of compounds **2-4** (100 MHz, DMSO-d_6).

Carbon	Compd. **2**	Compd. **3**	Compd. **4**	DEPT of compd. **4**
Aglycone				
2	156.44	156.42	156.42	C
3	133.25	133.38	133.98	C
4	177.51	177.54	177.59	C
5	161.28	161.34	160.97	C
6	98.76	98.77	98.95	CH
7	164.23	164.22	164.80	C
8	93.71	93.62	93.78	CH
9	156.33	156.28	157.40	C
10	104.05	104.07	103.65	C
1`	120.95	121.25	120.55	C
2`	130.93	115.31	115.47	CH
3`	115.12	144.91	144.84	C
4`	160.01	148.56	148.50	C
5`	115.12	116.29	117.67	CH
6`	130.93	121.71	120.91	CH
3-O-glucosyl				
1``	100.95	100.89		
2``	74.27	74.17		
3``	76.47	76.57		
4``	69.95	69.99		
5``	77.52	77.67		
6``	60.89	61.04		
3-O-ß-glucuronide-6``-ethyl ester				
1``			102.40	CH
2``			74.08	CH
3``			76.50	CH
4``			71.70	CH
5``			74.20	CH
6``			172.20	C
$-CH_2$			64.95	CH_2
$-CH_3$			15.19	CH_3

Table 2: The effect of I.V injection of total extract (400 mg/kg) and aqueous extract (800 mg/kg) of *Juglans nigra* L. leaves on the mean arterial blood pressure (MABP) in the normotensive anaesthetized rabbits.

Time after administration (min)	% Fall in MABP mmHg	
	Total extract	Aqueous extract
0	50.8 ± 2.45**	46.2 ± 4.48**
1	47.9 ± 2.12**	40.3 ± 4.56**
2	44.6 ± 1.12**	36.6 ± 6.61**
3	39.8 ± 2.05**	26.4 ± 6.69**
5	37.9 ± 2.00**	10.2 ± 1.62**
10	37.3 ± 3.39**	4.10 ± 2.20**
15	28.7 ± 2.70**	23.8 ± 2.45**
20	27.5 ± 3.01**	26.5 ± 2.32**
30	22.8 ± 3.90**	30.6 ± 8.06**
45	18.2 ± 2.20**	44.8 ± 4.70**
60	13.0 ± 1.50**	39.7 ± 3.12**
75	10.0 ± 1.44**	39.7 ± 3.12**
90	8.00 ± 1.01**	38.9 ± 3.20**
100	6.50 ± 1.41**	35.7 ± 3.50**
110	3.80 ± 1.40*	30.5 ± 2.70**
120	1.80 ± 0.93*	25.3 ± 2.60**
140	-	16.7 ± 3.10**
150	-	10.3 ± 2.90**
160	-	8.40 ± 3.50*

Value present the mean of 5 experiment ± S.E
* Significant from control values at $p > 0.05$
**Highly significant from control values at $p > 0.01$

Table 3: Determination of LD_{50} of total extract of *Juglans nigra* leaves.

Grade I.P. dose levels of test extract/kg		24 hours observed mortality	
No.	G.	Died/test	% observed mortality
1	4	0/6	0%
2	5	0/6	0%
3	7.5	0/6	0%
4	10	1/6	17%
5	12.5	1/6	17%
6	13	2/6	33%
7	14	3/6	50%
8	15	4/6	67%
9	18	6/6	100%

RESULTS AND DISCUSSION

The methanolic extract of *Juglans nigra* L. leaves was extracted with chloroform and the remaining aqueous layer was subjected to column chromatography on Diaion HP-20. The methanol eluate was repeatedly chromatographed on columns of silica gel to afford four glycosides **1-4**.

Compound **1** was identified as stigmasterol-3-O-glucoside by comparison of its physico-chemical data such as m.p, IR and co-chromatography with reference sample before and after acid hydrolysis. The chromatographic study of sugar in the aqueous fraction revealed that the sugar appeared as single spot corresponding to authentic glucose. The aglycone was identified as stigmasterol.

The UV spectrum of compound **2** indicated a flavone or C_3-OH substituted flavonol skeleton and the absence of ortho-dihydroxy groups at ring B.[18] The ^{1}H-NMR spectra showed two doublets at δ 8.03 (2H, d, J= 7.1 Hz, H-2`, H-6`), 6.9 (2H, d, J= 7.1 Hz, H-3`, H-5`) which are characteristic for 1,4-disubstituted benzene in kaempferol structure. This was further confirmed with ^{13}C-NMR data (Table 1), which are identical with those previously reported.[15] The ^{1}H-NMR spectrum of compound **2** showed two broad singlets at δ 6.2, 6.4 that are characteristic for H-6 and H-8 respectively. The glycosidic nature was confirmed by the appearance of anomeric proton doublets at δ 5.46 (J= 7.4 Hz). Acid hydrolysis for compound **2** gave glucose and kaempferol, which were identified by co-TLC with authentic samples. From the above data, it could be concluded that compound **2** is kaempferol-3-O-ß-glucoside.

A study of the effect of ionizing and complexing agents on the UV absorption of compound **3** revealed the presence of ortho-dihydroxy groups at ring B and a free hydroxyl group at C-7. The ^{1}H-NMR spectra of compound **3** showed two broad singlet at δ 6.19, 6.39 assigned to H-6 and H-8 respectively. The spectra showed also three aromatic protons at δ 6.77 (1H, d, J= 8.5 Hz, H-5`), 7.4 (1H, d, J= 8.5 Hz, H-6`) and 7.6 (1H, br.s, H-2`).

The glycosylation of compound **3** was confirmed from the appearance of one anomeric proton, doublet at δ 5.44 (J= 6.48 Hz). Acid hydrolysis of compound **3** gave glucose and quercetin which were identified by co-TLC with authentic sample. From the above data, it could be concluded that compound **3** is quercetin-3-O-ß-glucoside. The ^{13}C-NMR data listed in Table 1.

Compound **4** was isolated as a yellow amorphous powder. It showed a molecular ion peak at m/z $(M-H)^-$ 505 in negative FABMS that corresponding to the molecular formula $C_{23}H_{22}O_{13}$. The fragment at m/z 301 is derived from the aglycone part, while the loss of ethyl moiety gave an ion peak at m/z 476. It showed a brown fluorescence under UV light at 360 nm. UV data showed the presence of free 4`-OH group, a free 7-OH group and presence of ortho-dihydroxy group.[18] The $^1$H-NMR spectrum showed signals at $\delta$ 6.2 (s, H-6), 6.4 (s, H-8), 6.8 (d, J= 7.1 Hz, H-5`), 7.4 (d, J= 7.1 Hz, H-6`), 8.01 (s, H-2`). These signals are identical to those reported for quercetin derivative.[15] A broad band at δ 3.37 for the sugar protons was presented. The anomeric proton signal was found at δ 5.2 (J= 5.2 Hz), that indicating a ß-linkage.[18] The ^{1}H-NMR spectrum exhibited signal for the methyl group of the ethyl moiety at δ 1.1 (t, J= 7.1 Hz), while the methylene group was obscured by water signal. The ^{13}C-NMR signals were in full agreement to those reported for 3-substituted quercetin (Table 1).[19] The remaining ^{13}C-NMR signals were at δ 15.19 for methyl group, 64.95 for the methylene group, 102.4, 74.08, 76.5, 71.7, 74.2 and 172.2 for glucuronic acid moiety.[20] From the above mentioned data compound **4** was found to be quercetin-3-O-ß-glucuronide-6``-ethyl ester.

Result of biological screening

Both total and aqueous extracts from *J. nigra* leaves caused a significant decrease in systolic and diastolic blood pressure in the anaesthetized rabbits in a dose dependant manner. The total extract (400 mg/kg) dropped the blood pressure 50.8% and its hypotensive

effect was lasting and did not revert to control value till 2 hours, while in the case of aqueous extract after producing a hypotensive effect (42-46%) that persist for about 2-4 minutes, the blood pressure rises again to reach the control value and this persists for 10-15 minutes, then the blood pressure decreases again (40%) and the hypotensive effect presists for about 1.5-2 hours.

The mice showed a high tolerance to the total ethanolic extract, no mortality, no change in general behaviour or physiological activities even at a high dose 5 g/kg. However, in a dose of 7.5 g/kg the animals become anaesthetized with loss of wrighting reflex, with no mortality at a dose of 10 g/kg animals show spasms and began to die. LD_{50} value is found to be 14 g/kg (Table 3).

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