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Antifungal Quinoline Alkaloids from *Waltheria indica*

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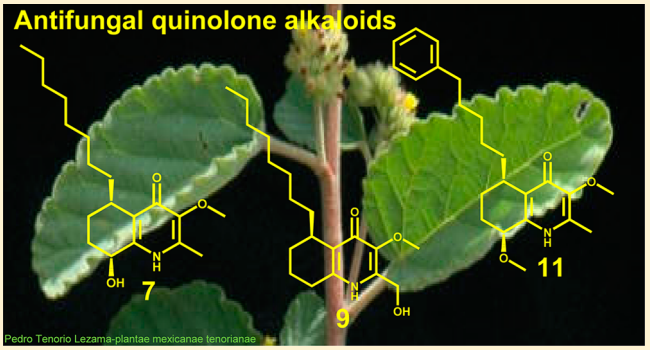
Supporting Information

ABSTRACT: Chemical investigation of a dichloromethane extract of the aerial parts of *Waltheria indica* led to the isolation and characterization of five polyhydroxymethoxyflavonoids, namely, oxyanin A (**1**), vitexicarpin (**3**), chrysosplenol E (**4**), flindulatin (**5**), 5-hydroxy-3,7,4′-trimethoxyflavone (**6**), and six quinolone alkaloids, waltheriones M−Q (**2**, **7**, **8**, **10**, **11**) and 5(*R*)-vanessine (**9**). Among these, compounds **2**, **7**, **8**, **10**, and **11** have not yet been described in the literature. Their chemical structures were established by means of spectroscopic data interpretation including ^{1}H and ^{13}C, HSQC, HMBC, COSY, and NOESY NMR experiments and UV, IR, and HRESIMS. The absolute configurations of the compounds were established by ECD. The isolated constituents and 10 additional quinoline alkaloids previously isolated from the roots of the plant were evaluated for their in vitro antifungal activity against the human fungal pathogen *Candida albicans*, and 10 compounds (**7**, **9**, **11**−**16**, **18**, **21**) showed growth inhibitory activity on both planktonic cells and biofilms (MIC ≤ 32 μg/mL). Their spectrum of activity against other pathogenic *Candida* species and their cytotoxicity against human HeLa cells were also determined. In addition, the cytological effect of the antifungal isolated compounds on the ultrastructure of *C. albicans* was evaluated by transmission electron microscopy.



Candida albicans is the most frequent human fungal pathogen causing predominantly superficial skin and mucosal infections. However, it can lead to life-threatening systemic infections in immune-compromised patients with a mortality rate greater than 50%.[1] Candidiasis are also due to the increasing emergence of other *Candida* species such as *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* that are identified frequently as human pathogens.[2] The frequency of invasive fungal infections and their resistance to antifungal therapy are increasing despite the development of new agents.[3] In particular, the growing number of clinical isolates resistant to azole drugs and the limited number of alternative options (polyenes and echinocandins) are of great concern. An important contributing factor to the virulence and resistance of *Candida* species (mostly *C. albicans*) to antifungal drugs is their ability to form biofilms. These structured microbial communities, embedded in an extracellular matrix, are characterized by increased resistance to antifungal therapy, and recent evidence suggests that the majority of infections produced by these pathogens are associated with biofilm growth.[4]

According to the World Health Organization, plants represent the best sources for obtaining a wide variety of drugs that could benefit a large population.[5] In the course of our ongoing project focused on the discovery of new natural antiparasitic compounds from *Waltheria indica* L. (Malvaceae),[6,7] extracts from this plant were screened for antifungal activity. In the present study, from the active dichloromethane extracts of the aerial parts, five flavonol derivatives (**1**, **3**−**6**) and six 4-quinolone alkaloids (**2**, **7**−**11**) were isolated and characterized. Among these, five compounds (**2**, **7**, **8**, **10**, **11**) have not yet been described in the literature and **9** is reported for the first time in *W. indica*. The antifungal activity of these compounds, and of 10 quinoline alkaloids (**12**−**21**) previously isolated from the roots, was determined against *Candida* spp.

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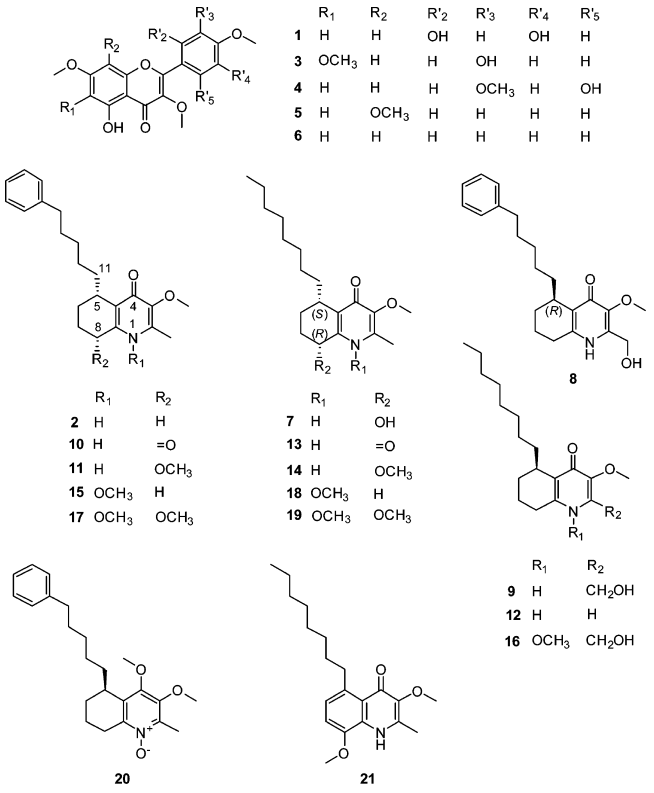

RESULTS AND DISCUSSION

In a preliminary screening experiment, a TLC bioautography assay[8] of the dichloromethane extract of the aerial parts from *W. indica*, at 200 μg/spot, showed significant inhibition of a hypersusceptible strain of *C. albicans* (DSY2621). This strain allows the detection of a small quantity of bioactive compounds from complex matrices. To localize the active compounds, the extract was microfractionated into 100 fractions using reversed-phase semipreparative HPLC. The fractions collected were dried and then evaluated with this TLC bioautography assay.

To obtain preliminary information on its chemical composition, the profiling of the dichloromethane extract of the aerial parts was established by UHPLC-PDA-TOFMS. The positive HRESIMS trace revealed 20 peaks in the chromatographic zone that showed growth inhibition of the hypersusceptible *C. albicans* strain in the TLC bioautography assay (Figure 1). Among them, 16 peaks displayed ions at *m/z* 340.2274 $[M + H]^+$ $C_{22}H_{30}NO_2$ (**2**), *m/z* 322.2378 $[M + H]^+$ $C_{19}H_{32}NO_3$ (**7** and **9**), *m/z* 356.2221 $[M + H]^+$ $C_{22}H_{30}NO_3$ (**8**), *m/z* 354.2065 $[M + H]^+$ $C_{22}H_{28}NO_3$ (**10**), *m/z* 370.2380 $[M + H]^+$ $C_{23}H_{32}NO_3$ (**11**, **15**, and **20**), *m/z* 306.2430 $[M + H]^+$ $C_{19}H_{32}NO_2$ (**12**), *m/z* 320.2223 $[M + H]^+$ $C_{19}H_{30}NO_3$ (**13**), *m/z* 336.2535 $[M + H]^+$ $C_{20}H_{34}NO_3$ (**14** and **18**), *m/z* 352.2485 $[M + H]^+$ $C_{20}H_{34}NO_4$ (**16**), *m/z* 400.2482 $[M + H]^+$ $C_{24}H_{34}NO_4$ (**17**), *m/z* 366.2642 $[M + H]^+$ $C_{21}H_{36}NO_4$ (**19**), and *m/z* 332.2222 $[M + H]^+$ $C_{20}H_{30}NO_3$ (**21**) and showed the presence of a nitrogen atom in their molecular formula, indicating the putative presence of alkaloids. After injection of reference compounds previously isolated from the roots of *W. indica*,[6] 10 known quinolone alkaloids were identified without ambiguity as 8-deoxoantidesmone (**12**), antidesmone (**13**), waltherione E (**14**), waltherione G (**15**), waltherione I (**16**), waltherione H (**17**), waltherione J (**18**), waltherione K (**19**), waltherione L (**20**), and waltherione F (**21**). In addition, four main peaks, which showed a characteristic PDA-UV spectrum of a flavonol, were observed with a pseudomolecular ion at *m/z* 375.1077 $[M + H]^+$ $C_{19}H_{19}O_8$ (**3** and **4**), *m/z* 359.1128 $[M + H]^+$ $C_{19}H_{19}O_7$ (**5**), and *m/z* 329.1021 $[M + H]^+$ $C_{18}H_{17}O_6$ (**6**).

A flash chromatographic separation was carried out to isolate most of the other metabolites with antifungal activity. This separation, combined with further semipreparative HPLC purifications, resulted in the isolation of 11 compounds (**1**–

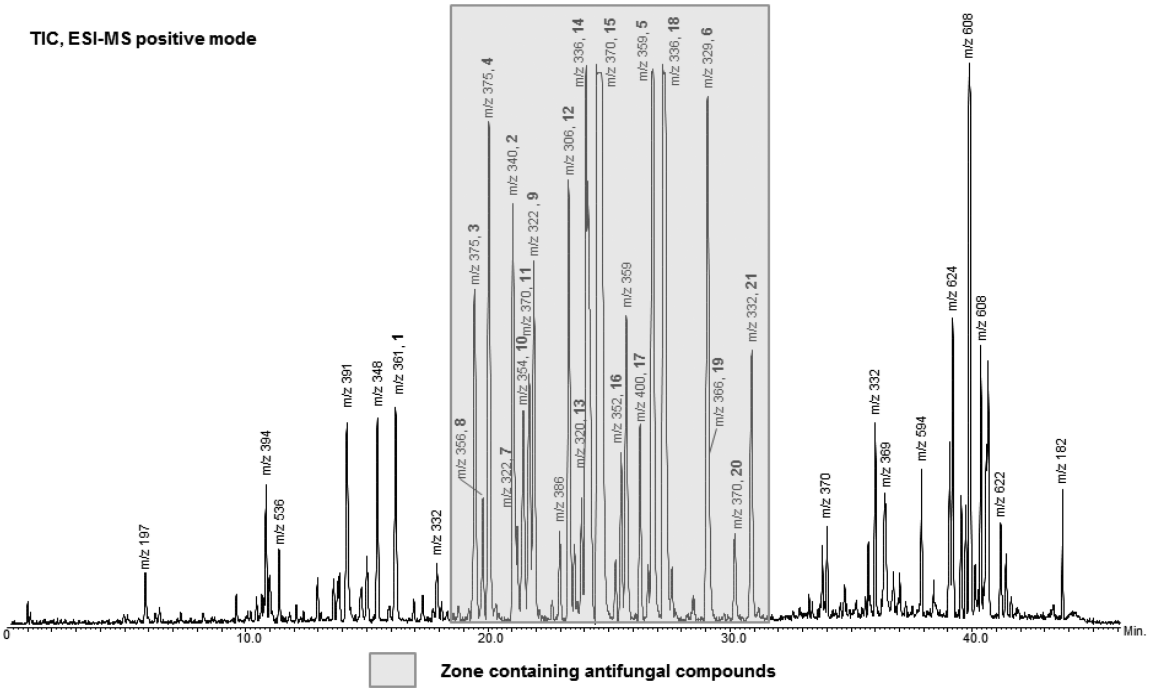

Figure 1. UHPLC-TOFMS metabolite profiling of the dichloromethane extract of *W. indica*. The chromatographic zone that presented growth inhibition of *C. albicans* in the TLC bioautography assay is highlighted.

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Table 1. ^{1}H NMR Data of Compounds 2, 7, 8, 10, and 11 (500 MHz, in CD_3OD, δ in ppm, J in Hz)

position	2	7	8	10	11
5	2.90, m	2.86, m	2.93, m	3.19, m	2.86, m
6	eq 1.89, brd (12.3) ax 1.49, brq (12.3)	eq 1.92, ddd (13.5, 4.9, 2.0) ax 1.67, tdd (13.5, 4.9, 2.0)	eq 1.91, brd (14.0) ax 1.53, tt (14.0, 4.2)	eq 2.20, ddt (14.1, 5.0, 2.2) ax 2.08, m	eq 1.93, ddt (13.9, 5.6, 2.6) ax 1.65, m
7	1.75, m	eq 2.01, ddt (13.5, 6.3, 2.0) ax 1.83, tdd (13.5, 10.0, 2.0)	1.79, m	ax 2.80, ddd (18.0, 14.7, 5.3) eq 2.53, ddd (18.0, 4.4, 2.5)	eq 2.13, dtd (11.3, 5.9, 2.3) ax 1.73, m
8	2.61, m	4.55, dd (10.0, 6.3)	eq 2.70, ddd (17.4, 6.0, 2.6) ax 2.65, dt (17.4, 10.5)		4.49, dd (9.3, 5.9)
11	1.71, m 1.23, m	1.76, m 1.31, m	1.72, m 1.24, m	1.68, m 1.49, m	1.75, m 1.28, m
12	1.45, m 1.40, m	1.43, m	1.43, m	1.58, m 1.46, m	1.44, m
13	1.36, m	1.31, m	1.36, m	1.40, m	1.34, m
14	1.63, m	1.31, m	1.63, m	1.67, m	1.63, m
15	2.59, t (7.6)	1.31, m	2.60, t (7.7)	2.61, t (7.5)	2.60, t (7.7)
16		1.29, m			
17	7.14, d (7.5)	1.31, m	7.16, m	7.17, d (7.5)	7.16, d (7.5)
18	7.22, t (7.5)	0.90, t (6.9)	7.22, t (7.5)	7.23, t (7.5)	7.23, t (7.5)
19	7.11, t (7.5)		7.12, m	7.12, t (7.5)	7.12, t (7.5)
20	7.22, t (7.5)		7.22, t (7.5)	7.23, t (7.5)	7.23, t (7.5)
21	7.14, d (7.5)		7.16, m	7.17, d (7.5)	7.16, d (7.5)
CH_3-2	2.30, s	2.34, s		2.39, s	2.34, s
CH_2OH-2			4.64, s		
OCH_3-3	3.75, s	3.76, s	3.78, s	3.82, s	3.75, s
OCH_3-8					3.47, s

Table 2. ^{13}C NMR Data of Compounds 2, 7, 8, 10, and 11 (125 MHz, in CD_3OD, δ in ppm)

position	2	7	8	10	11
2	140.2, C	140.5, C	141.9, C	142.9, C	141.1, C
3	145.7, C	145.9, C	144.3, C	148.7, C	146.2, C
4	174.3, C	[a]	[a]	[a]	174.2, C
5	32.3, CH	32.5, CH	32.2, CH	31.7, CH	32.6 CH
6	25.8, CH_2	23.8, CH_2	25.6, CH_2	25.0, CH_2	23.5, CH_2
7	18.0, CH_2	28.0, CH_2	17.8, CH_2	33.1, CH_2	23.6, CH_2
8	27.6, CH_2	67.3, CH	27.4, CH_2	194.8, C	76.1, CH
9	144.6, C	145.6, C	[a]	[a]	143.4, C
10	[a]	129.7, C	[a]	139.5	130.4, C
11	33.6, CH_2	33.3, CH_2	33.3, CH_2	31.4, CH_2	33.5, CH_2
12	28.8, CH_2	29.0, CH_2	28.5, CH_2	29.0, CH_2	28.9, CH_2
13	30.5, CH_2	30.4, CH_2	30.3, CH_2	30.1, CH_2	30.5, CH_2
14	32.8, CH_2	30.4, CH_2	32.6, CH_2	32.4, CH_2	32.8, CH_2
15	36.9, CH_2	30.4, CH_2	36.7, CH_2	36.6, CH_2	36.9, CH_2
16	144.0, C	32.8, CH_2	143.8, C	143.7, C	144.0, C
17	129.4, CH	23.4, CH_2	129.2, CH	129.1, CH	129.4, CH
18	129.2, CH	14.2, CH_3	129.0, CH	129.0, CH	129.2, CH
19	126.6, CH		126.3, CH	126.4, CH	126.6, CH
20	129.2, CH		129.0, CH	129.0, CH	129.2, CH
21	129.4, CH		129.2, CH	129.1, CH	129.4, CH
CH_3-2	13.5, CH_3	13.2, CH_3		13.4, CH_3	13.5, CH_3
CH_2OH-2			56.7, CH_2		
OCH_3-3	60.2, CH_3	59.8, CH_3	60.4, CH_3	59.6, CH_3	60.1, CH_3
OCH_3-8					56.4, CH_3

[a]Signal too weak to be measured.

11). Their structural elucidation was based on the analysis of the 1D and 2D NMR spectra and HRMS data.

Six known compounds were identified as oxyanin A (**1**),[9] vitexicarpin (**3**),[10] chrysosplenol E (**4**),[9] flindulatin (**5**),[11] 5-hydroxy-3,7,4′-trimethoxyflavone (**6**),[12] and vanessine (**9**).[13] Moreover, five new alkaloids were identified, and their structural elucidation is described below. NMR data (Tables 1 and 2) and 2D NMR experiments suggested a quinolone skeleton for these alkaloids.

The ^{1}H and HSQC NMR spectra of compound **2** exhibited very strong similarities to 8-deoxoantidesmone (**12**), previously isolated from the roots of the same plant.[6] Both compounds

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shared the same 3-methoxy-2-methyl-5,6,7,8-tetrahydroquinolin-4(1*H*)-one skeleton as revealed by the presence of signals for a methine at δ_C 30.8 (C-5), three methylenes at δ_C 25.8, 18.0, and 27.6 (C-6, C-7, and C-8, respectively), a methyl at δ_C 12.1 (CH_3-2), and a methoxy at δ_C 58.9 (OCH_3-3). The difference lies on the side chain at C-5 with a *n*-octane chain for 8-deoxoantidesmone and a pentylbenzene unit for **2**, as shown by the aromatic signals at δ_H 7.14 (2H, d, J = 7.5 Hz, H-17, 21), 7.22 (2H, t, J = 7.5 Hz, H-18, 20) and 7.11 (1H, t, J = 7.5 Hz, H-18), as well as the methylene signal at δ_H 2.59 (2H, t, J = 7.6 Hz, H-15). The absolute configuration at C-5 was established by comparison of the experimental ECD spectrum with previously published data of waltheriones E-L.[6] The experimental ECD spectrum (data not shown) exhibited positive and negative Cotton effects (CE) at 265 and 330 nm, respectively. Thus, the absolute configuration was established as (5*S*). Compound **2** was identified as 3-methoxy-2-methyl-5-(5-phenylpentyl)-5,6,7,8-tetrahydroquinolin-4(1*H*)-one and named waltherione M. The HRESIMS of **2** showed a protonated molecule at m/z 340.2274 $[M + H]^+$ (calcd for $C_{22}H_{30}NO_2$, 340.2276), indicating a molecular formula of $C_{22}H_{29}NO_2$ which is in agreement with the structure elucidated by NMR spectroscopy.

The HRESIMS of compound **7** exhibited a $[M + H]^+$ ion at m/z 322.2378 (calcd for $C_{19}H_{32}NO_3$, 322.2382), corresponding to the molecular formula, $C_{19}H_{31}NO_3$. In comparison with **2**, no phenyl ring at the terminal position of the side chain was observed but instead a methyl group at δ_H 0.90 (3H, t, J = 6.9 Hz, H-18) occurred at the terminal position of a *n*-octyl side chain. In addition, an extra hydroxy group was confirmed by NMR spectroscopy where an additional methine at δ_H 4.55 (1H, dd, J = 10.0, 6.3, H-8)/δ_C 67.3 was detected instead of the methylene on C-8. The value of the coupling constant (10 Hz) indicated an axial position of H-8 whereas the NOEs correlations from H-6ax to H-8ax and H-5, and from H-6eq to H-5 indicated the equatorial position of H-5 (Figure 2). The hydroxy group and the side chain were thus located on the same side of the molecule.

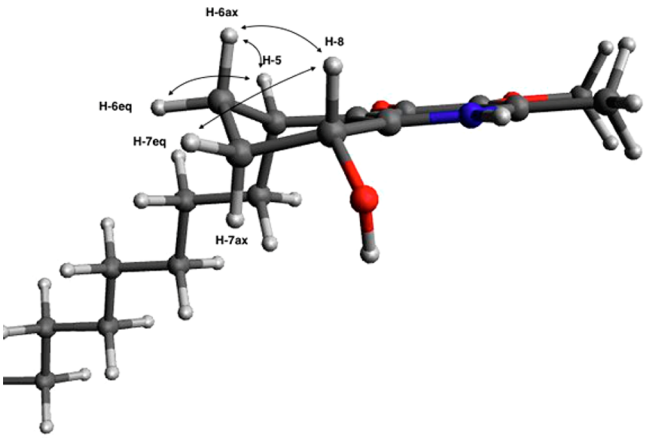

Figure 2. 3D structure of **7** and key NOEs correlations.

To establish the absolute configuration at C-5 and C-8, the ECD spectrum of **7** was measured and compared with data previously published.[6] The experimental spectrum showed two positive CEs at 226 and 265 nm and one negative CE at 285 nm (Figure 3). The good match between the experimental spectrum of **7** and published data for (5*S*,8*R*)-stereoisomers led to the conclusion that the absolute configuration of **7** is (5*S*,8*R*). Compound **7** (waltherione N) was established as 8-hydroxy-3-methoxy-2-methyl-5-octyl-5,6,7,8-tetrahydroquinolin-4(1*H*)-one.

The ^{1}H NMR spectrum of **8** demonstrated a close resemblance to that of **2**. The only difference observed was a hydroxymethyl at δ_H 4.64 instead of a methyl group. The HMBC correlations with C-2 (δ_C 142.9) and C-3 (δ_C 148.7) allowed positioning the methylene at C-2. The HRESIMS of **8** confirmed the presence of an additional oxygen atom (+ 16 amu) based on the protonated molecule detected at m/z 356.2221 $[M + H]^+$ (calcd for $C_{22}H_{30}NO_3$, 356.2225), corresponding to a molecular formula of $C_{22}H_{29}NO_3$. The ECD spectrum of **8** showed two negative CEs at 230 and 261 nm and one positive CE at 310 nm (Figure 3). As compared to compound **2**, the spectrum was opposite in sign. Given that **8** has one chiral center, the configuration at C-5 is (5*R*). Compound **8** (waltherione O) was assigned as 2-(hydroxymethyl)-3-methoxy-5-(5-phenylpentyl)-5,6,7,8-tetrahydroquinolin-4(1*H*)-one.

For compound **9**, the molecular formula $C_{19}H_{31}NO_3$ suggested by HRESIMS and the NMR data were consistent with the literature values of vanessine,[13] a 4-quinolone alkaloid isolated initially from *Waltheria douradinha*. However, analysis of the ECD spectrum of **9** (two negative CEs at 230 and 261 nm and one positive CE at 310 nm, Figure 3) led to the conclusion that **9** has an absolute configuration (5*R*) that is opposite to the configuration (5*S*) assumed for vanessine in the literature. Thus, compound **9** [(5*R*)-vanessine] was established as the (*R*)-enantiomer of vanessine.

Compound **10** showed a $[M + H]^+$ ion at m/z 354.2065 (calcd for $C_{22}H_{28}NO_3$, 354.2069), corresponding to a molecular formula of $C_{22}H_{27}NO_3$. When compared with the molecular formula, $C_{22}H_{29}NO_2$, of **2**, an additional oxygen atom minus two hydrogen atoms was evident for **10**. This difference could be explained by the HSQC spectrum of **10** where the methylene C-8 was missing when compared to **2**, and the HMBC spectrum where the H-6 and the H-7 signals correlated with a carbonyl at δ_C 194.8 (C-8). The ECD spectrum showed three positive CEs around 240, 280, and 310 nm and a negative CE at 355 nm, which was consistent with literature values for antidesmone (**13**).[14] Thus, the absolute configuration was established as 5*S*. The structure of **10** (waltherione P) was identified as 3-methoxy-2-methyl-5-(5-phenylpentyl)-1,5,6,7-tetrahydroquinoline-4,8-dione.

The HRESIMS of compound **11** displayed a $[M + H]^+$ ion at m/z 370.2380 (calcd for $C_{23}H_{32}NO_3$, 370.2382), in agreement with the molecular formula, $C_{23}H_{31}NO_3$, suggesting this compound to be an isomer of waltheriones G (**15**) and L (**20**). The ^{1}H and HSQC spectra of **11** indicated the presence of an extra methoxy group (3H, s, OCH_3-8) at δ_H 3.47 and δ_C 56.4, as compared to compound **2**. This methoxy group was positioned at C-8 (δ_C 76.1) based on the HMBC correlation. The absolute configurations of C-5 and C-8 were assigned as (5*S*,8*R*) by analysis of the experimental ECD spectrum (Figure 3). Alkaloid **11** (waltherione Q) was thus identified as (5*S*,8*R*)-3,8-dimethoxy-2-methyl-5-(5-phenylpentyl)-5,6,7,8-tetrahydroquinolin-4(1H)-one.

From the active antifungal zone (Figure 1), 16 main peaks corresponding to alkaloids were detected. Among these, ten compounds (**12**–**21**) were identified as quinoline alkaloids previously isolated from the roots of *W. indica*. The remaining six alkaloids were identified as five new 4-quinolone alkaloids

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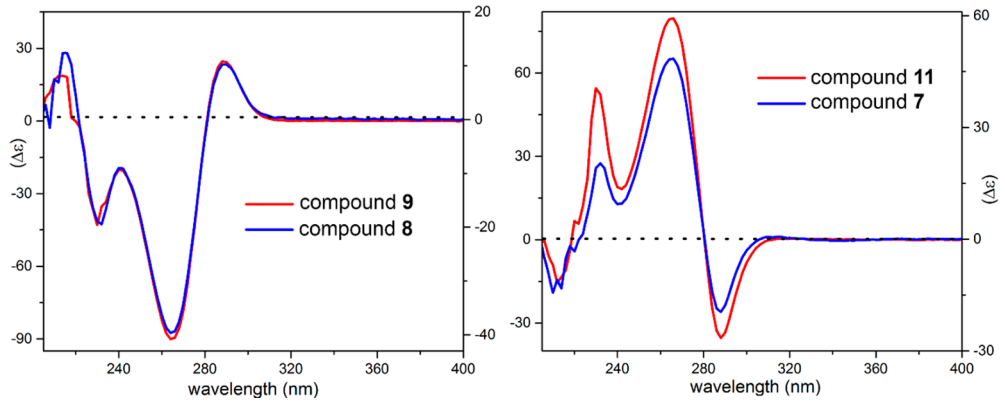

Figure 3. Experimental ECD spectra of **7**–**9** and **11**.

Table 3. Antimicrobial Activity of Selected Compounds Isolated from *Waltheria indica*

compound	*C. a*	*C. g*	*C. k*	*C. t*	*C. p*	*S. c*	biofilm	HeLa	SI
				MIC μg/mL[a]				IC_{50} μg/mL	
7	32	>32	32	>32	>32	>32	16	50.0	1.6
9	32	>32	32	>32	>32	>32	16	24.4	0.8
11	32	>32	>32	>32	>32	>32	32	11.9	0.4
12	16	>32	32	>32	32	>32	32	24.7	1.5
13	32	>32	16	>32	4	>32	16	23.4	0.7
14	4	>32	>32	>32	>32	>32	8	20.0	5.0
15	32	32	32	32	2	16	32	9.5	0.3
16	32	>32	>32	>32	>32	>32	16	26.6	0.8
18	16	32	16	32	2	16	16	16.1	1.0
21	8	>32	>32	>32	>32	>32	16	14.4	1.8
fluconazole	0.5	>128	32	4	4	16	>256	>100	>200
caspofungin	0.3	0.1	0.1	0.5	1.0	0.3	0.1	>100	>400

[a]List of strains: *C. a*: *Candida albicans*, *C. g*: *Candida glabrata*, *C. k*: *Candida krusei*, *C. t*: *Candida tropicalis*, *C. p*: *Candida parapsilosis*, *S. c*: *Saccharomyces cerevisiae*, biofilm: *C. albicans*. Cytotoxicity was measured on HeLa cells. SI (selectivity index) = IC_{50} HeLa cells/MIC for *C. albicans*.

(**2**, **7**, **8**, **10**, **11**) and the (*R*)-enantiomer of vanessine (**9**). In addition, four flavonoids were isolated (**3**–**6**) from the active zone and a fifth one (**1**), although outside of the antifungal zone, was also isolated because it was described previously as active against *C. albicans*.[9]

The biological activity of these 21 compounds was evaluated by a standard antifungal susceptibility assay (Eucast method)[15] using broth microdilution toward a *C. albicans* wild type strain. The Minimum Inhibitory Concentrations (MIC) were determined, and 10 out of 21 exhibited antifungal activity (MIC ≤ 32 μg/mL). The data obtained are summarized in Table 3.

To further characterize their antifungal properties, the spectrum of activity of the 10 active compounds was determined using different pathogenic *Candida* species (*C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*) and a related nonpathogenic yeast (*Saccharomyces cerevisiae*). Interestingly, the compounds exhibited heterogeneous antifungal potencies. Compounds **15** and **18** showed the broadest activity spectrum, with a MIC ≤ 32 μg/mL against all yeast strains tested, while several other compounds (**11**, **14**, **16**, **21**) were active only against *C. albicans*. It seems that *C. glabrata*, *C. tropicalis*, and *S. cerevisiae* are less susceptible to the compounds than *C. albicans* and, to a lesser extent, than *C. krusei* and *C. parapsilosis*.

It is important to mention that all antifungal activities were obtained in an acidic culture medium (pH 4.6) and could not be detected at neutral pH. This pH-dependent activity may be explained by a protonation of the molecules that could facilitate their import and/or activity into the fungal cells as reported for other drugs.[16] However, in the case of 4-quinolone alkaloids, protonation of the molecule seems to be difficult at pH 4.6, as the 4-pyridone nucleus possesses a pK_a of 3.3.[17] In addition, in silico calculations showed that the percentages of nonionized species at pH 4.0 are close to 100% for all these alkaloids except for **20**.[18] According to this observation, acidic conditions may induce changes in *Candida* cells with their environment (membrane, metabolism) more than modifications of the antifungal alkaloids themselves (protonation).

Biofilms are complex cell populations that develop on surfaces with an intrinsic resistance to many antifungal drugs.[19] The 10 compounds that were tested on *C. albicans* biofilms formed in vitro all reduced by more than 50% the metabolic activity of mature biofilms at concentrations similar to their planktonic MIC values (≤32 μg/mL). This antibiofilm activity is interesting, as most inventoried antifungal agents tend to exhibit biofilm MIC values that are much higher than their planktonic MIC values.[20] Further experiments could be performed to assess the effect of the compounds on the initial step of biofilm formation, which is the morphological switch from the yeast to the hyphal form.

In previous publications,[5,6] eight out of the 10 compounds (**11**–**16**, **18**, **21**) were evaluated for their antitrypanosomal activity. This questioned if the activity of these compounds on yeast is specific or if they display a general cytotoxic effect. The compounds were tested against Gram-negative and Gram-positive bacteria (*E. coli*, *P. aeruginosa*, and *S. aureus*), and no

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significant activity was detected (MIC > 32 μg/mL) (data not shown). Then, cytotoxicity was assessed using a standard in vitro toxicity protocol[21] on HeLa cells. All compounds exhibited an IC_{50} ranging from 9.5 to 50 μg/mL. A selectivity index (SI) could then be determined (SI = IC_{50}/MIC *C. albicans*), and the compounds showed a weak selectivity (between 0.3 and 5) when compared to those of known antifungal drugs (>100), demonstrating some nonspecific and toxic biological activities.

The cytological effects of waltheriones E (**14**) and G (**15**) were assessed by electron microscopy. The potential alterations of these compounds on the ultrastructure of *C. albicans* wild-type CAF2-1 were evaluated on thin sections by transmission electron microscopy (TEM). The yeast was grown on yeast nitrogen base (YNB) medium at pH 4.6 and treated with these two compounds at the MIC concentration, with only DMSO as a positive control and alone as the negative control. Nontreated CAF2-1 cells, with or without DMSO, showed no lesions on cell walls, cell membranes, and organelles, such as mitochondria, the nucleus, and nuclear membrane, as well as a large number of ribosomal particles across the overall cytoplasm (Figure 4A and B). In comparison to the positive and negative controls, which exhibited the same growth and cytological morphology, CAF2-1 cells treated by compounds **14** and **15** showed, after 4 h, the same accumulation of dark corpuscles within the nucleus as well as the punctual disruption of the nuclear membrane (Figure 4C and D), but organelles such as mitochondria were still present. After 18 h, important alterations and breakage zones of the plasmic membrane were observed (Figure 4E and F). In the same cells, an important disorganization of the cytoplasmic content was observed, where the mitochondria, nucleus and nuclear membrane, and the Golgi apparatus or ribosomes were not visible. Contrary to what was shown after miconazole treatment,[8] a reference drug, no accumulation of lipid-like bodies was observed in vacuoles or dark material in the parietal zone or highly contrasted membranes. These results suggested that the waltheriones exerted different cytological effects on *C. albicans* and should further be investigated from a mechanistic point of view at the molecular level.

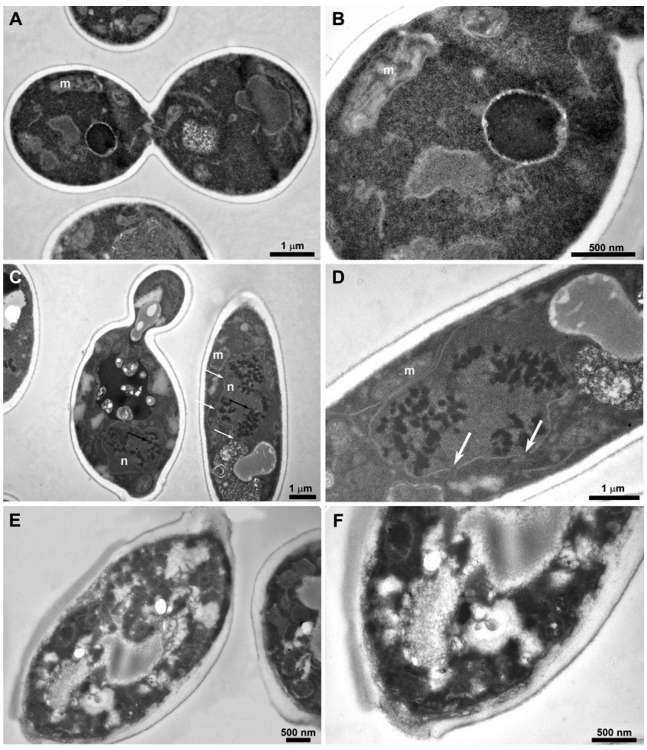

Figure 4. Ultrastructure of *C. albicans* CAF2-1 with and without treatment with waltherione G (**15**) as observed by transmission electron microscopy. (A) Control with DMSO treatment. (B) Detail of A with intact organelles and multiple ribosomes. (C) Cells treated with waltherione G for 4 h. Black arrows show dark corpuscles observed within the nucleus, and white arrows show punctual disruption of the nuclear membrane. (D) Detail of C. (E) Cells treated with waltherione G for 18 h with no recognizable organelles or cell structures. (F) Detail of E. m: mitochondria; n: nucleus.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1030 (Easton, MD, USA) polarimeter (EtOH, *c* in g/100 mL). The ECD spectra were recorded on a JASCO J-815 CD spectrometer in MeOH. UV spectra were recorded on a PerkinElmer Lambda-25 UV−vis spectrophotometer (Wellesley, MA, USA) in MeOH. IR spectra were measured on a PerkinElmer Spectrum 100 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 MHz NMR (Palo Alto, CA, USA) instrument. Chemical shifts are reported in parts per million (δ) using the residual CD_3OD signal (δ_H 3.31; δ_C 49.0) as internal standards for ^1H and ^{13}C NMR, respectively, and coupling constants (*J*) are reported in Hz.

UHPLC-PDA-TOF-MS metabolite profiling of the extract was performed on a Micromass-LCT Premier time-of-flight (TOF) mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface and coupled to an Acquity UPLC system hyphenated with an Acquity photodiode array (PDA) detector (Waters). The ESI conditions were as follows: capillary voltage 2800 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 20 L/h, desolvation gas flow 600 L/h. Detection was performed in the positive ion mode with an *m/z* range of 150−1300 Da and a scan time of 0.5 s in the W-mode. The MS was calibrated using sodium formate. Leucine enkephalin (Sigma-Aldrich, Steinheim, Germany) was used as an internal reference at 2 μg/mL and infused through a Lock Spray probe at a flow rate of 10 μL/min aided by a second LC pump. The UV-PDA spectrum was recorded between 210 and 500 nm (step 2 nm). The separation was performed on an Acquity BEH C_{18} UPLC column (150 mm × 2.1 mm i.d.; 1.7 μm, Waters), using an optimized gradient (MeCN and H_2O both containing 0.1% formic acid) of 15% to 60% MeCN in 30 min, 60% to 90% in 5 min, then 90% to 98% in 5 min and followed by a washing step with 98% MeCN for 2 min. After the washing step, the column was equilibrated with 15% MeCN for 14 min before the next injection. The flow rate was set to 0.4 mL/min, the temperature to 25 °C, and the injection volume 1 μL.

Fractionation was performed on a Puriflash 4100 preparative chromatographic system (Interchim, Montluçon Cedex, France) equipped with a quaternary pump, a PDA detector, and a fraction collector. Semipreparative chromatography was performed on an Armen Spot System (Saint-Avé, France) with a Kinetex Axia Core−Shell C_{18} column (5 μm, 250 × 21.2 mm; Phenomenex, Torrance, CA, USA).

Plant Material. The aerial parts of *Waltheria indica* were collected between June 2012 and February 2013 in Zinder (Niger). The identification was confirmed by Didier Roguet (Botanical Garden of Geneva). Voucher specimens are deposited at the Botanical Garden of Geneva (no. 19003).

Extraction and Isolation. Air-dried aerial parts (2.0 kg) were powdered and extracted with 8 L of CH_2Cl_2 (3 × 24 h) at room temperature. After filtration, the CH_2Cl_2 extract was evaporated to dryness. The residue (3.1 g) was mixed with 8 g of Celite 577 (Fluka, AG, Switzerland) and introduced into a cartridge for a dry load injection. Fractionation of the extract was performed using two flash

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chromatography columns connected in series (PF-C_{18}HQ/120 g, 15 μm C_{18}, Interchim). Fractionation was carried out with the mobile phase MeCN and H_2O, both containing 0.1% formic acid in an optimized gradient mode: 30% to 35% MeCN in 206 min, 35% to 40% in 81 min, and finally 40% to 100% in 72 min. The flow rate was set to 16 mL/min, and the UV detection was simultaneously performed at 220, 254, and 360 nm. The flash chromatographic separation yielded 290 fractions, which were analyzed individually by UHPLC-MS. Fractions 83 and 84 yielded **1** (3.7 mg), fractions 96−100 yielded **2** (20.9 mg), fractions 221−223 yielded **3** (3.3 mg), fractions 224−230 yielded **4** (5.3 mg), fractions 266−268 yielded **5** (12.9 mg), and fraction 285 yielded **6** (9.7 mg). Fractions 87−93, 101−103, 134−146, 149−158, and 160−167 were selected for further purification. The final fractionation steps were performed by semipreparative HPLC using a Kinetex Axia Core−Shell C_{18} column (5 μm, 250 × 21.2 mm; Phenomenex) using MeCN/H_2O/0.1% formic acid as solvents for an isocratic elution. The flow rate was set to 25 mL/min, and UV absorbance was at 220 nm. Fractions 87−93 (43.0 mg) yielded **7** (3.0 mg) (28% MeCN); fractions 101−103 (20.1 mg) yielded **8** (1.3 mg) (30% MeCN); fractions 134−146 (30.6 mg) yielded **9** (2.5 mg) (34% MeCN), fractions 149−158 (32.0 mg) yielded **10** (1.9 mg) (34% MeCN), and fractions 160−167 (57.7 mg) yielded **11** (9.7 mg) (37% MeCN).

*Oxyanin A (**1**):* yellow needles; HRESIMS *m*/*z* 361.0917 $[M + H]^+$ (calcd for $C_{18}H_{17}O_8$, 361.0923); for other spectroscopic data, see ref 9.

*Waltherione M (**2**):* pale yellow oil; $[\alpha]_D^{22}$ +16 (*c* 0.1, EtOH); UV (MeOH) λ_{max} (log ε) 219 (3.4), 266 (2.3) nm; ECD (MeOH, *c* 0.15 mM, 0.1 cm) $[\theta]_{223}$ = +11 042, $[\theta]_{265}$ = +12 662, $[\theta]_{302}$ = +1481, $[\theta]_{344}$ = −890; IR ($CHCl_3$) ν_{max} 2930, 1712, 1613, 1493, 1435 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 340. 2274 $[M + H]^+$ (calcd for $C_{22}H_{30}NO_2$, 340.2276).

*Vitexicarpin (**3**):* yellow needles; HRESIMS *m*/*z* 375.1077 $[M + H]^+$ (calcd for $C_{19}H_{19}O_8$, 375.1080); for other spectroscopic data, see ref 10.

*Chrysosplenol E (**4**):* yellow needles; HRESIMS *m*/*z* 375.1077 $[M + H]^+$ (calcd for $C_{19}H_{19}O_8$, 375.1080); for other spectroscopic data, see ref 9.

*Flindulatin (**5**):* yellow needles; HRESIMS *m*/*z* 359.1128 $[M + H]^+$ (calcd for $C_{19}H_{19}O_7$, 359.1131); for other spectroscopic data, see ref 11.

*5-Hydroxy-3,7,4′,-trimethoxyflavone (**6**):* yellow needles; HRESIMS *m*/*z* 329.1021 $[M + H]^+$ (calcd for $C_{19}H_{17}O_6$, 329.1025); for other spectroscopic data, see ref 12.

*Waltherione N (**7**):* pale yellow oil; UV (MeOH) λ_{max} (log ε) 220 (2.7), 273 (2.5) nm; ECD (MeOH, *c* 0.16 mM, 0.1 cm) $[\theta]_{231}$ = +12 672, $[\theta]_{266}$ = +31 153, $[\theta]_{288}$ = −12 578; IR ($CHCl_3$) ν_{max} 2970, 1490 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 322.2378 $[M + H]^+$ (calcd for $C_{19}H_{32}NO_3$, 322.2382).

*Waltherione O (**8**):* pale yellow oil; UV (MeOH) λ_{max} (log ε) 218 (2.7), 273 (2.4) nm; ECD (MeOH, *c* 0.16 mM, 0.1 cm) $[\theta]_{230}$ = −25 219, $[\theta]_{263}$ = −57 988, $[\theta]_{287}$ = +15 842; IR ($CHCl_3$) ν_{max} 2930, 1496 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 356.2221 $[M + H]^+$ (calcd for $C_{22}H_{30}NO_3$, 356.2225).

*(R)-Vanessine (**9**):* pale yellow oil; HRESIMS *m*/*z* 322.2378 $[M + H]^+$ (calcd for $C_{19}H_{32}NO_3$, 322.2382); for other spectroscopic data, see ref 13.

*Waltherione P (**10**):* pale yellow oil; UV (MeOH) λ_{max} (log ε) 218 (2.4), 247 (2.4), 338 (1.8) nm; ECD (MeOH, *c* 0.16 mM, 0.1 cm) $[\theta]_{232}$ = −12 350, $[\theta]_{264}$ = −25 516, $[\theta]_{291}$ = +6371; IR ($CHCl_3$) ν_{max} 2929, 1696, 1611, 1554, 1505, 1452 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 354.2065 $[M + H]^+$ (calcd for $C_{22}H_{28}NO_3$, 354.2069).

*Waltherione Q (**11**):* pale yellow oil; $[\alpha]_D^{22}$ +33 (*c* 0.5, EtOH); UV (MeOH) λ_{max} (log ε) 225 (3.7), 274 (2.4) nm; ECD (MeOH, *c* 0.14 mM, 0.1 cm) $[\theta]_{232}$ = +39 480, $[\theta]_{268}$ = +57 870, $[\theta]_{289}$ = −25 444; IR ($CHCl_3$) ν_{max} 2927, 2855, 1716, 1619, 1504, 1453 cm^{-1}; ^1H and ^{13}C NMR, see Tables 2 and 3; HRESIMS *m*/*z* 370.2380 $[M + H]^+$ (calcd for $C_{23}H_{32}NO_3$, 370.2382).

TLC Bioautography Assay. The *C. albicans* mutant DSY2621 was constructed by targeted deletions of genes encoding membrane efflux transporters and calcineurin subunit A.[22] A 10 μL amount, corresponding to 40 μg, of each microfraction was spotted manually on the TLC plate (silica gel 60 F_{254}; Merck, Germany). Then, the TLC plate without elution was submitted to a bioautography assay as previously described.[8] Briefly, malt agar (malt extract, 30 g/L; peptone from soymeal, 3 g/L; agar−agar, 15 g/L; Merck) for *C. albicans* was used as the solid medium for the overlays. The molten medium was maintained in a water bath at 45 °C. The optical density (OD) of the *C. albicans* culture was measured at 630 nm with a UV/vis spectrophotometer (Synergy H1, Biotek, Winooski, VT, USA, equipped with Gen 5.2 software). A dilution was made to obtain an inoculum of 8 × 10^5 cells/mL (an OD of 1 at 630 nm, corresponding to approximately 10^7 cells/mL). The suspension was prepared immediately before carrying out the test.

TLC plates were placed on a hot plate maintained at 37 °C. Approximately 20 mL of the inoculum (DSY2621) was distributed rapidly over the TLC plate with a sterile pipet. After solidification of the medium, the plates were incubated overnight at 37 °C in polyethylene boxes lined with moist chromatography paper. The bioautograms were sprayed with an aqueous solution (2.5 mg/mL) of methyl thiazolyl tetrazolium chloride (MTT, Fluka) and incubated for 6 h at 37 °C. Clear inhibition zones were observed against a purple background.

Antifungal Assay. The strains used in the study were *C. albicans* CAF2.1, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 75, *C. parapsilosis* ATCC 22019, *C. glabrata*,[23] and *S. cerevisiae* BY4741. Antifungal susceptibility tests were carried out based on EUCAST protocols[15] with slight modifications. Briefly, *Candida* strains were cultivated overnight at 30 °C under constant agitation in yeast extract peptone dextrose (YEPD). The cultures were then diluted to a density of 2 × 10^5 cells/mL in yeast nitrogen base medium containing glucose at a final concentration (w/v) of 2% (pH 4.6). The compounds were dissolved in DMSO to a final concentration of 10 mg/mL. Twofold serial dilutions were performed in a 50 μL volume per well, and 150 μL of inoculum was added. A final concentration of 1% DMSO was present in each well. Drug-free cultures and sterility controls were included in each 96-well plate. The plates were incubated at 34 °C for 24 h. The MICs were determined with a spectrophotometer plate reader set at 450 nm and defined as the drug concentration at which the optical density was ⩽50% of that of the test-compound-free culture. For *S. cerevisiae*, the medium (YNB) was complemented with Complete Supplement Mixture (Mpbio, Santa Ana, CA, USA) according to the supplier's instructions.

Antifungal susceptibility tests on biofilms were conducted according to a published protocol[24] with 48 h of biofilm formation and 48 h of antifungal treatment.

Cytotoxicity Assay. Cytotoxicity measures were performed according to the sulforhodamine B standard procedure.[21] HeLa cells (ATCC CCL-2, Manassas, VA, USA) were cultured in DMEM + 10% FBS, at 37 °C, 5% CO_2. The 96-well plates were filled with a seeding density of 10^4 cells/well. After 24 h of growth (day 1), cells were washed twice with phosphate-buffered solution (PBS), and 2-fold serial dilutions (starting at 100 μg/mL) of the compounds were added to the cells and incubated for 48 h. The starting amount of cells was monitored by fixing the cells at day 1. At day 3, cells were all washed twice with PBS and then fixed and labeled as described in the protocol.[21] OD was measured at 492 nm, and the percentage of control cell growth could be determined using the following formula: $(OD\ treated\ cells_{day3} - OD_{day1})/(OD\ DMSO_{day3} - OD_{day1}) \times 100$. Inhibitory concentration 50 (IC_{50}) corresponds to the concentration at which 50% of cell growth was inhibited. It was calculated by applying curve-fitting methods (nplr R package) on a dose−response curve.

Electron Microscopy. The *C. albicans* CAF2-1 strain was grown in 10 mL of YNB liquid cultures (50 mL plastic tubes, 37 °C, 2 h). At this time, waltheriones E and G (1 mg/mL in DMSO) were added at a concentration of respectively 2 and 1 μg/mL, and the cultures were grown during 18 h to evaluate their cytotoxic effects. The samples were taken after 4 and 18 h and centrifuged (10 min, 1300*g*, room temperature), the supernatant was discarded, and the resulting pellet

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was prepared according to Roland and Vian.[25] Briefly, the pellets were prefixed with a solution of 3% glutaraldehyde−2% paraformaldehyde in 0.07 M phosphate buffer (pH 7), embedded in 2% agarose, and postfixed with a solution of 1% OsO_4. They were then dehydrated in a graded series of ethanol solutions [30−50−70−95−100% (v/v)] and embedded in LR white resin (14381-UC; London Resin Company). After polymerization for 24 h at 60 °C, thin (0.08 μm) sections were cut and stained with 2% uranyl acetate followed by lead citrate according to Reynolds.[26] The thin sections were observed with a transmission electron microscope (Philips CM10) equipped with a Mega View II camera. Controls were performed in the same way without waltherione E (**14**) or G (**15**) or with DMSO alone.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00896.

^{1}H and ^{13}C NMR spectra of compounds **2** and **7**−**11** (PDF)

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Notes

The authors declare no competing financial interest.

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