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Investigation of the Coumarin Content of *Cassia nodosa* Leaves Growing in Egypt

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Four natural coumarins were isolated from the leaves of *Cassia nodosa*. The structures of these coumarins were established by spectroscopic data mainly ^{1}H NMR, ^{13}C NMR and EIMS. Hydro alcoholic extract of *Cassia nodosa* showed a potent effect in lowering blood sugar levels in normal and streptozotocin induced diabetic model rats, when compared to controls.

Key Words: *Cassia nodosa*, Coumarins.

INTRODUCTION

Cassia nodosa, family; *Caesalpinioideae*/*Caesalpiniaceae* is a flowering tree from genus cassia which is known mainly for its landscaping attributes[1], Cassias are ornamental plants of great beauty[1,2]. *Cassia nodosa* has pretty pale-green leaves 2 inches (5 cm) long[3], produces a mass of gorgeous flowers, with petals at first pale red, changing to dark red, then palling again to pink[2,4]. The flower stems are red and grow in whorls[4,5]. The buds and flowers are deep pink fading to white and each is somewhat pointed at the tip[5]. It is also known as *Cassia agnes*, *Cassia renigera Wall*, pink shower trees and apple blossom tree[6-8].

Flavonoids, D-3-O-methyl chiro-inositol sugar and chromone named as 5,4'-dihydroxy-7-methyl-3-benzyl chromone were previously isolated from the leaves of *Cassia nodosa*[9-11]. Fifteen compounds were obtained from the volatile oil of *Cassia nodosa* flowers[10] in addition to a new compound, nodolidate which isolated from the flowers of *Cassia nodosa* and characterized as (-)-7-acetoxy-9,10-dimethyl-1,5-octacosanolide[12]. Nodoside,a new anthraquinone glycoside and a mixture of fatty acids, hydrocarbons, campesterol and sitosterol along with ceryl alcohol were also isolated from flowers[12-14].

There are no enough reports regarding the active constituents of the different parts of *Cassia nodosa*, nor their pharmacological activities, so, the aim of this work is to investigate some of the active ingredients of the leaves of *Cassia nodosa* growing in Egypt, as well as study of some of its biological activities.

EXPERIMENTAL

Cassia nodosa leaves were collected (September 2001) from El-Orman garden, Giza, Egypt. The plant was identified by Dr. Mohammed El-Gebaily, lecturer of plant Taxonomy, Department of Natural and microbial products, NRC, Cairo, Egypt.

General procedures: Melting points were determined on Koffler's hot stage microscope and were uncorrected; UV spectra were obtained on Schimadzu 160UV spectrometer in methanol solution. NMR spectra was recorded in DMSO-d_6 as a solvent on a Varian NMR spectrometer 300 MHz with TMS as an internal standard and chemical shifts were in ppm. EI mass spectra were performed using a Helwlet Packard 5890 mass spectrometer. Column chromatography using silica gel 60 (Merck), TLC was performed on precoated silica gel 60 plates (Merck).

Extraction and isolation: The dried leaves of *Cassia nodosa* (2 Kg) were exhaustively extracted (3 times) with chloroform:methanol (1:1) mixture at room temperature, evaporation to dryness in vacuum of the combined chloroform: methanol extracts afforded a dark green residue (110 g).

The crude extract was subjected to silica gel column chromatography with a gradient elution of petroleum ether, chloroform, ethyl acetate and methanol, (100 mL fraction each). Fractions with the same thin layer chromatography (TLC) profile were combined together yielding 10 fractions.

Fraction No. 1 eluted with 100 % petroleum ether was further purified by silica gel column chromatography using solvent mixtures of increasing polarity (petroleum ether,

petroleum ether:ethyl acetate to pure ethyl acetate). From this fractionation compound No. 1 (C1, 32 mg) was isolated from the fraction eluted with 65 % pet. ether:35 % ethyl acetate.

Fraction No. 6 eluted with 50 % pet. ether and 50 % ethyl acetate was further fractionated on VLC (vacuum liquid chromatography) column (5 cm × 30 cm, 100 g silica gel) and eluted with a gradient system (benzene-ethyl acetate-methanol), from these fractionation second isolated coumarin was obtained (C2, 9.4 mg).

Fraction No. 8 eluted with 70 % ethyl acetate and 30 % pet. ether was further purified by preparative TLC using 65 % benzene and 35 % ethyl acetate to yield third isolated compound (C3, 7.4mg).

Fraction eluted with 100 % ethyl acetate was subjected to silica gel column chromatography, Pharmadex LH-20 and RP C-18 to yield compound **4**, which was isolated as light yellow oil.

Pharmacological studies:

Preparation of plant extracts: Air-dried powder (1 Kg) of leaves of the plant was extracted by percolation at room temperature with 70 % ethyl alcohol. The extract was concentrated under reduced pressure and finally dried in vacuum desiccator. The residue was dissolved in distilled water and filtered. The filtrate was evaporated to dryness. The dried mass (46.2 g) was suitably diluted with normal saline and used in the experiment.

Animals: Healthy rats (100-150 g, 3-4 months old) were housed in cages at an ambient temperature of 25 ºC and 45 % relative humidity. The animals were kept on standardized diet and tap water was freely available. For experimental purpose the animals were kept fasting overnight for 18 h, but allowed free access to water. Induced diabetes was produced by intraperitoneal injection of 50 mg/Kg of streptozotocin (Sigma) in citrate buffer (pH 3.4) and blood samples were collected 24, 48, 72 and 96 h after streptozotocin treatment. Stable hyperglycemia (280-300 mg/dl) was produced 72 h after streptozotocin treatment. Blood sugar and urine examination for glucose were estimated for the establishment of the diabetic state. The blood samples were collected from the orbital plexus and blood sugar was estimated by the glucose oxidase method[15].

For the purpose of the experiments, the rats were divided into the two following groups; group 1, normal rats; group 2, streptozotocin-induced diabetic rats. Six rats were used for each dose and treated as follows:

Group-1: Normal rats: In this group of rats, various doses (50, 100, 200 and 400 mg/Kg, p.o.) of the leaf extract was administered after drawing the initial sample (at 0 h). Further samples of blood were collected 2 h after each dose administration. Control animals were treated similarly but with normal saline instead of leaf extract, various doses of tolbutamide (20, 40, 60 and 80 mg/Kg, p.o.) were given to normal rats and blood samples were collected at 0 and 2 h after tolbutamide administration.

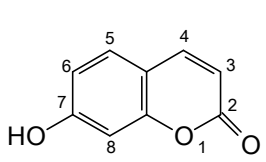
Group-2: Induced diabetic model rats: Varying doses (50, 100, 200 and 400 mg/Kg, p.o.) of the leaf extract were given to induce diabetic model rats after drawing the initial sample. Further samples of blood were collected after 2 h of each dose administration. Varying doses (20, 40, 60 and 80 mg/Kg, p.p.) of tolbutamide were given to induced diabetic model rats and blood samples were collected at 0 and 2 h after tolbutamide administration.

Acute toxicity study: Albino mice of either sex of 10 animals per group and weighing 20-25 g were administered dreaded doses (250, 500, 1000, 2000, 4000 and 8000 mg/Kg, p.o.) of the leaf extract. After administration of the leaf extract the mice were observed for gross behavioural, neurologic, autonomic and toxic effects continuously for 24 h and then at 6 h interval for 24 h[16]. The toxicological effects were observed in terms of mortality expressed as LD_{50}. The number of animals dying during a 24 h period was noted[17]. The LD_{50} of the extract was calculated[18].

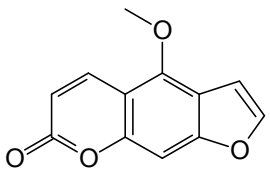
In the present study it was observed that the leaf extract of *Cassia nodosa* possesses significant dose-dependant blood sugar lowering activity in normal and induced diabetic model rats. The blood sugar-lowering activity of the extract was comparable to that of tolbutamide in normal and induced diabetic model rats and observed that the plant extract was 3.76 times less potent in normal and 4.65 times less potent in induced diabetic model rats than tolbutamide.

RESULTS AND DISCUSSION

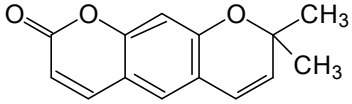
Compound 1: Isolated as off white crystals, R_f 0.45 with solvent system (toluene:ether, 1:1), showed blue fluorescence in both UV and visible light, melting point 226-233 ºC (decomposes). The fragmentation of compound (**1**) showed peaks specific for compounds containing hydroxyl group[19]. The most intense peak were at 162 (M^+), 134 (M^+ - 28) loss of one CO group, 106 (M^+ - 56) loss of 2 CO groups and 78 (M^+ - 84), thus the three oxygen atoms in compound **1** are evolved successively in the form of CO. ^{1}H NMR showed signals at 6.11 (1H, d, *J* = 9.5 Hz, H-3), 6.60 (1H, s, H-8), 6.66 (1H, d, *J* = 9.5 HzH-7), 7.30 (1H, d, *J* = 9.5 Hz, H-5), 7.65 (1H, d, *J* = 9.5 Hz, H-4), 9.9 (1H, s, OH). On the basis of the above data, the structure of compound **1** was elucidated as umbelliferone.



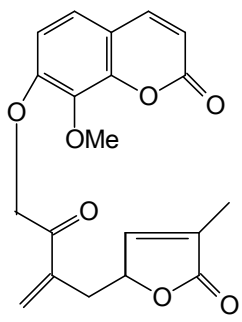
Umbelliferone



Bergapten



Xanthyletin



Hekumarone

Compound 2: Isolated as white needle crystals, R_f 0.52 with solvent system (50 % *n*-hexane:50 % $CHCl_3$), showed a yellowish green fluorescence under UV light, m.p. 185-188 ºC, ^{1}H NMR showed signals at δ ppm: 3.85 (3H, S, OCH_3), 6.2 (1H, d, *J* = 9.9, H-3), 7.01 (1H, d, *J* = 2.4 Hz, H-6 furan), 7.12 (1H, S, H-8), 7.40 (1H, d, *J* = 2.30 Hz, H-7 furan) and 8.12 (1H, d, *J* = 9.9 Hz, H-4) which were found to be identical to published data[20,21] of bergapten, so, compound No. **2** was identified as bergapten.

Compound 3: UV spectrum of third isolated compound in distilled methanol between 400-200 nm gives λ_{max}: 160 and 340 nm; λ min: 250 and 290 nm. NMR spectrum in $CdCl_3$ at 200 MHz showed the presence of two methyl groups at δ 1.46 ppm (singlet), two signals corresponding to two protons which can be attributed to a double bound conjugated to a carbonyl group. Two further doublets (*J* = 10 Hz) as well as two singlet of one proton each at δ 6.71 and δ 7.01 ppm suggested the presence of dimethyl chromene unit on an aromatic ring possessing two protons in *para* position. The above data strongly favoured as structure of coumarin with an annelated dimethyl chromene ring. This was further confirmed by a carbonyl absorption in the infrared at 1720 cm^{-1} (KBr).

The mass spectra indicated a molecular weight of 228 in agreement with a molecular formula of $C_{14}H_{12}O_3$. Comparison of above data with literature[21,22] confirmed the structure of xanthyletin.

For compound 4: The ^{1}H NMR spectrum (Table-1) showed resonances for characteristic doublets at δ H 6.33 and 7.65 (each *J* = 9.5 Hz) and δ H 6.97 and 7.20 (each *J* = 8.6 Hz), corresponding to H-3, H-4, H-5 and H-6 in the coumarin nucleus. Additional signals in the spectrum were consistent with three olefinic protons δ 6.37, 6.09 (each 1H, s) and 7.01 (1H, d, *J* =1.6 Hz), one oxymethylene group δ 5.57 (2H, s), two methylene protons δ 2.80, 2.62 and one olefinic methyl δ 1.77 could be seen. IR bands at 1,753, 1,748 and 1,730 cm^{-1} were indicative of the presence of three carbonyl groups, suggesting a ketonic group and a lactone in the side chain.

The ^{13}C NMR spectrum (Table-1) showed, in addition to the resonances of the carbons belonging to the coumarin nucleus, ten other signals arising from one methyl, one oxymethylene, one methylene, one oxygen-bearing methine, four olefinic and two carbonyl carbons that could only be located on the side-chain, confirmed the presence of a C_{10} terpenoid side-chain attached to the coumarin skeleton[23]. The connection of the side chain to C-7 on the coumarin nucleus was revealed by observation of a three-bond correlation between the oxymethylene proton signals (H-1') and carbon C-7 at δC 153.5 s. The presence of only one methoxyl signal at δ H 4.05, the MS spectra also showed characteristic 8-OMe coumarin fragment ion at m/z 193 corresponding to loss of side chain[24]. The molecular formula $C_{20}H_{18}O_7$ deduced from ^{13}C NMR spectrum and MS which exhibited a molecular ion at m/z 396 $(m-1)^+$, indicated 12º of unsaturation, strong UV band at λ_{max} 256 and 319, IR band at 1.730 cm^{-1} indicated the presence of 7,8- deoxygenated coumarins[23,25].

TABLE-1
^{1}H AND ^{13}C NMR DATA FOR COMPOUNDS **4** (δ IN ppm, *J* IN Hz)

	δH	δC
2	/	159.2 (s)
3	6.33 (d, 9.5)	112.7 (d)
4	7.65 (d, 9.5)	142.9 (d)
5	7.20 (d, 8.6)	122.3 (d)
6	6.97 (d, 8.6)	109.3 (d)
7	/	153.5 (s)
8	/	135.6 (s)
9	/	113.4 (s)
10	/	147.7 (s)
1'a, 1'b	5.58 (s)	69.6 (t)
2'	/	193.8 (s)
3'	/	139.6 (s)
4'a, 4'b	2.80 (dd, 14.1, 4.8) 2.62 (dd, 14.1, 9.4)	33.5 (t)
5'	5.12 (m)	78.2 (d)
6'	7.00 (d, 1.6)	147.6 (d)
7'	/	128.8 (s)
8'	/	172.7 (s)
9'	1.77 (s)	9.3 (q)
10'a, 10'b	6.37 (s), 6.09 (s)	128.3 (t)
OMe	4.05 (s)	60.0 (q)

Careful analysis of the ^{1}H and ^{13}C NMR data of Table-1 showed that the signal patterns were in good agreement with hekumarone.

The results of pharmacological activity showed that the alcoholic extract of *Cassia nodosa* leaves possessed significant blood sugar lowering effect in normal and treptozotocin-induced diabetic model rats and was dose dependent (Table-2). The potency ratio of *Cassia nodosa* leaf extract against tolbutamide in normal and induced diabetic model rats is presented in Table-2. In acute toxicity studies, no gross behavioural, neurologic and autonomic effects were observed in mice and the acute LD_{50} value was found to be 4750 mg/Kg, p.o. (Table-3). The isolated coumarines had no effect on blood sugar in normal and treptozotocin-induced diabetic model rats.

TABLE-2
EFFECT OF *Cassia nodosa* LEAF EXTRACT ON BLOOD SUGAR LEVEL OF NORMAL AND DIABETIC RATS

Treatment	Dose (mg/Kg p.o.)	Normal rats blood sugar (mg/dl)		Diabetic rats blood sugar (mg/dl)	
		0 h	2 h	0 h	2 h
Saline (mL/Kg, p.o.)	2	106.6 ± 4.3	104.4 ± 5.2	292.6 ± 9.5	288.5 ± 9.3
Leaf extract	50	103.3 ± 4.2	77.7 ± 4.3**	295.5 ± 11.3	251.0 ± 8.6
	100	101.2 ± 3.6	63.1 ± 3.7***	285.7 ± 8.3	190.8 ± 6.5***
	200	97.8 ± 4.6	43 ± 4.2***	281 ± 9.2	146.3 ± 6.5***
	400	106 ± 5.2	40.3 ± 3***	292.3 ± 6.7	106 ± 7.5***
Tlobutamide	20	101.4 ± 4.7	74 ± 4.5 **	287.6 ± 7.5	222.5 ± 8.2***
	40	104.6 ± 4.3	58.1 ± 3.7***	293.6 ± 7.5	187.4 ± 7.5***
	60	106.7 ± 4.6	46.3 ± 4.3***	291.5 ± 6.3	134.4 ± 8.3***
	80	103.7± 5.2	31.1 ± 4.6 ***	283.7 ± 7.2	95.9 ± 5.6***

Results are mean of six observation ± SEM. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

TABLE-3
COMPARATIVE EFFECT OF BLOOD SUGAR LOWERING ACTIVITY OF *Cassia nodosa* AND TOLBUTAMIDE

Treatment	Dose (mg/Kg, p.o.)	Normal rats			Diabetic rats	
		Blood sugar reduction (%)	LD_{50} (mg/kg, p.o.)	ED_{50} (mg/kg, p.o.)	Blood sugar reduction (%)	ED_{50} (mg/kg, p.o.)
Saline (mL/Kg, p.o.)	2	–	–	–	–	–
Cassia nodosa	50	25	4570	180	15	210
	100	39			33	
	200	56			48	
	400	62			64	
Tolbutamide	20	29	–	47	22	49
	40	44			36	
	60	57			54	
	80	71			67	

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