Original Article

A new furoquinoline alkaloid with antifungal activity from the leaves of *Ruta chalepensis* L.

Ahmed Emam[1,*], Mohamed Eweis[2], Medhat Elbadry[3]

[1] *Department of Biochemistry, Faculty of Agriculture, Fayoum University, Fayoum, Egypt;*
[2] *Department of Botany, Faculty of Science, Cairo University, Cairo, Egypt;*
[3] *Department of Agricultural Microbiology, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.*

ABSTRACT: Bioassay-guided separation with an eye toward antifungal activity led to the isolation of the new alkaloid 5-(1̀,1̀-dimethylallyl)-8-hydroxy-furo[2-3-b] quinoline (1) and the known bis-coumarin daphnoretin (2) as the active constituents of the chloroform extract obtained from the leaves of *Ruta chalepensis*. The structures of the metabolites were elucidated on the basis of their spectral characteristics (NMR, UV, and MS) and were compared with the literature. The antifungal activity of the isolated compounds was evaluated against the phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii*, and *Fusarium solani*, which cause root-rot and wilt diseases in several economically important food crops such as potato, sugar beet, and tomato.

Keywords: Furoquinoline alkaloid, bis-coumarin, *Ruta chalepensis*, Rutaceae, phytopathogenic fungi

1. Introduction

The increasing demand for food by the world's rapidly expanding population is exacerbated by the inevitable and substantial loss of crops due to plant diseases. Phytopathogenic fungi are among the most serious agriculture pests, and synthetic fungicides continue to be the least expensive and thus the most common approach for crop protection (*1*). Despite the effectiveness of these fungicides, the widespread use of chemicals has triggered public concern and scrutiny, mainly due to their persistence in the environment, toxicity to mammals and humans, harmful effects on non-target organisms, and induction of resistance and development of resistant strains of pathogens (*2*). Additionally, the number of new pesticides in the last years has decreased mainly due to rising development and registration costs (*2*). As these facts demonstrate, there is an urgent need for new, safer, and ecologically compatible antifungal agents.

Over the past few years, significant efforts have been made to evaluate the effectiveness and safety of plant extracts and/or their metabolites for use in controlling plant diseases. Screening of plant extracts allows fast detection of potential sources of new bioactive molecules that can have applications in medicine or the control of agricultural pests (*3*).

Ruta chalepensis L. (syn. *Ruta bracteosa* DC., *Ruta angustifolia* Pers.), an evergreen shrub with the common name "Egyptian rue" indigenous to the Mediterranean region, is now cultivated in many parts of the world (*4*). The biological activities of *R. chalepensis* are frequently utilized in herbal therapy and the plant is used as a promoter of menstruation, treatment for hypertension, a topical treatment for ear aches and headaches, and an external treatment in the form of a skin antiseptic and insect repellent (*5*,*6*). Rue's active ingredients have antifungal and insecticidal properties that could prove beneficial to agriculture as well (*7*,*8*). Previous phytochemical investigation of this plant resulted in the isolation of a number of alkaloids and coumarins (*9*-*11*). Using an aqueous extract of the plant's leaves, a recent study isolated two flavonoids, which are thought to be responsible for its antioxidant activity (*12*), and two furoquinoline alkaloids, which are thought to be responsible for its antibacterial activity (*13*).

The objective of the present study was to detect and isolate new metabolites with antifungal activity from a chloroform extract of *R. chalepensis* leaves and then evaluate their inhibition of *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Fusarium solani* since these phytopathogenic fungi cause root-rot and wilt diseases in several economically important crops.

2. Materials and Methods

2.1. *General experimental procedures*

Analytical and preparative thin-layer chromatography

**Address correspondence to:*
Dr. Ahmed M. Emam, Department of Biochemistry, Faculty of Agriculture, Fayoum University, Fayoum 63514, Egypt.
e-mail: maoudimam@yahoo.com

(TLC) was carried out on Merck precoated silica gel plates (F_{254} thickness: 0.25 mm and 2.0 mm, respectively). Spots were visualized under ultraviolet (UV) light (254 and 365 nm) and by spraying with 30% H_2SO_4 in methanol followed by heating at 105°C for 5 min and by spraying with modified Dragendorff's reagent to detect alkaloids. The preliminary phytochemical screening of the purified compounds for saponins, flavonoids, alkaloids, tannins, and glycosides was performed on TLC plates according to the methods described (*14*). ^1H, ^{13}C, and heteronuclear multiple bond correlation (HMBC) nuclear magnetic resonance (NMR) spectra were recorded in dimethyl sulfoxide (DMSO)-d_6 on a Varian Mercury VXR 300 spectrometer (300 MHz for ^1H and 75 MHz for ^{13}C; Varian, Inc., Palo Alto, CA, USA). NMR experiments were performed at the Central Laboratory of the Faculty of Science, Cairo University, Giza, Egypt. UV spectra were recorded on a Cecil 3000 Series spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). Mass spectra (MS) were recorded on a GC/MS QP 100 EX Shimadzu Mass spectrometer (Shimadzu, Kyoto, Japan) at 70 eV. The MS experiments were carried out at the Macro-Analytical Center, Faculty of Science, Cairo University.

2.2. *Plant material*

Leaves of *R. chalepensis* (Rutaceae) were collected in the flowering stage in June 2004 from plants growing on the experimental farm of the Faculty of Agriculture, Cairo University, Giza, Egypt. Plant taxonomists in the Botany Department, Faculty of Science, Cairo University confirmed the taxonomic identification of the plant species. A vouchered specimen (R.C. 30) was deposited in the herbarium of the Department of Biochemistry, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.

2.3. *Extraction and isolation*

Powdered air-dried leaves (350 g) were extracted three times overnight with 80% ethanol (700 mL each time) at room temperature (25 ± 2°C). After filtration, the combined extracts were evaporated under reduced pressure to yield 55.2 g of residue. A portion of the ethanol extract (40 g) was dispersed in water (150 mL) and partitioned with chloroform (3 × 50 mL) to remove the chloroform-soluble components (Fr A, 6.5 g). The aqueous layer was freeze-dried (33.5 g) and then extracted with chloroform:methanol:water (70:30:5, v/v; 150 mL). After centrifugation, both the supernatant and the precipitate were dried under reduced pressure to yield 4.6 g (Fr B) and 28.8 g (Fr C), respectively. Fractions A, B, and C were subsequently tested for their antifungal activity.

Bioactive fraction A (6.5 g) was subjected to chromatography in order to isolate antifungal components as follows: 6.0 g of Fr A were loaded on a chromatographic column (2 cm × 60 cm) packed with silica gel (230-400 mesh, 150 g, Merck & Co., Inc., Whitehouse Station, NJ, USA) and eluted with a gradient of chloroform:methanol:water (100:0:0, 90:10:0, 85:15:0, 70:30:5, 60:40:5, and 0:100:0, v/v; 200 mL for each eluent). Ten fractions of each eluent were collected. Because of their similarities in TLC, the collected fractions were combined with 17 fractions that were further tested for antifungal activity. Two fractions (1 and 2) exhibited strong antifungal activity. Fraction 1 eluted with 100% chloroform (441 mg) was further purified on silica gel with chloroform:benzene (50:50, v/v) as the mobile phase to provide six fractions (A'-F'). Fraction A' (341.8 mg) containing the major metabolite was further purified on a Sephadex LH 20 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) with methanol followed by preparative TLC with chloroform:benzene (60:40, v/v) to yield 275 mg of pure active compound **1**. Fraction 2 also eluted with 100% chloroform (870 mg) was further purified on silica gel using chloroform:benzene (80:20, v/v; 400 mL). On the basis of their similarities in TLC, 40 fractions were combined with 10 fractions (G'-P'). Fraction I' (410.2 mg) was further purified several times on Sephadex LH 20 with methanol as the eluent followed by preparative TLC with chloroform:benzene (70:30, v/v) to give 226 mg of pure active compound **2**.

2.4. *Test organisms*

Fungicidal activity tests were performed with *R. solani, S. rolfsii*, and *F. solani*. These fungi had been isolated from diseased sugar beet roots and identified and their pathogenicity had been confirmed (*15*). The cultures were maintained on malt extract agar (MEA) medium (malt extract 20 g, peptone 5 g, and agar 15 g per liter of culture) and covered with phosphate buffer (pH 6.5) at 4 ± 1°C (*16*).

2.5. *Preliminary fungicidal activity tests*

The disk diffusion technique was used to test the fungicidal activity of the aqueous ethanol extract of *R. chalepensis* leaves and its fractions against each of the three phytopathogenic fungi on MEA medium at 25 ± 1°C (*17*).

2.6. *Evaluation of the antifungal activity of the isolated compounds*

2.6.1. *Percent germination of sclerotia*

Sclerotia of *R. solani* and *S. rolfsii*, produced on potato dextrose agar (PDA), were surface-disinfected by soaking them for 5 min in bromine/water (1:400, w/v)

to kill hyphal extensions, washed thoroughly with distilled water, and dried. Ten sclerotia per Petri dish of each pathogen were plated on an agar surface (1.5%, w/v) supplemented with relevant amounts of each purified compound to produce concentrations in the range of 10-40 µg/mL in the medium (*15*). The dishes were incubated at 27°C for 16 h for *R. solani* and 30 h for *S. rolfsii*, and the percentage of germinated sclerotia was determined and the average length of hyphal elongation was measured at 45× magnification using a calibrated micrometer in the microscope eyepiece. Five plates were prepared for each treatment and the means were compared.

2.6.2. *Percent germination of conidial spores*

Glass slides were supported on a glass rod in Petri dishes lined with moist filter paper. One mL of spore suspension (1×10^5 conidia/mL) of *F. solani* in an aqueous solution with the desired compound concentration or with distilled sterile water (DSW) as a control (check) was placed on each slide. Slides were then incubated at 27°C for 16 h in complete darkness. The percent germination was assessed and the average length of the germ tubes was measured at 45× magnification using a calibrated micrometer in the microscope eyepiece (*18*). Five slides were used per each treatment and the means were compared.

2.6.3. *Production of sclerotia and conidiospores*

Each purified compound was mixed aseptically with MEA medium in amounts calculated to produce the required concentration and then poured into Petri dishes. The fungi *R. solan*i and *S. rolfsii* that produced sclerotia and *F. solani* that produced conidiospores were grown in the dark on PDA at 27°C for 4 days. Four-mm diameter agar plugs were removed from the leading edges of colonies with a sterile cork borer, and one such plug was placed in the center of a 90-mm Petri dish (plate) containing MEA medium of the required concentration. Plates were wrapped with parafilm and incubated at 27°C for 9 days (*15*). For *R. solani* and *S. rolfsii*, the number of sclerotia produced per plate was visually counted for each treatment. For *F. solani*, the number of spores produced was calculated with a hemacytometer (*15*). Five plates were prepared for each treatment and the means were compared.

2.6.4. *Dry mass of mycelia*

Each purified compound was mixed aseptically with the malt extract broth MEB medium in amounts calculated to produce concentrations of 10-40 µg/mL and dispensed in 50 mL aliquots into 250-mL Erlenmeyer flasks. The fungi *R. solani*, *S. rolfsii*, and *F. solani* were grown in the dark on PDA at 27°C for 4 days. A four-mm diameter agar plug was removed and incubated at 27°C for 9 days as described above. The mycelia were harvested, dried to constant weight at 80°C, and then the dry mass yield and final pH value were recorded. Five flasks were prepared for each treatment and the means were compared. The natural antifungal agent chitosan (9012-76-4; Sigma-Aldrich, St Louis, MO, USA) at a concentration of 20 µg/mL was used as a positive control.

2.7. *Statistical analysis*

Experiments were conducted 5 times, and the results obtained were submitted to analysis of variance. Significance was expressed as the least significant difference (LSD) at levels of 5 and 1%.

3. Results and Discussion

The ethanol extract (80%) of the *R. chalepensis* leaves exhibited antifungal activity against the three phytopathogenic fungi *R. solani*, *S. rolfsii*, and *F. solani* when tested with the disk diffusion technique. The inhibition zone diameter was 10, 18, and 12 mm, respectively. The active ethanol extract (80%) of *R. chalepensis* leaves was fractionated into three fractions with chloroform (Fr A, 6.5 g), and then extracted with chloroform:methanol:water to yield 4.6 g (Fr B) and 28.8 g (Fr C), respectively. Fractions A, B, and C were tested for antifungal activity with the disk diffusion technique. Among the tested fractions, only the chloroform-soluble component (fraction A') possessed antifungal activity against the three pathogenic fungi. Bioactivity-guided purification of this fraction with chromatography led to the isolation of two compounds, **I** (275 mg, 4.58%) and **II** (226 mg, 3.77%), in pure form.

Metabolite **1** was obtained as a colorless amorphous powder and had a positive color reaction with modified Dragendorff's reagent on TLC, suggesting an alkaloid. Electron ionization MS revealed a molecular ion peak (M^+) at *m/z* 253 (100%), indicating a molecular formula of $C_{16}H_{15}NO_2$, which agreed with the results of elemental analysis. The UV spectrum of this compound indicated a conjugated aromatic system (287 and 234 nm), and this was confirmed by the NMR spectra.

The furoquinoline skeleton of this compound was established because of the presence of 6 aromatic, 2 quaternary (δ 138.7 and 158.8 ppm), and 3 olefinic carbons (δ 98.2, 106.5 and 150.6 ppm) as well as the presence of 3 olefinic proton signals at δ 7.03 (1H, d, J = 2.1 Hz), 8.04 (1H, d, J = 2.01 Hz), characteristic of furan ring protons (H-3 and H-2), and at δ 7.59 (1H, s; H-4) along with two adjacent aromatic protons coupled with each other at δ 7.93 ppm (2H, d, J = 6.3 Hz; H-6 and 7). The presence of only two aromatic protons

indicated that all other carbons were substituted. These data matched the furoquinoline skeleton of many alkaloids isolated from the Rutaceae family (*19-21*).

The presence of a dimethyl allyl group was indicated by resonances in the ^{1}H-NMR spectrum corresponding to a pair of geminal methyls at δ 1.14 (6H, s) and a vinylic group at δ 5.05 (2H, ddd, J = 1.2, 10.5, 17.4 Hz) and 6.14 (1H, dd, J = 11.1, 17.4 Hz) as well as by comparison with published metabolites having the same functionality (*22*).

The presence of a hydroxyl group was established by elemental analysis as well as by the presence of a bathochromic shift in the UV spectrum upon addition of sodium acetate. The position of this hydroxyl group was established by comparison to chemical shifts reported in the literature (*19-21*).

The ^{1}H and ^{13}C-NMR data for this compound were similar to those for a furoquinoline alkaloid (robustine) isolated from *Philotheca deserti* belonging to the family Rutaceae (*21*) but differed due to the absence of the methoxy group at C-4 and the presence of the 1̀,1̀-dimethylallyl group at C-5. On the basis of these findings, the furoquinoline alkaloid (**I**) was deduced to be 5-(1̀,1̀-dimethylallyl)-8-hydroxy-furo [2-3-b] quinoline (**1**).This structure was confirmed by heteronuclear correlations obtained from HMBC data (Table 1).

Metabolite **2** was obtained as a colorless amorphous powder. The mass spectrum had a molecular ion peak at m/z 352 in accordance with the molecular formula $C_{19}H_{12}O_7$. This compound was identified as the bis-coumarin daphnoretin by comparing its ^{1}H and ^{13}C- NMR data with those previously reported for daphnoretin isolated from the Rutaceae family (*23,24*). This is the first report of daphnoretin from *R. chalepensis*.

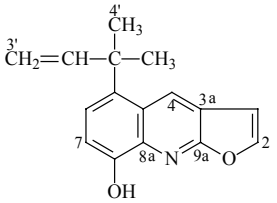
The antifungal efficacy of the isolated compounds 5-(1̀,1̀-dimethylallyl)-8- hydroxy-furo [2-3-b] quinoline (compound **1**) and daphnoretin (compound **2**) against the three phytopathogenic fungi was studied *in vitro* and the results are shown in Table 2. The data obtained indicated that the percent germination (G %) of *R. solani* and *S. rolfsii* sclerotia decreased with an increasing concentration of the two compounds. Metabolite **2** was responsible for strong inhibition of sclerotia or spore germination (79.83%, 83.7%, and 88%) whereas lower inhibition in the range of 70.03%, 76.7%, and 70.7% was achieved with compound **1** for *R. solani*, *S. rolfsii*, and *F. solani*, respectively, at a concentration of 20 µg/mL. However, *S. rolfsii* was found to be more sensitive to compounds **1** and **2** than the fungus *R. solani* was.

The average length of hyphal extensions (L_h µm) and dry mass yield (D_m mg) were also affected. Inhibition increased proportionally with the concentration of compounds **1** and **2**. Compound **2** was found to be more effective than compound **1** in this respect.

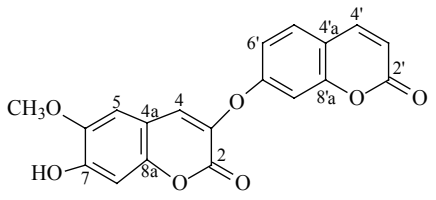
The number of *R. solani* and *S. rolfsii* sclerotia produced per plate at different concentrations was

Table 1. NMR data for compound 1 (in DMSO-d_6)

Atom No.	^{13}C	^{1}H	HMBC
1	–	–	
2	150.6	8.04 (d, 2.1)	C3, C3a
3	106.5	7.03 (dd, 0.9, 2.1)	C2 , C9a, C4
3a	138.7	–	
4	98.2	7.59 (s)	C3a, C4a, C9a, C8a, C5, C3
4a	131.7	–	
5	124.2	–	
6	120.3	7.93 (d, 6.3)	C5, C7, C1', C8, C4a
7	115.5	7.93 (d, 6.3)	C6, C8, C5, C8a
8	155.1	–	
8a	145.3	–	
9	–	–	
9a	158.8	–	
1	43.3	–	
2	111.9	6.14 (dd, 11.1, 17.4)	C'1, C'3, C5, C'4, C'5
3	147.5	5.05 (ddd, 1.2, 10.5, 17.4)	C2', C1'
4	25.9	1.41 (s)	C1', C5, C2'
5	25.9	1.41 (s)	C1', C5, C2'



Compound **I**



Compound **II**

Table 2. Effect of metabolites 1 and 2 on the germination (G%), average length of hyphal extensions (L_h), dry mass yield (D_m), and production of sclerotia of *R. solani* and *S. rolfsii* and macroconidia of *F. solani*

Treatment	Metabolite 1 (μg/mL)				Metabolite 2 (μg/mL)			
	G%	L_h (μm)	D_m (mg)	Number of sclerotia or macroconidia*	G%	L_h (μm)	D_m (mg)	Number of sclerotia or macroconidia*
R. solani								
Positive control	47.2	707.5	869.1	48				
Negative control	71.4	877.1	1108.1	63	71.4	877.1	1108.1	68
10	38.1	574.1	791.2	44	22.6	385.2	587.3	23
20	21.4	380.2	583.4	22	14.4	130.2	241.2	13
30	13.2	128.7	234.1	14	4.5	98.2	160.2	7
40	4.8	101.2	171.3	7	–	–	–	–
LSD at 1%	3.8	12.8	16.5	6.1	3.9	11.8	15.3	6.0
LSD at 5%	1.4	7.6	8.7	2.8	1.5	7.5	7.8	3.1
S. rolfsii								
Positive control	42.4	502.7	470.4	500				
Negative control	58.6	682.4	676.1	591	58.6	682.4	676.1	591
10	26.4	383.1	352.1	339	14.2	190.2	182.1	140
20	13.6	198.4	181.2	145	9.5	140.2	131.2	110
30	10.5	147.3	135.6	115	3.8	62.2	84.1	80
40	3.4	61.2	82.1	85	–	–	–	–
LSD at 1%	4.3	14.1	16.7	6.2	4.4	11.2	10.7	6.2
LSD at 5%	1.5	8.6	8.7	2.6	1.6	7.3	6.2	2.6
F. solani								
Positive control	44.8	9.1	301.8	260.7				
Negative control	60.1	13.6	399.2	332.7	60.1	13.6	399.2	332.7
10	21.3	8.9	229.7	196.1	14.5	7.8	181.2	158.2
20	17.6	7.6	190.2	159.6	7.6	5.3	142.1	130.2
30	10.9	5.9	160.1	131.1	5.4	3.9	110.1	93.2
40	8.9	4.0	122.1	100.2	–	–	–	–
LSD at 1%	4.1	2.4	9.6	12.5	4.0	2.5	8.3	12.1
LSD at 5%	2.2	1.3	5.5	7.2	2.1	1.2	4.6	7.6

* Data represent the number of sclerotia per plate of *R. solani* and *S. rolfsii* or macroconidia (× 10^4/mL) of *F. solani.*

reduced proportionally with increased concentrations of compound **1** or compound **2**. Compound **2** was responsible for a respective reduction in sclerotia of 80.8% and 81.4%, whereas compound **1** was responsible for a respective reduction in *R. solani* and *S. rolfsii* of 65.07% and 75.4% at a concentration of 20 μg/mL.

The number of conidial spores of *F. solani* per plate decreased with increasing concentrations of compound **1** or compound **2**. Maximum reduction was observed with a concentration of 30 μg/mL of metabolite **2**. Compound **2** reduced the conidial spores by 60.8% while **1** was responsible for a 52% reduction in *F. solani* at a concentration of 20 μg/mL.

Furoquinolines and coumarins are widely distributed in the plant kingdom and are present in notable amounts in the Rutaceae family. These classes have been reported to exhibit a wide array of interesting biological activities (antiplatelet aggregation, cytotoxic activity, inhibition of various enzymes, and antiviral, antibacterial, and antifungal activity) (*25*,*26*).

Daphnoretin has been found to have antineoplastic activity against Ehrlich ascites carcinoma (*23*), but no prior reports have noted its fungicidal activity against the phytopathogenic fungi *R. solani*, *S. rolfsii*, and *F. solani.*

The current data suggest that alkaloids and coumarins might play an important role in the rue plant's chemical defense against plant pathogens. In the future, the two isolated metabolites can be used as a starting point to provide natural product-based fungicides that control pathogens affecting economically significant plants.

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