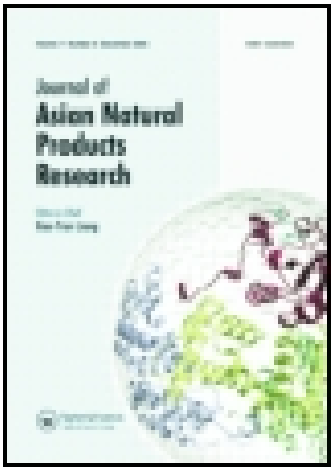

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Chemical constituents of Osyris alba and their antiparasitic activities

Hala I. Al-Jaber [a], Ibrahim M. Mosleh [b], Abdallah Mallouh [c], Omar M. Abu Salim [c] & Musa H. Abu Zarga [a]

[a] Chemistry Department, Faculty of Science, University of Jordan, Amman, Jordan

[b] Department of Biological Sciences, Faculty of Science, University of Jordan, Amman, Jordan

[c] Al-Kuds Medical Laboratories, Zarka, Jordan

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NOTE

Chemical constituents of *Osyris alba* and their antiparasitic activities

Hala I. Al-Jaber[a], Ibrahim M. Mosleh[b]*, Abdallah Mallouh[c], Omar M. Abu Salim[c] and Musa H. Abu Zarga[a]

[a]*Chemistry Department, Faculty of Science, University of Jordan, Amman, Jordan;* [b]*Department of Biological Sciences, Faculty of Science, University of Jordan, Amman, Jordan;* [c]*Al-Kuds Medical Laboratories, Zarka, Jordan*

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Phytochemical investigation of *Osyris alba* L. (Santalaceae) of Jordanian origin resulted in the isolation and identification of one new pyrrolizidine alkaloid, osyrisine (**1**), together with 16 other known compounds. The structures of all compounds were established on the basis of spectroscopic analysis. Osyrisine, catechin, and catechin-3-*O*-α-L-rhamnopyranoside exhibited a significant level of antiparasitic activity against two parasites, *Entamoeba histolytica* and *Giardia intestinalis*.

Keywords: osyrisine; *Osyris alba*; pyrrolizidine alkaloids; catechin; catechin-3-*O*-α-L-rhamnopyranoside; antiparasitic activity

1. Introduction

Amoebiasis and giardiasis are among the most important diarrheal infections in several regions of the world. Metronidazole, the drug preferred by most clinicians for the treatment of these infections, has a mutagenic activity in eukaryotic cells [1] and carcinogenicity in animals [2]. No carcinogenic effect has yet been demonstrated in humans [3], but its use during pregnancy is avoided by many physicians.

Although drug resistance to *Entamoeba* and *Giardia* does not appear to be a serious problem, occasional reports of failure with metronidazole and the reported variations in drug sensitivities of isolates [4] may be alarming. Because of the limited number of drugs available against anaerobic protozoal parasites, there is a serious need for new active compounds. Analysis of natural sources is an alternative strategy to develop such compounds. The phytochemical investigation of plant species used traditionally in the treatment of the two diseases resulted in the isolation and characterization of a variety of bioactive chemical compounds [5].

Osyris alba (Santalaceae) is a widely spread dioecious shrub growing wild in southern Europe, North Africa, Southeast Asia and in different localities in Turkey, Jordan, and Palestine [6]. This plant is known to be a root hemiparasite having a wide range of hosts including forest trees, perennial woody herbs, and fruit trees [7]. Previous phytochemical studies on this plant revealed that it contains alkaloids, carbohydrates, amino acids, fatty acids, and phenolics [8]. An investigation concerning the alkaloid constituents revealed the concomitant occurrence of quinolizidine

*Corresponding author. Email: i.mosleh@ju.edu.jo

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and pyrrolizidine alkaloids in *O. alba* [6]. The use of the plant *O. alba* in traditional medicine seems to be rare and its use against dysentery has never been mentioned in the literature. However, some Jordanian traditional healers claim the successful use of this plant in the treatment of amoebic dysentery. The presence of different types of alkaloids in this plant seemed to support its unusual use in traditional medicine in Jordan. Driven by these findings, bioactivity-guided fractionation of extracts of the whole plant material was performed and the activity of each preparation on axenic strains of *Entamoeba histolytica* and *Giardia intestinalis* (=*lamblia*) was tested.

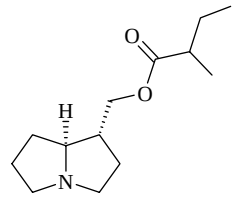
2. Results and discussion

2.1 Bioguided fractionation

The crude methanol extract of the dried, ground, and defatted plant material was partitioned according to the procedure described in Section 3 into hexane (C), an aqueous methanol (A), butanol (B), and water (W) extracts. The aqueous methanol (A) and butanol (B) extracts were found to be bioactive. Both extracts were fractionated as described in Section 3 and antiparasitic activity tests were performed for each fraction. The results showed that fractions AV, BIV–BVII were active. Chromatography of these fractions afforded six compounds identified as catechin (**2a**) [8], engeletin (**6**) [9], chysin A (**5**) [6], osyrisine (**1**), catechin-3-*O*-α-L-rhamnopyranoside (**2b**) [10], and mannitol (**3**), respectively. The structures of all these compounds were determined by their spectral data including EI-MS, FAB-MS, UV, IR, 1D and 2D NMR.

The bio-inactive fractions AII–AIV and BIII afforded 12 known compounds identified as: the flavonoids, salvigenin (**7**), pachypodol (**8**) [11], kumatakillin (**9**) [12], penduletin (**10**) [12], jecidiene (**11**) [13], and kampferol-7-methyl ether (**12**) [13]; two fatty acids: dotriacontanoic acid (**13**) and 2-octenoic acid (**14**); β-sitosteryl glucoside (**15**); triterpenoids ursolic acid (**16**) and oleanolic acid (**17**); and *p*-hydroxybenzoic acid (**4**). All these compounds, except for mannitol (**3**), chysin A (**5**), and *p*-hydroxybenzoic acid (**4**), are reported for the first time from *O. alba*.

The new compound osyrisine (**1**) (Figure 1) was isolated as a yellow oil from the bioactive fractions AV and BIV. The high-resolution mass spectra (HR-MS) of compound **1** showed a molecular ion peak at *m/z* 226.1802 $[M + H]^+$, corresponding to the molecular formula $C_{13}H_{23}NO_2$. The IR spectrum of compound **1** revealed the presence of ester carbonyl at 1732 cm^{-1}. The ^1H NMR ($CDCl_3$) spectrum of this compound (Table 1) showed resonances for two methyl groups, one of which appeared as a triplet at δ 0.84 ($J = 7.4$ Hz) while the other as a doublet at δ 1.07 ($J = 7.0$ Hz). The appearance of several aliphatic signals in the range δ 1.2–2.8 was indicative of a bicyclic pyrrolizidine nucleus. An interesting feature of the ^1H NMR spectrum of this compound was the two signals located at δ 4.04 (dd, $J = 8.1$, 11.1 Hz, H-9_α) and 4.14 (dd, $J = 7.0$, 11.1 Hz, H-9_β), which were assigned to the diastereotopic protons of the oxymethylene group. The ^{13}C NMR spectrum of osyrisine revealed the presence of 13 carbon signals including one ester carbonyl carbon at δ 176.6, one oxymethylene carbon at δ 63.9, one nitrogen-bearing methine carbon at δ 66.4, and two nitrogen-bearing methylene carbons at δ 53.7 and 55.8. The remaining



1

Figure 1. Structure of osyrisine (**1**).

Table 1. ^{1}H and ^{13}C NMR spectral data for compound **1** in $CDCl_3$.

No.	δ_H (J in Hz)	δ_C
1	2.62 (1H, m)	40.2
2	1.63 (2H, m)	27.0
3_α	2.73 (1H, br t, $J = 9.4$)	53.7
3_β	3.21 (1H, dt, $J = 6.3$, 10.0)	
5_α	2.52 (1H, dt, $J = 6.4$, 10.0)	55.8
5_β	3.42 (1H, br t, $J = 10.0$)	
6, 7_α	1.87 (3H, m)	26.8
7_β	1.69 (1H, m)	26.4
8	3.78 (1H, m)	66.4
9_α	4.04 (1H, dd, $J = 8.1$, 11.1)	63.9
9_β	4.14 (1H, dd, $J = 7.0$, 11.1)	
$1'$	–	176.6
$2'$	2.33 (q, $J = 7.0$)	41.1
$3'_\alpha$	1.43 (1H, m)	26.2
$3'_\beta$	1.61 (1H, m)	
$4'$	0.84 (3H, t, $J = 7.4$)	11.7
$5'$	1.07 (3H, t, $J = 7.0$)	16.6

carbon signals were assigned to two methyl, four methylene, and one methine carbons. Further analysis of COSY, HMBC, and HMQC spectra of compound **1** suggested that the compound consisted of a pyrrolizidine moiety (C-1–C-9) esterified to 2-methylbutanoic acid (C-$1'$–C-$5'$) through the hydroxymethylene group (C-9). The interesting long-range correlations observed in the HMBC spectrum (Figure 2) between the oxymethylene protons at C-9 with the carbonyl carbon (C-$1'$) and the methine carbons C-1 and C-8 confirmed the location of the ester linkage at C-9. The chemical shifts for all protons and carbons

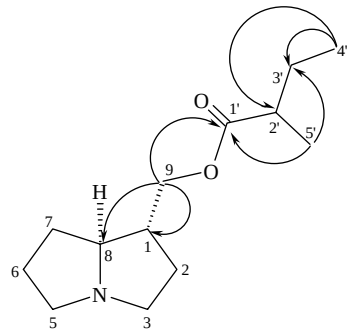


Figure 2. Main HMBC correlations in **1**.

of the pyrrolizidine moiety in osyrisine (**1**) were comparable to those of known pyrrolizidine alkaloids [6,14].

The alkaline hydrolysis of compound **1** gave 1-hydroxymethyl pyrrolizidine moiety with an optical rotation of $[\alpha]_D^{25} + 7.5$ ($c = 0.2$, MeOH), which is in good agreement with the optical rotation of the known pyrrolizidine alkaloid (+)-laburnine ($[\alpha]_D^{25} + 9.1$). These results confirm the stereochemistry of the pyrrolizidine nucleus in osyrisine as (1*S*, 8*R*) [15].

Further confirmation of the structure of osyrisine (**1**) was gained from its mass spectrum, which showed the correct molecular ion peak at *m/z* 225 and a base peak at *m/z* 124 resulting from the loss of the ester moiety from the molecular ion.

2.2 Antiparasitic activity

The IC_{50} values for the three compounds, osyrisine (**1**), catechin (**2a**), and

Table 2. The biological activity of compounds **1**, **2a**, **2b** and metronidazole against the parasites, *E. histolytica* and *G. intestinalis*, and cancer (Hep-2) and nonmalignant (Vero) cells.

	$IC_{50} \pm$ SD μg/ml		$IC_0 \pm$ SD (growth as in negative control) μg/ml	
Compounds	*E. histolytica*	*G. intestinalis*	Hep-2 cells	Vero cells
1	7.10 ± 0.36	7.57 ± 0.35	597.00 ± 11.53	596.66 ± 7.64
2a	17.67 ± 2.5	19.00 ± 1.73	444.33 ± 5.51	299.33 ± 8.62
2b	29.67 ± 2.5	29.67 ± 2.01	596.00 ± 6.93	596.00 ± 3.46
Metronidazole	0.80 ± 0.04	0.86 ± 0.02	186.33 ± 8.62	194.67 ± 5.03

catechin-3-*O*-α-L-rhamnopyranoside (**2b**), and the standard drug, metronidazole, against the tested parasites are shown in Table 2. Among the purified compounds, the new pyrrolizidine alkaloid, osyrisine, was the most potent (around 7 μg/ml). Although its activity was around eight times less than the standard drug, its cytotoxicity against Hep-2 cell lines and non-malignant (Vero) cells was around three times less (Table 2). The other two purified biologically active compounds were also non-cytotoxic to the two cell lines at concentrations much greater than the anti-parasitic concentrations (Table 2). This cytotoxicity pattern of the three biologically active compounds indicates potentially safe alternative therapeutic agents against the susceptible pathogens [16].

Interestingly, the three compounds exhibited a similar activity against *G. intestinalis* and *E. histolytica* (Table 2), indicating that each compound affects both the parasites by a similar mechanism of action. Regardless of this mechanism, the importance of such biologically active, non-cytotoxic compounds, especially osyrisine (**1**), lies in their potential contribution to overcome any emerging problem of resistance of pathogens to the standard drug [5]. It is noteworthy that the low cytotoxicity of osyrisine **1** is consistent with the previous reports about the non-cytotoxicity of pyrrolizidine alkaloids with a saturated necine nucleus [17].

3. Experimental

3.1 General experimental procedures

The IR spectra were recorded on a Thermo-Nicolet Nexus 870 FT-IR spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker DPX-300 MHz spectrometer with TMS as an internal standard. ^{13}C NMR spectra were recorded at 75.5 MHz. EI-MS were recorded using a Finnigan MAT TSQ-70, JMS 600 or MAT-112 S spectrometer at 70 eV and ion source temperature equal to 200°C. HR-MS were measured in positive ion mode using electrospray ionization (ESI) technique on a Bruker APEX-2 instrument. The optical rotation was recorded on a Perkin-Elmer polarimeter 141. Column chromatography was performed on silica gel 60 (0.063–0.200 mm; Fluka, Steinheim, Germany) or silica gel S (Ridel dehaën, Seelze-Hannover, Germany). Purification of the compounds was achieved by routine TLC on silica gel G-UV$_{254}$ glass plates (0.25 mm; Macherey-Nagel, Easton, PA, USA). Compounds were visualized under UV light and spraying with sulfuric acid–anisaldehyde spray reagent followed by heating at 120°C. Alkaloids were detected by spraying with iodoplatinic acid spray reagent.

3.2 Plant material

O. alba was collected during the flowering period in July 2001 from the Salt area (25 km northwest of Amman, Jordan). The plant was identified by Prof. Sawsan Al-Oran, Department of Biological Sciences, University of Jordan, Amman, Jordan. A voucher specimen of the plant has been kept in the herbarium of the Department of Biological Sciences, University of Jordan.

3.3 Extraction and isolation

The dried plant (7.0 kg) was defatted by soaking in petroleum ether and then was repeatedly soaked in methanol at room temperature (50 liters, four times, 7 days each). The residue obtained upon removal of MeOH under reduced pressure (1.0 kg) was partitioned between chloroform and water. The dried chloroform layer was further partitioned between hexane and 10% aqueous methanol, furnishing the aqueous methanol extract (A, 125 g) and hexane extract (C, 100 g). The water layer was extracted with *n*-butanol to afford the butanol extract (B, 375.4 g) and the water extract (W, 355.6 g).

Fractions A and B were successively subjected to CC and the isolated compounds were purified by TLC. The aqueous methanol extract (A, 125 g) was chromatographed on a silica gel S (63–230 mesh; Fluka) column eluted with a gradient of MeOH/$CHCl_3$ of increasing polarity to give five fractions (AI–AV). Each fraction was purified by a combination of CC, TLC, and recrystallization using suitable solvent systems for elution. Fraction AII (2.3 g) afforded compound **13** (361 mg). Fraction AIII (32.33 g) afforded a mixture of compounds **16** and **17** (164 mg), **7** (36 mg), **14** (12 mg), **8** (11 mg), **9** (6 mg), **10** (43 mg), and **11** (15 mg). Fraction AIV (6.4 g) afforded compound **12** (145 mg). Fraction AV (7.0 g) afforded compounds **15** (1000 mg) and **1** (50 mg).

The butanol extract (B, 375.4 g) precipitated a white pinkish solid upon treatment with distilled methanol. The solid was collected and the mother liquor was chromatographed following the same procedure described for fraction A to give eight fractions (BI–BVIII). Each fraction was further purified by a combination of column chromatography and TLC using suitable solvent systems. Fraction BIII (2.0 g) afforded compound **4** (45 mg). Fraction BIV (7.6 g) afforded compounds **2a** (400 mg), **6** (145 mg), **1** (30 mg), and **5** (75 mg). Fractions BV (29 g) and BVI (15.0 g) afforded compound **2b** (900 and 100 mg, respectively). Fraction BVII (15.0 g) afforded compound **3** (2870 mg).

Namely, fraction AV (7.0 g) was introduced into a silica gel column (34.5 × 4.8 cm) packed in ethyl acetate and eluted with a gradient of ethyl acetate/methanol of increasing polarity. Then, the mobile phase was changed into $CHCl_3$–MeOH mixture (85:15, v/v) saturated with ammonia to elute the alkaloid. The crude fraction containing the alkaloid was evaporated, treated with 10% aqueous ammonia solution and then extracted with $CHCl_3$ (3 × 20 ml). The organic layer was collected and evaporated under vacuum affording impure alkaloid. Final purification was achieved on TLC using the solvent mixture hexane–diethyl amine (90:10, v/v) to give the pure alkaloid as a yellow oil (50 mg). Similarly, the same compound was obtained from fraction BIV following the same procedure (30 mg).

*3.3.1 Osyrisine (**1**)*

Yellow oil (80 mg). $[\alpha]_D + 45.2$ ($c = 0.001$, MeOH). IR (film): 1732 (C=O). ^1H NMR spectral data: see Table 1, ^{13}C NMR spectral data, see Table 1. EI-MS *m/z* (%): 225 $[M]^+$(17), 124 (100), 122 (11), 95 (10), 83 (37), 55 (16), 41 (43); HR-ESI-MS: 226.1806 $[M + H]^+$ (calcd for $C_{13}H_{24}NO_2$, 226.1802).

*3.3.2 Alkaline hydrolysis of **1***

Compound **1** (7 mg) was hydrolyzed by refluxing with 5.0 ml of 0.10 N NaOH in 5.0 ml methanol for 3 h. The mixture was cooled, diluted with water, and extracted with $CHCl_3$ (3 × 4 ml). The chloroform layer was evaporated to give 2 mg of 1-hydroxymethyl pyrrolizidine alkaloid. The optical activity of the product was $[\alpha]_D^{25} + 7.5$.

3.4 Biological materials

E. histolytica HK-9 strain (ATCC number 30015) was cultured in LYI-S-2 medium supplemented with antibiotics. *G. intestinalis* WB strain (ATCC number 30957) was grown in a modified YI-S medium with antibiotics. Both parasites were cultivated in 15 ml screw-capped borosilicate glass tubes containing 13 ml of medium. The tubes were incubated on a 15° horizontal slant at 36–37°C. Culture maintenance and subculturing were performed as described previously [18]. *Entamoeba* and *Giardia* were harvested

from confluent cultures by the chilling of the tubes in ice for 5–10 min to detach cells, followed by centrifugation at 800 *g* for 5 min.

3.5 Antiparasitic activity

The antiamoebic and antigiardial activities of the plant fractions and metronidazole (the reference drug) were tested as previously described [19]. Briefly, the plant preparations and metronidazole were dissolved in dimethyl sulfoxide (DMSO), then in medium and filter-sterilized. Twofold dilutions ranging from 50 to 0.2 μg/ml were prepared in a final volume of 15 ml to exclude air from the tube. Each tube was inoculated with 20,000 cells of the parasite under testing (*Entamoeba* or *Giardia*). Each one of the plant preparations was assayed in duplicate in each of three independent experiments. In each assay, the appropriate controls were performed, including one without any plant preparation and another with metronidazole as the positive control. The biological activity of the plant preparations was evaluated by counting the parasites in each tube using the standard hemacytometer. In each count, trypan blue was employed to distinguish live from dead parasites [20]. The 50% inhibitory concentration (IC_{50}) was employed as a parameter for biological activity. The IC_{50} is the concentration of tested plant preparations or metronidazole which cuts the number of parasites to half that in the negative control.

3.6 Cytotoxicity assay

The cytotoxicity of plant preparations and metronidazole was investigated on Hep-2 and Vero cells using the standard cytotoxicity assay and the trypan blue exclusion method as described before [19]. Briefly, 100 μl portions of each cell suspension were added to the wells of 96-well plates, incubated for 24 h, and the medium in each well was then replaced with 150 μl of fresh medium. Solutions of the plant preparations and metronidazole were dissolved in DMSO, prepared in medium, and filter-sterilized. Then, 150 μl of twofold serial dilutions of each of the plant preparations and the reference drug starting at a concentration of 2000 μg/ml in the culture medium were prepared in the plates. After 48 h incubation, the number of cells in each well was determined using a hemacytometer. Each tested plant preparation or metronidazole was assayed in duplicate in each of three independent experiments. In each assay, the negative controls (without any compound or reference drug) were included in duplicates. IC_0 was the concentration at which the number of cells in the tube was more than or equal to that of the negative control (IC_0 = no inhibition of cell growth).

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