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A coumarin with an unusual structure from *Cuphea ignea*, its cytotoxicity and antioxidant activities

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Phenolic metabolite profiling using two dimensional paper chromatographic analysis (2 DPC) was used for assaying the complex mixture of phenolics of an aqueous ethanol aerial part extract of *Cuphea ignea* (Lytheraceae). A coumarin with a rare structure, namely, 7-hydroxy 3-methoxy coumarin 5-*O*-β-glucopyranoside was isolated from the investigated extract. The structure was elucidated by conventional methods and spectral analysis, including one and two dimensional NMR (1D and 2D NMR), as well as by interpretation of the spectra obtained by high resolution electrospray ionization mass technique (HRESIMS). The rare coumarin significantly inhibited reactive oxygen species production with an ED_{50} value of 6.31±1.64 µg/ml and 5.78±0.66 µg/ml as determined by the the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the oxygen radical absorption capacity (ORAC) assay respectively. The isolated coumarin presented a cytotoxic activity assessed by using the neutral red assay (NRU) against lung cancer cell line (H23) with IC_{50} of 40.38±2.75 µg/ml.

1. Introduction

Phenolics are an important type of substrates in the areas of natural products because they possess a wide range of biological activities (Wagner 1979). Therefore, we initiated research during which some selected folk medicinal plants were subjected to comprehensive phytochemical and biological investigations of their phenolics to evaluate their bioactivities and to isolate and identify some of them, specifically those which might possess unique structural features. Unique structures are of a major interest because they offer new chemical models to be taken as targets for comprehensive biological investigations and the subsequent simple chemical modifications or synthesis of them aiming to achieve new plant derived drugs. (e.g. Nawwar et al. 1997, 2012; Hussein et al. 2006). During the course of the present work, we applied the 2-DPC technique to determine the profile of the phenolics existing in the aqueous ethanol aerial part extract of *Cuphea ignea* and to discover compounds which might possess new structural features. Consequently, we isolated a novel coumarin that possesses an oxygen function at its carbon number 3, a feature which is quite rare among the natural coumarins (Venugopala et al. 2013), from the investigated *Cuphea ignea* (Lythraceae) extract, whereby there were no previous reports in concern with its phenolics. Also, we evaluated the antioxidant capacities using the DPPH and the ORAC method and assessed the cytotoxicity against the keratinocyte cell line HaCaT, normal lung cell line (MRC-9), non-small-cell lung cancers (Non-SCLC) (H460 and H23), human hepatocellular carcinoma cell line (Huh-7) and colorectal adenocarcinoma cell line (HCT-116) using the neutral red assay (NRU) for that extract and for the new natural coumarin (**1**), as well. *Cuphea ignea*, called Firecracker plant or Cigar Flower, is native to Mexico and the West Indies. It is a rounded, densely branched, bushy, evergreen sub-shrub that grows 20-30 cm tall and wide. Small, tubular, bright red flowers bloom singly in the leaf axils from late spring to frost along stems crowded with pointed, lance-shaped to ovate, dark green leaves (to 1.5 cm long). Each flower consists of a narrow, tubular, red calyx (no corolla) tipped with a thin white rim and two tiny purple-black petals, all of which is purportedly suggestive of a lit cigar (cigarette or firecracker). Flowers are attractive to humming birds and butte technique.
(Royal Horticultural Society)

2. Investigations, results and discussion

2.1. Isolation and structure elucidation of coumarin 1

The search for new, potentially biologically active compounds becomes more efficient after sorting out all the known structures in that extract. According to the received 2-DPC analytical data the phenolics of the aqueous ethanol whole plant extract of *Cuphea ignea* (Lythraceae) were found to be best fractionated over a polyamide S_6 column eluted by MeOH/H_2O mixtures of decreasing polarities, a process which afforded ten column fractions. An amorphous material was separated from a concentrate of fraction 3 (desorbed from the column by 30 % aqueous MeOH) on standing overnight at room temp. Crystallization of this material from EtOH yielded an off-white amorphous powder (**1**) of chromatographic properties, color reactions and UV absorption maxima in MeOH (λ_{max} nm: 260, 273, 358) similar to those of coumarins (Rafie 1985). Compound 1 exhibited an $[M-1]^-$ ion at *m/z* 369.3010, in the negative ESI-mass spectrum, corresponding to a *Mr* of 370 Dalton. The molecular formula was concluded to be $C_{16}H_{17}O_{10}$ from its negative HRESI-MS, which showed an $[M-1]^-$ ion at *m/z* 369.30107 (calculated for $C_{16}H_{17}O_{10}$: 369.300816). The ^1H NMR spectrum of (**1**) showed five signals and the correlations recognized in the hetero nuclear single quantum coherence (HSQC) spectrum together with the attached proton test (APT) measurement and the recorded ^{13}C NMR spectrum allowed the definition of these protons as follows: three sp^2 methine protons, two of which are arranged in an AX system at δ (ppm) 6.18 (d, J = 2 Hz) and 6.21 (d, J = 2 Hz), together with a methine proton singlet at δ (ppm) 7.22. In addition, a fourth anomeric methine sugar proton resonance was recognized at δ 4.7

Table 1: NMR spectral data of coumarin 1

Number	δ H [1] (J, Hz)	δ[13]C and APT	HMBC*
2		160.93 (quaternary)	
3		146.00 (quaternary)	
4	7.22 (s)	107.21 (methine)	2, 5, 9
5		156.89 (quaternary)	
6	6.21 (d, 2 Hz)	96.86 (methine)	10, 8,
7		158.89 (quaternary)	
8	6.19 (d, 2 Hz)	92.07 (methane)	10, 6
9		156.39 (quaternary)	
10		105.29 (quaternary)	
1'	4.7 (d, 7.5 Hz)	101.30 (methine)	5
2'		73.53 (methine)	
3'		77.19 (methine)	
4'		70.25 (methine)	
5'		77.53 (methine)	
6'		61.17 (methylene)	
OMe	3.7 (s)	55.72 (OMe)	3

(d, J = 7.5 Hz). The spectrum also showed a fifth singlet, integrated to three protons resonating at δ ppm 3.7, thus being assigned to a methoxyl signal. The pattern of proton signals in the aromatic region of the ^{1}H NMR spectrum when incorporated in the above given analytical data including the measured APT was reminiscent of a α-benzopyrone system, bearing di-*meta* hydroxyl group in its aromatic ring and a substituent at its C-3 pyrone carbon, thus leaving a protonated carbon at position number 4 of the pyrone moiety (singlet at δ 7.22 ppm). This view was supported by ^{13}C, homonuclear chemical shift correlation (COSY), HSQC and the hetero nuclear multi bond connectivity HMBC) NMR data (Table 1). Among the sixteen carbon signals recognized in the APT spectrum of **1** six possessed chemical shift values consistent with 5,7-dioxygenated aromatic moiety in benzopyrone in which position 5 is substituted by a glucoside moiety whose 6 carbons were found resonating at δ ppm: 101.3 (C-1'), 73.19 (C-2'), 77.19 (C-3'), 70.25 (C-4'), 77.53 (C-5'), 61.17 (C-6'). The data obtained from the NMR experiments for the remaining three carbon signals number 2, 3 and 4 (in the pyrone moiety) were found to be best interpreted in terms of: 2 (C=O), 3 (C-OMe) and 4 (-C=). The latter is connected to the aromatic carbon number 10 (see formula) thus forming the molecule of **1** in which the methoxyl carbon was located at δ 55.72 ppm. Among the ^{3}J correlations recognized in the HMBC spectrum, one was found correlating the methine H-4 proton signal at δ 7.22 to the carbonyl C-2 carbon signal at δ 160.93 and to the aromatic carbons C-5 and C-9 signals at δ 156.89 and δ 156.39, respectively. Another ^{3}J correlations correlated the anomeric H-1' glucose proton signal at δ 4.7 to C-5 of the aromatic moiety at δ 156.89 ppm and another correlated the methoxyl protons singlet at δ 3.7 ppm to the sp2 C-3 carbon signal at δ 146.00. Detailed direct correlations observable in the HMBC spectrum are given in Table 1. In the ^{1}H spectrum, the resonances of the AX system at δ 6.19 (^{13}C at 92.07 ppm, assignable to C-8) and 6.22 ppm (^{13}C at 69.86 ppm, assignable to C-6) together with the lowfield olefinic proton singlet resonance at δ 7.22 (^{13}C at 104.21 ppm, assignable to C-4) all are attributable to the trioxygenated benzopyrone **1**. Besides, the splitting of the two AX proton signals into two individual doublets each of J = 2 Hz confirmed its *meta* configuration. The β configuration of the glucoside moiety was derived from its C-l chemical shift at δ 101.3 ppm (Garcia-Granados et al. 1980) and the chemical shift values of all

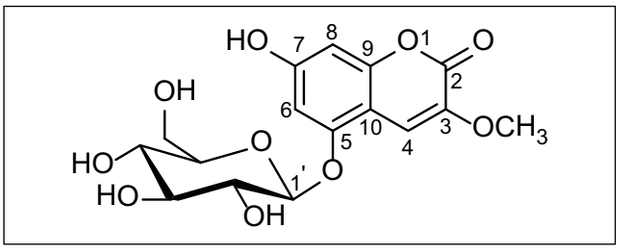

Fig.: 7-Hydroxy 3-methoxy coumarin 5-O-β-glucopyranoside (**1**)

the glucoside carbons confirmed the pyranose form of this moiety. These and the above given data finally confirmed the structure of compound **1** to be 7-hydroxy 3-methoxy- coumarin 5-O-β-glucopyranoside, a new coumarin, which has not been previously reported to occur in nature.

2.2. Biological activity

In order to get information about biological activities of *C. ignea* the extract and the new coumarin were tested for radical scavenging activity by DPPH and by ORAC assays and for cytotoxicity on HaCaT keratinocytes, normal lung cell line (MRC-9), non-small-cell lung cancers (Non-SCLC) (H460 and H23), human hepatocellular carcinoma cell line (Huh-7) and colorectal adenocarcinoma cell line (HCT-116) by neutral red assay (NRU). The ED_{50} of the extract for radical scavenging activity in DPPH assay was 11.41±1.37 µg/ml and in ORAC assay 13.21±2.56 µg/ml. 7-Hydroxy 3-methoxy coumarin 5-*O*-β-glucopyranoside considerably contributed to this effect with an ED_{50} value of 6.31±1.64 µg/ml and 5.78±0.66 µg/ml in the DPPH and ORAC assay, respectively. The latter value is lower than that of the positive control Trolox which had an ED_{50} of 27.0±13.41 µg/ml. The IC_{50} values for cytotoxicity of the extract and the new coumarin are given in Table 2.The vehicle in which the test samples were dissolved had no influence on the measured parameters. Using etoposide (positive control for cytotoxicity) viability of the three tested cell lines were reduced to 40 to 60%. The extract and the new coumarin showed the highest cytotoxic activity against a lung cancer cell line (H23) with IC_{50} of 32.44±5.23 µg/ml and 40.38±2.75 µg/ml, respectively.

3. Experimental

3.1. General

NMR spectra were acquired in DMSO-d_6 on a Bruker Avance 400 NMR spectrometer, at 400 MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ^{1}H and ^{13}C APT, and two dimensional HSQC and HMBC spectra. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to DMSO-d_6 and were converted to TMS scale by adding 39.49. High resolution ESIMS spectra were measured on a Finnigan LTQ-FTMS (Thermo Electron, Bremen, Germany) (Department of Chemistry, Humboldt-Universität zu Berlin). ORAC measurements were performed on a FLUOstar Omega Microplate Reader - BMG LABTECH. UV recording was made on a Shimadzu UV–Visible-1601 spectrophotometer. Chromatographic analysis (PC) was carried out on Whatman No. 1 paper, using solvent systems: (1) H_2O; (2) 6% HOAc; (3) BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, upper layer). For 2- DPC, solvent 2 was used for the first way and solvent 3 for the second way.

3.2. Plant materials

Samples of *Cuphea ignea* were collected from a garden, 25 km north of Cairo, on May, 2016 and identified by Prof. Salwa Kawashty at the Department of Phytochem-

Table 2:Cytotoxicity (IC50 µg/mL) of *Cuphea ignea* extract and the new isolate (1)

	HaCaT keratinocytes	Colon cancer (HCT 116)	liver carcinoma cell line (Huh-7)	Normal lung cell line (MRC-9)	Non-SCLC (H460)	Non-SCLC (H23)
Extract	397.34±19.83	70.88 ± 0.62	98 ± 2.91	483.65 ±13.43	37.76 ± 3.41	32.44 ± 5.23
7-hydroxy 3-methoxy coumarine 5-*O*-β-glucopyranoside (**1**)	220.52 ±28.83	59.29±6.21	66.39±2.39	340.67±22.21	45.56±1.61	40.38±2.75

istry and Plant Systematic, National Research Centre (NRC), Cairo, Egypt. A voucher specimen (C 182) has been deposited at the herbarium of the NRC.

3.3. Preparation, fractionation of extract and isolation of coumarin 1

A fresh whole plant sample of *C. ignea* (2 kg) was extracted with hot EtOH/H_2O (3:1, 3 times, each with 3 l, for 8 h, under reflux). The solvent was evaporated under reduced pressure. The resulting dry sticky material thus left (201 g), dissolved in 150 ml H_2O, was applied to a Polyamide S_6 chromatographic column (Riedel-de Haen, Seelze, Hannover, Germany) and eluted with H_2O, followed by H_2O/MeOH mixtures of decreasing polarity to yield ten major fractions (I – X). Fraction I, eluted with H_2O (22 g), fraction II, eluted with 10 % aqueous MeOH (3.1 g), fraction III, eluted with 30 % aqueous MeOH (3.6 g), fraction IV, eluted with 40 % aqueous MeOH (2.7 g), fraction V, eluted with 50 % aqueous MeOH (1.9 g), fraction VI, eluted with 60 % aqueous MeOH (3.3 g), fraction VII, eluted with 70 % aqueous MeOH (2.5 g), fraction VIII, eluted with 80 % aqueous MeOH (4.1 g), fraction IX eluted with 90 % aqueous MeOH (2.1 g), fraction X, eluted with MeOH (4.7 g). Concentration of the eluted solution of column fraction number 3 and leaving to stand overnight afforded a crude sample of compound **1** (67 mg) which is filtered on and crystallized from EtOH to afford pure sample of **1** (54 mg).

3.4. Identification of coumarin 1

Compound **1** (41 mg) was obtained as an off-white powder; *Rf*-values: 0.47 (H_2O), 0.56 (6 % HOAc), 0.73 (BAW). UV λ_{max} nm in MeOH: 260, 273, 358. HRESI-FTMS (negative ions) of **1**: $[M - 1]^-$ ion at *m/z* 369.3010, in the negative ESI-mass spectrum, *Mr* = 370 dalton. Molecular formula: $C_{16}H_{17}O_{10}$ from its negative HRESI-MS: $[M–1]^-$ ion at *m/z* 369.30107 (calculated for $C_{16}H_{17}O_{10}$: 369.300816). NMR: ^{1}H, APT, HSQS and HMBCP: Table I.

3.5. Biological assays

3.5.1 Determination of radical scavenging activity (DPPH assay)

The estimation was done according to the method of Brand-Williams and Cuvelier (1995). DPPH (2,2-diphenyl-1-pycrylhydrazyl), a stable radical, is reduced after reaction with an antioxidant compound and its absorbance at 517 nm is then reduced. The reaction mixture contained 500 µl of test extract, 375 µl ethanol and 125 µl of a 1 Mm freshly prepared DPPH solution in ethanol. Different concentrations of test samples were prepared while the final concentration of DPPH in the reaction mixture was 0.125 mM. After incubation of the mixture at 37 °C for 30 min in the dark the absorbance was measured at 517 nm. Blank samples contained the same amount of methanol and DPPH solution. All experiments were carried out in triplicate. Ascorbic acid was used as a positive control. Percentage radical scavenging activity of samples was calculated using the radical scavenging activity:

$$(\%) = \frac{A_{blank} - A_{Sample}}{A_{blank}} 100$$

ED_{50} values, the concentration of the substrate that causes 50% loss of the DPPH activity (color), were calculated for the standard and the extract from a graph plotted for the % inhibition against the concentration in µg/ml.

3.5.2. Oxygen radical absorbance capacity (ORAC assay)

Reactive oxygen species, ROS are generated by the thermal degradation of 2,2¨-azobis(2-amidinopropane) dihydrochloride (AAPH) and quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of fluorochrome (Lucas-Abellán et al. 2008). Avitamin E derivate, 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), was used as positive control. Test compounds **5** and **6** were dissolved in phosphate buffered saline (10 mM, pH 7.4) and investigated for their antioxidant capacity. Experiments were done in black 96-well plates. In each well of a 96-well Plate 150 µl fluorescein (final concentration: 2.5 nM), 25 µl Trolox (final concentrations: 0.78 – 25 µM) or 25 µl test compound were pipetted in quadruplicate. The plate was allowed to equilibrate at 37 °C for 30 min. After this time, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 s; first to determine the background signal. After three cycles, 25 µl AAPH (final concentration: 60 mM) were added manually in each well with a multi-channel-pipette. This was done as quickly as possible since the ROS generator displays immediate activity after addition. Fluorescence measurements were continued for 90 min. Half life time of fluorescein was determined using MS Excel software.

3.5.3. Cytotoxicity assay

Non tumorigenic HaCaT keratinocytes, normal lung cell line (MRC-9), non-small-cell lung cancers (Non-SCLC) (H460 and H23), human hepatocellular carcinoma cell line (Huh-7) and colorectal adenocarcinoma cell line (HCT-116) were obtained from the Vaccera (Giza, Egypt). Cells were cultured in RPMI 1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 8 % fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) and antibiotics (100 U/ml penicillin/100 µg/ml streptomycin; Sigma Aldrich, Taufkirchen, Germany) at 95% humidity, 5% CO_2 and 37 °C. HaCaT cells were subcultured twice a week and regularly tested for mycoplasma. Cytotoxicity of test samples against the four cell lines was investigated using the neutral red uptake (NRU) assay (Lindl et al. 1989). After 24 h cultivation in 96 well plates (3 or 8 x 10^3 cells/well) medium was removed and cells were exposed for 72 h to various concentrations (max. 500 µg/ml) of test samples. After removal of the medium wells were washed with HBSS (Hanks Balanced Salt Solution, PAA). Cells were than incubated for 3 h with 100 µl 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, Merck, Darmstadt, Germany, stock solution 3.3 µg/ml; working solution 33 ng/ml). Medium was removed and wells were washed twice with HBSS. Afterwards cells were lysed with 100 µl of 1% acetic acid in 50% EtOH. Finally, after 45 min optical density was measured at 450 nm in a plate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany). The IC_{50} values were defined from obtained dose-response curves and expressed in mean±SD. All samples were tested in duplicate. Etoposide (Alexis Biochemicals, ≥ 98 % purity) was used as positive control.

Conflicts of interest: None declared.

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