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Antileishmanial and antifungal acridone derivatives from the roots of *Thamnosma rhodesica*

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Abstract

Eight furanocoumarins, one coumarin and four acridone derivatives have been identified in the roots of *Thamnosma rhodesica* (Rutaceae). Rhodesiacridone, one of these acridone derivatives, is reported here for the first time. Its structure was elucidated by spectrometric methods including ESI-HR, EI, DCI mass spectrometry, ^{1}H, ^{13}C and 2D NMR experiments. This novel compound showed activities against the intracellular form of a human pathogen, the protozoan parasite *Leishmania major*. Two known acridone related compounds, gravacridonediol and 1-hydroxy-10-methylacridone, exhibited activities against the intracellular form of the same parasite and the fungus *Cladosporium cucumerinum*, respectively.

Keywords: *Thamnosma rhodesica*; Rutaceae; Acridone derivatives; Coumarins; Furanocoumarins; *Leishmania major*; *Cladosporium cucumerinum*

1. Introduction

The *Thamnosma* genus (11 species) is a remarkably disjunct Afro-American genus that belongs to the Rutaceae family. *Thamnosma rhodesica* (Bak. f.) Mendonça, is one of the 6 species that grows on the African continent (Thulin, 1999). Previously coumarins, linear furanocoumarins and alkaloids have been isolated from *T. montana* Torr. and Frem. and *T. texana* Torr., two American species (Chang et al., 1976). Very few studies (Campbell et al., 1990; Roos et al., 1998) have been performed so far on the *Thamnosma* species from African origin and none of them concerning *Thamnosma rhodesica*, a perennial woody-based herb or shrublet densely gland-dotted that grows up to 60 cm tall. The plant is distributed in Southern and Western Zimbabwe and in Botswana where it occurs in dry bushland and open woodland at altitudes between 1000 and 1400 m. *Thamnosma rhodesica* is traditionally used in Zimbabwe as an ant and flea repellent and smoked for the relief of chest conditions (Medonça, 1963). In the course of our search for new bioactive lead compounds from higher plants, the dichloromethane extract of the roots of *Thamnosma rhodesica* was found to show a marked activity against the phytopathogenic fungus *Cladosporium cucumerinum*. The present work describes the phytochemical investigation of this extract in relationship with some of its biological properties, and identifies specific molecules active against ***both*** *C. cucumerinum* and the intracellular protozoan parasite *Leishmania major*, one of the agents of cutaneous leishmaniasis.

2. Results and discussion

A HPLC/UV dereplication based on the specificity of both retention times and characteristic UV spectra of reference compounds enabled the on-line identification of some coumarins and furanocoumarins including xanthotoxin, psoralen, bergapten, umbelliferone directly in the dichloromethane extract. From these measurements, several other unidentified compounds with UV

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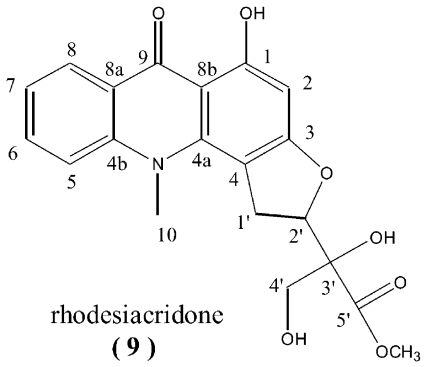
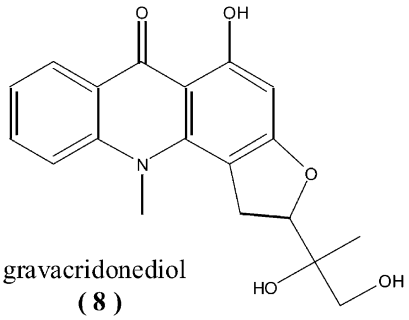


rhodesiacridone
(9)



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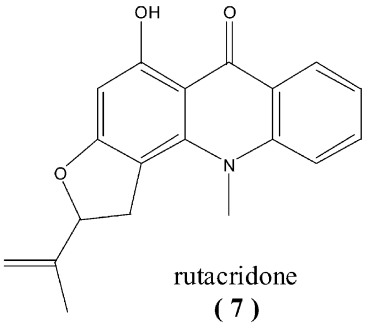
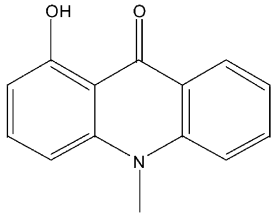
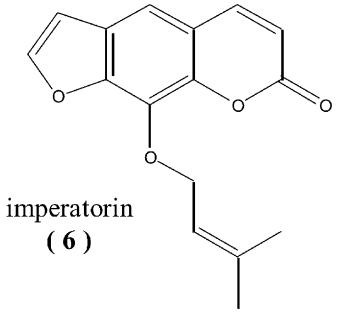
(8)



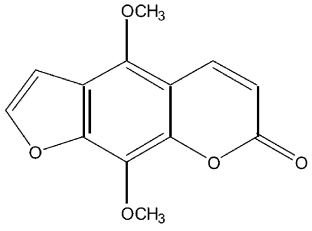
rutacridone
(7)



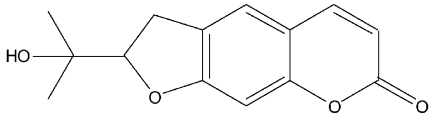
1-hydroxy-10-methylacridone
(1)



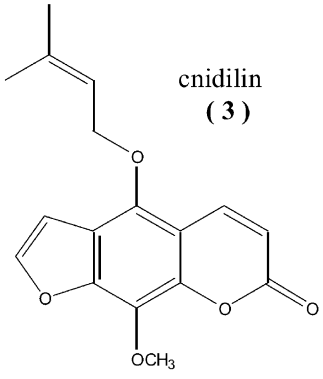
imperatorin
(6)



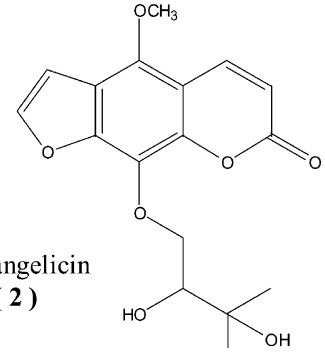
isopimpinellin
(5)



marmesin
(4)



cnidilin
(3)



byakangelicin
(2)

spectra characteristic of furanocoumarins could be observed together with another category of compounds possessing an unusual UV spectral pattern with maxima at 250 (sh), 273, 300 and 334 nm or at 242 (sh), 263, 315 and 345 nm. The phytochemical investigation of this extract was eventually carried out giving the priority to the isolation of the compound active against *Cladosporium cucumerinum*. It appears after a first separation step that the antifungal product was one of the substances possessing a UV spectrum not attributable to coumarin or furanocoumarin derivatives. An activity-guided fractionation led to the isolation of the antifungal product that was identified as a known acridone alkaloid named 1-hydroxy-10-methylacridone (**1**), previously isolated from the roots of *Ruta graveolens* and *Boenninghausenia albiflora,* both plants belonging to the Rutaceae family (Reisch et al., 1971; Scharlemann, 1972). This structural identification suggested that all UV detected compounds of the extract should be either furanocoumarins/coumarins or acridone alkaloids.

The major constituents of the extract in terms of UV absorption were isolated and led to the identification of known furanocoumarins including byakangelicin (**2**), cnidilin (**3**), marmesin (**4**), isopimpinellin (**5**) and imperatorin (**6**). The structure of these compounds were established by spectrometric methods including 1D- and 2D-NMR, EI-MS and DCI-MS. The interest was then focused on the isolation of other acridone derivatives following a UV-guided strategy. The structure determination of these compounds allowed the characterisation of rutacridone (**7**) and gravacridonediol (**8**), two known acridone alkaloid derivatives. Rutacridone was previously isolated from *Ruta graveolens* and *Ruta chalepensis* (Rutaceae) (Reisch et al., 1978) and gravacridonediol was reported from the root tissue cultures of *Ruta graveolens* (Rósza et al., 1976).

In addition to these products, another acridone derivative (**9**) was isolated using the same UV-guided strategy. The presence of a major ion observed at *m*/*z* 386 $[M+H]^+$ after a loop injection in the APCI mode indicated a molecular weight of 385 for **9** that was confirmed by a ESI-HRMS analysis which enabled assignment of a molecular formula of $C_{20}H_{19}NO_7$. The ^1H and ^{13}C NMR spectra (Table 1) gave evidence for a 10-methylacridin-9(10*H*)-one pattern oxygenated in positions 1,3 and substituted in position 4. As in the case of rutacridone (**7**) and gravacridonediol (**8**), the substituents in positions 3 and 4 were proved to be part of a dihydrofuran moiety associated with protons at δ 4.11 and 3.90 (H-1′a and H-1′b, 2H), δ 5.69 (H-2′) and ^{13}C resonances at δ 33.4 (C-1′) and δ 84.8 (C-2′). ^1H NMR signals at δ 4.60, 4.66 (H-4′a,b, 2H) and 3.79 (CH_3-6′, 3H) in combination with ^{13}C NMR resonances at δ 81.7 (C-3′), 66.3 (C-4′), 174.4 (C-5′) and 52.9 (6′-CH_3) gave evidence for a methyl 2,3-dihydroxypropanoate chain. The identity of this subunit was confirmed by long-distance heteronuclear correlations observed between protons of the methyl group at δ 3.79 (6′-CH_3) and the carbonyl function at 174.4 (C-5′) as well as between the protons of the hydroxymethylene subunit at δ 4.60, 4.66 (H-4′a,b) and ^{13}C resonances at 174.4 (C-5′) and δ 81.7 (C-3′). This chain was demonstrated to be connected to C-2′ through HMBC correlations observed between the quaternary carbon at δ 81.7 (C-3′) and ^1H NMR signals at δ 4.11 and 3.90 (H-1′a,b). The gathered spectroscopic data discussed above led to the structure elucidation of compound **9** that is to our knowledge a novel acridone alkaloid named rhodesiacridone.

Table 1
^1H and ^{13}C NMR data of compound **9** (in pyridine-d_5)

No	^1H NMR	^{13}C NMR
1	–	166.8
2	6.58 *s*	92.7
3	–	168.2
4	–	101.4
4a	–	142.8
4b	–	144.3
5	7.32 (*dd*, *J* = 8.8, 1.6 Hz)	115.6
6	7.64 (1H, *ddd*, *J* = 8.6, 8.0, 1.6 Hz)	134.2
7	7.26 (1H, *ddd*, *J* = 8.8, 7.9, 1.6 Hz)	121.7
8	8.62 (*dd*, *J* = 7.9, 1.6 Hz)	126.4
8a	–	121.9
8b	–	106.8
9	–	181.8
10-CH_3	3.66 *s*	37.7
1′	4.11 (*dd*, *J* = 14.2, 7.6 Hz) 3.90 (*dd*, *J* = 14.2, 9.5 Hz)	33.4
2′	5.69 (*dd*, *J* = 9.5, 7.9 Hz)	84.8
3′	–	81.7
4′	4.60 (*dd*, *J* = 10.7, 4.4 Hz) 4.66 (*dd*, *J* = 10.7, 4.4 Hz)	66.3
5′	–	174.4
6′-CH_3	3.79 *s*	52.9

All isolated compounds were tested against *Leishmania major* (Mauël, 1984) and *Cladosporium cucumerinum* (Homans and Fuchs, 1970): rutacridone displayed slight toxicity at 10 μM concentration against the extracellular, free-living promastigotes, but not against amastigotes within macrophages (Table 2). Gravacridonediol and rhodesiacridone at the same concentration also demonstrated some toxicity against the free parasite, but interestingly, the effect against intracellular amastigotes was far more pronounced. The products were not toxic for macrophages at the same concentration. In these tests, the reference compound amphotericin B showed high toxicity against both the extracellular and the intracellular parasite developmental stages. All other compounds were inactive against both targets.

This is the first report of acridone alkaloids in an African species of the *Thamnosma* genus, giving chemotaxonomic evidence of the close relationship existing

Table 2
Antileishmanial and antifungal activities of the isolated compounds from the roots of *Thamnosma rhodesica*

	Leishmania major promastigote[a]		*Leishmania major* amastigote[b]		*C. cucumerinum*[c]
1	90.0±5.0	95.5±3.8	n.t.	n.t.	10
2	90.7±1.4	98.8±4.6	n.t.	n.t.	–
3	74.6±5.5	92.7±5.2	n.t.	n.t.	–
4	97.7±1.7	96.5±1.1	n.t.	n.t.	–
5	99.1±1.8	97.6±3.2	79.0±4.2	91.7±4.5	–
6	70.5±5.0	83.0±1.9	n.t.	n.t.	–
7	34.9±1.5	69.9±2.8	88.0±5.1	82.0±4.0	–
8	54.0±1.1	97.2±2.2	9.5±1.0	58.0±3.1	–
9	30.7±3.2	96.0±1.8	6.2±0.7	48.6±2.7	–
Amphotericin B	0.2±0.04	71.9±4.4	0.4±0.02	0.5±0.03	n.t.
Nystatine	n.t.	n.t.	n.t.	n.t.	0.2

n.t. not tested. – not active.

[a] Survival (%) of free-living *L. major* promastigotes exposed to 10 μM (left column) and 1 μM (right column) of the indicated compounds.

[b] Survival (%) of intracellular *L. major* following exposure of parasitized macrophages to 10 μM (left column) and 1 μM (right column) of the indicated compounds.

[c] Minimal amount (μg) of compound to inhibit growth on a silica gel TLC plate.

between the American and African members of this genus.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR: Varian Unity Inova NMR instrument, Palo Alto, CA, USA. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD at 500.00 and 125 MHz, respectively. TMS: int. standard. UV: Varian DMS 100S UV-VIS spectrophotometer. UV spectra were recorded in MeOH. $[\alpha]_D$: Perkin-Elmer-241 polarimeter. TLC: silica gel 60 F_{254} Al sheets (Merck) using petrol ether–EtOAc 1:1. CC: silica gel 60 (70–200 μm, 750 × 65 mm i.d.; SDS), MPLC: RP-18 Lichroprep (40–63 μm; 270 × 25 mm i.d.; Merck). LPLC: Lobar RP-18 columns (LiChroprep 40–63 μm, 310 × 25 mm i.d.; Merck). Sephadex LH-20 (600 × 40 mm i.d.; Pharmacia). EI-MS and D/CI-MS: Finnigan MAT TSQ-700 triple stage quadrupole instrument. HPLC/UV/DAD with a Nova-Pak RP-18 column (4 μm; 250 × 3.9 mm i.d.; Waters) using a MeOH-H_2O gradient (15:85 – 100:0) in 35 min. The detection was performed at 210 and 254 nm. Semi-preparative HPLC was performed with a Shimadzu LC-8 pump equipped with a Knauer UV detector using a μBondapak® C_{18} prepacked radial-compression column (10 μm, 25 × 100 mm; Waters).

3.2. Plant material

The roots of *Thamnosma rhodesica* (Bak. f.) Mendonça were collected in February 1996 between Bulawayo and Beitbridge, Zimbabwe. Vouchers are deposited at the National Herbarium at Harare, Zimbabwe and at the Institut de Pharmacognosie et Phytochimie, Lausanne, Switzerland (No. 96106).

3.3. Extraction and isolation

The air-dried powdered roots of *T. rhodesica* were extracted at room temperature with dichloromethane to afford 3.45 g of extract. This extract was first fractionated by column chromatography on silica gel with a petrol ether–EtOAc gradient (8:1–2:1) giving fractions A – U. Fraction G was separated by medium pressure liquid chromatography (MPLC) with MeOH-H_2O gradient (45:55–65:35). Fractionation and purification have been realized by gel filtration on Sephadex LH-20 using CH_2Cl_2–MeOH (1:1) yielded 12 mg of compound **1**, 35 mg of compound **3** and 13 mg of compound **7**.

Fractions T, Q, I were separated by low pressure liquid chromatography (LPLC) with MeOH-H_2O gradient yielding respectively 3 mg of compound **2,** 4 mg of compound **4** and 3 mg of compound **5.** Further purification by semi-preparative chromatography using a μBondapak® C_{18} (10 μm, 25×100 mm) radial compression column with MeOH–H_2O (58:42) as the mobile phase led to the isolation of compound **6** (52 mg). Fraction U was also separated by semi-preparative chromatography giving compounds **8** (5 mg) and **9** (4 mg) with MeOH–H_2O (46:54) as the mobile phase.

3.3.1. 1-Hydroxy-10-methylacridone (*1*)

Amorphous yellow powder; UV λ_{max}^{MeOH} nm (log ϵ): 200 (4.15), 216 (4.02), 262 (4.46), 312 (3.67), 408 (3.63); EI-MS: (70eV, RIC) 225 $[M]^+$ (86), 201 (28).

*3.3.2. Byakangelicin (**2**)*

Amorphous yellow powder; $[\alpha]_D$ −13.3° (MeOH, *c* 0.1) [lit. Martinez et al., 1967, +23.8° (pyridine, *c* 0.0034)]; UV λ_{max}^{MeOH} nm (log ϵ): 222 (4.36), 240 (4.10), 248 (4.09), 269 (4.16), 313 (4.01); EI-MS: (70eV, RIC) 334 $[M]^+$ (20), 332 (100), 217 (81). D/CI-MS *m/z* (rel. int.): 352 $[M+NH_4]^+$ (100), 334 $[M]^+$ (20).

*3.3.3. Cnidilin (**3**)*

Amorphous yellow powder; UV λ_{max}^{MeOH} nm (log ϵ): 223 (4.51), 241 (4.24), 248 (4.23), 268 (4.34), 312 (4.16); EI-MS: (70eV, RIC) 232 (100), 231 (40), 217 (94), 203 (16), 188 (28), 160 (52). D/CI-MS *m/z* (rel. int.): 318 $[M+NH_4]^+$ (100), 301 $[M+H]^+$(64) .

*3.3.4. Marmesin (**4**)*

Amorphous yellow powder; $[\alpha]_D$ −15.8° (MeOH, *c* 0.1) [lit. Abu-Mustafa et al., 1961, +25° ($CHCl_3$)]; UV λ_{max}^{MeOH} nm (log ϵ): 223 (4.06), 334 (4.14); EI-MS: (70eV, RIC) 246 $[M]^+$ (50), 188 (74), 187 (100). D/CI-MS *m/z* (rel. int.): 264 $[M+NH_4]^+$ (70), 247 $[M+H]^+$(100), 204 (78), 187 (42) .

*3.3.5. Isopimpinellin (**5**)*

Amorphous yellow powder; UV λ_{max}^{MeOH} nm (log ϵ): 222 (4.47), 241 (4.21), 248 (4.22), 268 (4.31), 312 (4.14); D/CI-MS *m/z* (rel. int.): 264 $[M+NH_4]^+$ (100), 247 $[M+H]^+$(40).

*3.3.6. Imperatorin (**6**)*

Amorphous yellow powder; UV λ_{max}^{MeOH} nm (log ϵ): 218 (4.65), 248 (4.58), 300 (4.29); D/CI-MS *m/z* (rel. int.): 288 $[M+NH_4]^+$ (100), 271 $[M+H]^+$(20), 220 (16).

*3.3.7. Rutacridone (**7**)*

Amorphous yellow powder ; $[\alpha]_D$ −44.9° (MeOH, *c* 0.1) [lit. Reisch et al., 1967, −43° ($CHCl_3$, *c* 0.003)]; UV λ_{max}^{MeOH} nm (log ϵ): 212 (4.35), 227 (4.30), 249 (4.56), 273 (4.72), 300 (4.35), 332 (4.00), 398 (3.83); EI-MS: (70eV, RIC) 307 $[M]^+$ (100), 292 (40), 278 (60), 250 (36), 167 (36). D/CI-MS *m/z* (rel. int.): 308 $[M+H]^+$ (100).

*3.3.8. Gravacridonediol (**8**)*

Amorphous yellow powder; $[\alpha]_D$ −111.1° (MeOH, *c* 0.1); UV λ_{max}^{MeOH} nm (log ϵ): 227 (3.42), 249 (3.65), 273 (3.78), 300 (3.44), 332 (3.08), 398 (2.91); APCI-MS (positive mode) *m/z* : 342 $[M+H]^+$ (100).

*3.3.9. Rhodesiacridone (**9**) Methyl 2,3-dihydroxy-2-(5-hydroxy-11-methyl-6-oxo-1,2,6,11-tetrahydrofuro[2,3-c]acridin-2-yl) propanoate*

Amorphous yellow powder; $[\alpha]_D$ −47.7° (MeOH, *c* 0.1); UV λ_{max}^{MeOH} nm (log ϵ): 249 (3.96), 265 (sh, 4.01), 272 (4.09), 299 (3.73), 330 (3.44), 397 (3.18) ; ^1H NMR (500 MHz, $CDCl_3$): see Table 1. ^{13}C NMR (125 MHz, $CDCl_3$): see Table 1. ES-HRMS (positive mode) *m/z* : 386.1253 ($C_{20}H_{20}NO_7$ $[M+H]^+$, requires 386.1240).

3.4. Biological assays

3.4.1. Antifungal assay against C. cucumerinum

This direct bioautographic test against *C. cucumerinum* was developed by Homans et al. (1970) and is briefly described as follows: Geometric dilutions were obtained from freshly prepared stock solutions of isolated and reference compounds at a concentration of 1 mg/ml in an appropriate solvent. A volume of 10 µl of these solutions were applied on the on silica gel 60 F_{254} Al sheet TLC plates (Merck) using graduated capillaries. After application, of the samples the TLC plates were developed in petrol ether–EtOAc (1:1) solvent system and thoroughly dried for complete removal of solvents. The plates were then sprayed with a suspension of *C. cucumerinum* in a nutritive medium and incubated for 2–3 days in polystyrene boxes with a moist atmosphere. Clear inhibition zones appeared against a dark grey background. Nystatin (Sigma) was used as reference compound.

3.4.2. Antileishmanial assay against Leishmania major

This assay was performed as described by Mauël (1984).

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