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Antiprotozoal and cytotoxic naphthalene derivatives from *Diospyros assimilis*

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Dedicated to Prof. Dr. Peter G. Waterman on the occasion of his 60th birthday.

Abstract

Chemical investigation of the roots of *Diospyros assimilis* had led to the isolation and characterization of six naphthalene derivatives, two 2-naphthaldehyes, namely 4-hydroxy-3,5-dimethoxy-2-naphthaldehyde **1**, 4-hydroxy-5-methoxy-2-naphthaldehye **2**, its related isomer 5-hydroxy-4-methoxy-2-naphthaldehyde **3** and three commonly occurring naphthoquinones, diospyrin **4**, 8′-hydroxyisodiospyrin **5** and the simple monomer, plumbagin **6**. Their chemical structures were established by detailed NMR investigations including ^{1}H and ^{13}C NMR, HSQC, HMBC and NOESY experiments. In addition, the naphthalene derivatives **1–5** were evaluated for their in vitro antiprotozoal activity against protozoan parasites belonging to the genera *Trypanosoma*, *Leishmania* and *Plasmodium*. Among the tested compounds, naphthaldehyde **1** showed moderate inhibition of the growth of the parasites, *T. brucei*, *T. cruzi*, *L. donovani* with IC_{50} values of 19.82, 12.28 and 38.78 μM and displayed cytotoxicity towards rat skeletal myoblasts (L-6 cells) with IC_{50} of 174.94 μM, while **2** and **3** were found to be comparatively less active to **1**. The dimeric quinones **4** and **5** exhibited good activity against *T. brucei* and *L. donovani* with IC_{50} of 1.12 and 8.82 μM and 12.94 and 16.66 μM respectively.

Keywords: *Diospyros assimilis*; Ebenaceae; Antiprotozoal Naphthalene derivatives; Naphthaldehydes; Naphthoquinones; Cytotoxicity

1. Introduction

The genus *Diospyros*, family Ebenaceae, consists of woody shrubs and trees distributed in the tropical and sub-tropical regions of the world. Around 500 species are known world wide, of which, 41 species are native to India (Sastry, 1952; George Watt, 1956). Many *Diospyros* species have also been reported to exhibit interesting biological and pharmacological properties. Extracts of several *Diospyros* spp. have been used in Indian traditional systems of medicine such as Ayurveda, the African folklore and Chinese medicine for the treatment of whooping cough, leprosy, snake bites, scabies, skin eruptions, dysentry, eye infections, menstrual troubles, abdominal pains, wounds, ulcers, chest pains and gonorrhoea (George Watt, 1956). Due to their medicinal importance, extensive phytochemical studies have been carried out on more than 130 species of *Diospyros* (Mallavadhani et al., 1998) and have revealed the widespread presence of mono and dimeric naphthoquinones, naphthalene derivatives and lupane triterpenes (Hegnauer, 1989). *Diospyros assimilis* Bedd., also known as royal timber, is regarded in the sub-continent as one of the major ebony-producing trees and found growing in the Western Ghats from the Konkan coast to the hills of Travancore in Southern India (Gamble, 1997). To date, there has been no report on the chemical constituents or biological properties of this tree.

Quinonoid compounds form one of the major classes of natural products that possess significant biological

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activities (Fournet et al., 1992; Sepúvelda-Boza and Cassels, 1996 and Akendengue et al., 1999). The most interesting characteristics are their biological activities against parasitic protozoa of the genera *Leishmania*, *Trypanosoma* and *Plasmodium* (Croft et al., 1985; Koumaglo et al., 1992; Perez et al., 1997; Likhitwitayawuid et al., 1998). As part of our continued search for new bio-active compounds from natural sources (Ganapaty et al., 2004; Ganapaty et al., 2005; Ganapaty et al., 2006a; Ganapaty et al., 2006b), we have examined for the first time, a locally available species, *D. assimilis* for the presence of new antiprotozoal compounds. The present study led to the isolation and structure elucidation of two new 2-naphthaldehydes **1**, **2** and four known compounds **3**–**6** and their activity towards *Leishmania, Trypanosoma* and *Plasmodium* parasites. This report is the first of its kind on the antiprotozoal activity of polyoxygenated 2-naphthaldehydes from nature.

2. Results and discussion

The crude chloroform extract from the roots of *D. assimilis* was subjected to a succession of chromatographic procedures, including silica gel column chromatography and preparative TLC to afford two new 2-naphthaldehydes, namely 4-hydroxy-3,5-dimethoxy-2-naphthaldehyde **1**, 4-hydroxy-5-methoxy-2-naphthaldehye **2**, its related isomer 5-hydroxy-4-methoxy-2-naphthaldehyde **3** (Harper et al., 1970) and three commonly occurring naphthoquinones, diospyrin **4** (Yoshida and Mori, 2000), 8′-hydroxyisodiospyrin **5** (Baker et al., 1998) and the simple monomer, plumbagin **6** (Fallas and Thomson, 1968). All structures were elucidated using 1D and 2D NMR spectroscopic data and by comparison with those reported in the literature (see Fig. 2).

Compound **1** was obtained as yellow needles, mp 151–152 °C. The HR-EI mass spectrum showed a molecular ion at *m/z* 232.0733, suggesting a molecular formula $C_{13}H_{12}O_4$. It exhibited UV characteristics of a typical naphthalene aldehyde (Brown and Thomson, 1965) with

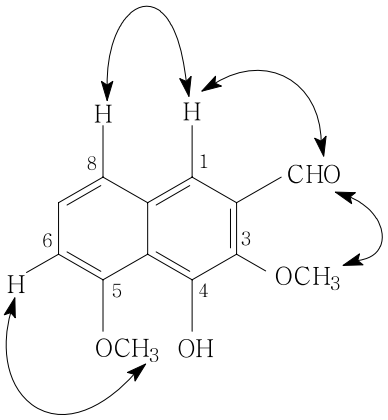

Fig. 1. NOESY interactions of **1** (Molecular mass: *m/z* 232.07).

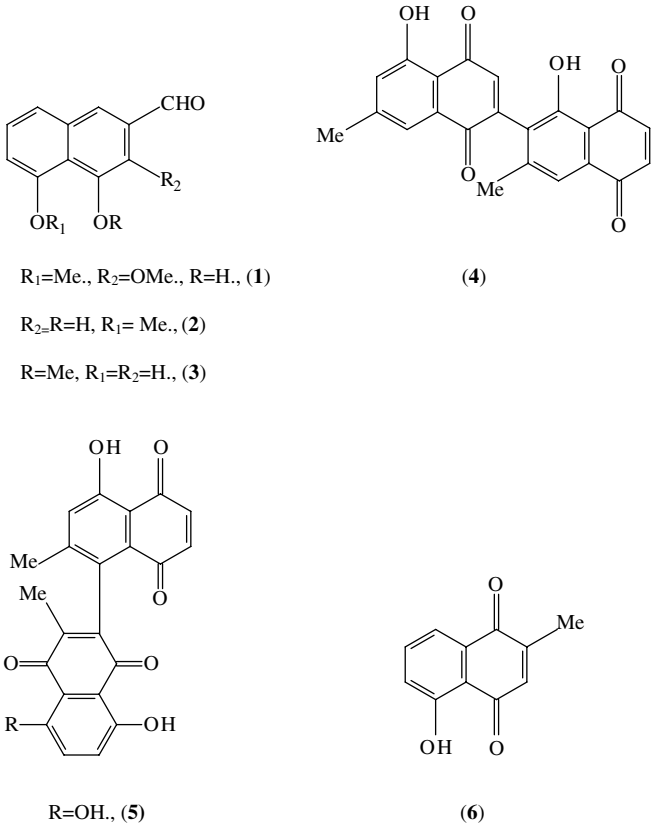

Fig. 2. Naphthalene derivatives from *D. assimilis* **1–6.**

absorption bands at 231, 291 and 390 nm. The ^{1}H NMR spectral data (Table 1) suggested a 2-naphthaldehyde structure with a highly oxygenated "A" ring with signals corresponding to two methoxyls (δ_H 4.06 and δ_H 4.11), a hydroxyl substituent (δ_H 9.46) and an aldehyde group (δ_H 10.53). In addition, the spectrum also displayed signals for four aromatic methines corresponding to H-1 (δ_H 7.85) of the A ring and three other signals resonating at δ_H 6.89, 7.32 and 7.51 assignable to H-6, H-7 and H-8 protons. Hence, the second methoxyl resonating at δ_H 4.11 must be located at C-5 (B ring) of a 3,4-oxygenated-2-naphthaldehyde.

Assignment of structure **1** was accomplished by HMBC correlations and confirmed by 2D-NOESY experiments that revealed interactions between H-1 and H-8, the aldehyde proton and H-1 and a first methoxyl, and H-6 and the second methoxyl (see Fig. 1). The downfield signal for a methine at H-1 (δ_H 7.85) showed clear ^3J correlations with the aldehyde carbon (δ_C 191.0), the methine carbon at C-8 (δ_C 124.0), the quaternary carbons C-3 (δ_C 144.1) and C-4a (δ_C 119.2) and on this basis, C-3 was assigned the position of the first methoxyl. The upfield methine at H-6 (δ_H 6.89) showed ^2J couplings with carbons C-5 (δ_C 155.8), C-7 (δ_C 125.7) and a ^3J correlation with a quaternary carbon at C-4a (δ_C 119.2) supporting that the second methoxyl must be located at C-5 of a 3,4-oxygenated-2-naphthaldehyde. Compound **1** was therefore characterized as the new 4-hydroxy-3,5-dimethoxy-2-naphthaldehyde.

Table 1
^{1}H, ^{13}C NMR and HMBC spectral data (run in $CDCl_3$, δ in ppm) of naphthaldehydes (**1–3**)

C/H	**1**		**2**		**3**		Observed HMBC[A]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{2}J	^{3}J
1	7.85 *s*	119.7[a]	7.82 *d* (1.2)	125.1	7.92 *d* (1.3)	130.7		CHO, C-3, C-4a, C-8
2		129.7		136.0[a]		134.6		
3		144.1	7.31 *d* (1.2)	107.0	7.23 *d* (1.3)	98.9	C-2	CHO, C-1
4		147.1		155.8[b]		157.5		
4a		119.2[a]		118.4		117.8		
5		155.8		156.4[b]		155.0		
6	6.89 *d* (7.7)	106.7	6.96 *d* (7.8)	107.4	7.08 *m*	114.7	C-5,C-7	C-4a
7	7.32 *t* (7.9)	125.7	7.44 *t* (7.9)	127.2	7.48 *m*	129.2	C-6	C-5, C-8a
8	7.51 *d* (8.3)	124.0	7.59 *d* (8.3)	123.6	7.48 *m*	121.0	C-8a	C-6
8a		132.1		136.3[a]		136.0		
CHO	10.53 *s*	191.0	10.07 *s*	192.3	10.10 *s*	191.9	C-2	C-1, C-3
OH	9.46 *s*	–	9.44 *s*	–	9.30 *s*	–		
3-OMe	4.07 *s*	62.1	–	–	–	–		
4-OMe	–	–	–	–	4.14 *s*	56.7	C-4	
5-OMe	4.11 *s*	56.5	4.11 *s*	56.5	–	–	C-5	

[a,b] Signals within a column are interchangeable. Coupling constants "*J*" (parentheses) in Hertz.
[A] Assignments were confirmed by 2D NMR experiments (^{1}H–^{1}H COSY, HSQC and 2D-NOESY).

Interestingly, the related isomer, 6-hydroxy-4,5-dimethoxy-2-naphthaldehyde has been reported from both *Diospyros ebenum* (Brown and Thomson, 1965) and *D. quiloensis* (Harper et al., 1970).

Compound **2** was obtained as yellow needles, m.p. 85–90 °C. The HR-EIMS showed a molecular ion at *m/z* 202.0627 corresponding to the molecular formula $C_{12}H_{10}O_3$. In the ^{1}H NMR (Table 1), the spectroscopic data of **2** closely resembled those of **1** except for the presence of signals corresponding to the *meta*-coupled protons, H-1 and H-3 of the A ring that appeared at δ_H7.82 and 7.31 ($J = 1.2$ Hz), respectively. Furthermore, the spectrum also showed the presence of a lone methoxyl at δ_H 4.11 (C-4) and a slightly up field hydroxyl resonance at δ_H 9.44. Compound **2** was found to be isomeric with the known, 5-hydroxy-4-methoxy-2-naphthaldehyde **3**, previously reported from the heart-wood of *D. quiloensis* (Harper et al., 1970). Accordingly, **2** was characterized as the new 4-hydroxy-5-methoxy-2-naphthaldehyde on the basis of a full 2D ^{1}H-^{13}C study (HSQC, HMBC) with the position of the methoxyl, H_3 at C-5 being confirmed by a strong NOE interaction to H-6. Although, 4-hydroxy-5-methoxy-2-naphthaldehyde has been previously reported through synthesis (Harper et al., 1970), this study constitutes the first isolation of **2** from nature. Complete ^{1}H and ^{13}C NMR for naphthaldehydes **1–3**, together with their HSQC ($^{1}J_{CH}$) and HMBC (^{2}J, $^{3}J_{CH}$) spectral data are presented in Table 1.

The naphthalene derivatives **1–5** (Table 2) were studied in vitro to inhibit the parasites, *Trypanosoma brucei rhodesiense* (STIB 900 strain, trypomastigote stage), *Trypanosoma cruzi* (Tulahuen strain C2C4, trypomastigote stage), *Leishmania donovani* (strain MHOM-ET/67/L82, amastigote stage) and *Plasmodium falciparum* (K1 strain, IEF stage, chloroquine resistant) using established procedures (Baltz et al., 1985; Orhan et al., 2006; Weniger et al., 2006; Brun and Schonenberger, 1979; Matile and Pink, 1990). Among the naphthalene derivatives tested, naphthaldehyde **1** showed moderate inhibition of the growth of the parasites, *T. brucei, T. cruzi* and *L. donovani* with IC_{50} values of 19.82, 12.28 and 38.78 μM, while the two isomeric naphthaldehydes, **2** and **3** showed very low response against all the three parasites. All three naphthaldehydes were marginally active against the K1 strain of *P. falciparum*. To compare the effect on the parasites with toxicity towards rat skeletal myoblasts (L-6 cells), the cytotoxic activities of the isolated compounds against the L-6 cell lines were evaluated (Page et al., 1993; Ahmed et al., 1994) and naphthaldehydes **1–3** displayed cytotoxicity with IC_{50} values of 174.94, 313.76 and 315.74 μM, respectively.

Table 2
Antiprotozoal and cytotoxic activity (IC_{50} values (μM) of naphthalene derivatives (**1–5**)[a]

Compounds	IC_{50} values (μM)				
	T. b. rhod	*T. cruzi*	*L. don. axenic*	*P. falc.* K1	L-6 cells
1	19.82	12.28	38.78	21.54	174.94
2	>50	>50	>50	24.74	313.76
3	>50	>50	>50	24.74	315.74
4	1.12	40.90	8.82	5.85	6.38
5	12.94	>50	16.66	8.94	9.30
Standards	0.005	1.69	0.47	0.19	0.048

Standards used: melarsoprol (*T.b. rhodesiense*), benznidazole (*T. cruzi*), miltefosine (*L. donovani*), chloroquine (*P. falciparum*) and podophyllotoxin (L-6 cells, cytotoxicity).
[a] Values indicate the inhibitory concentration of a compound/standard in μM necessary to achieve 50% growth inhibition (IC_{50}). Data shown are values from two replicate experiments.

The dimeric quinone **4** exhibited high inhibition of the growth of *T. brucei* and *L. donovani* with IC_{50} values of 1.12 and 8.82 μM, while the dimer, **5** was less active compared to **4** with IC_{50} values of 12.94 and 16.66 μM. Both

4 and **5** exhibited moderate activity against *P. falciparum* with IC_{50} values of 5.85 and 8.94 μM but showed toxicity towards L-6 cell lines with IC_{50} values of 6.38 and 9.30 μM, respectively. The tested compounds were found to be relatively less active in comparison with standard drugs like melarsoprol (*T.b. rhodesiense*), benznidazole (*T. cruzi*), miltefosine (*L. donovani*), chloroquine (*P. falciparum*) and podophyllotoxin (L-6 cells, cytotoxicity).

3. Conclusions

D. assimilis yields significant amounts of both mono and dimeric range of naphthalene derivatives which at present is exclusive to this taxon, but fairly widespread in *Diospyros*. At present, only two species are known to produce polyoxygenated 2-naphthaldehydes, *D. ebenum,* the other 'Royal' timber of India and the African crocodile bark tree, *D. quiloensis*. The later is known to produce the bulk of the methoxylated 2-naphthaldehydes. However, to our present knowledge oxygenation on the basic naphthalene aldehyde skeleton seem to be confined to C-3/4/5 as demonstrated by the isolation of three, 2-naphthaldehydes **1–3** from *D. assimilis*, while *D. ebenum* (Brown and Thomson, 1965; Gupta and Dhar, 1969; Sankaram and Reddy, 1984) and *D. quiloensis* (Harper et al., 1970) have shown slightly different patterns with oxygenation at C-4/5/6 and C-4/5/6/8 respectively. The 2-naphthaldehydes **1–3** isolated in this study are allied to the monomeric juglone, plumbagin **6**, via: a simple oxidation of the one-carbon substituent present at a β-position, while the dimeric quinones **4** and **5** are clearly derived from the isomer, 7-methyljuglone.

The results obtained from our in vitro bioassays have shown that the dimeric quinones **4** and **5** displayed high inhibition of growth of *T. brucei* and *L. donovani*, while moderate activity was observed against *P. falciparum* and low in vitro activity against *T. cruzi.* Naphthalene derivative **1** was found to be moderately active. It was by far the most active of the three naphthaldehydes. All the tested compounds displayed cytotoxicity towards rat skeletal myoblasts (L-6 cells). This is the first report on the antiprotozoal activity of polyoxygenated-2-naphthaldehydes.

4. Experimental

4.1. General

Melting points were recorded on a Cipla I-28, digital apparatus and were uncorrected. Silica gel (Acme) 60–120 mesh for column chromatography and silica gel (Acme) 100–200 mesh were used for preparative thin layer chromatography. Spots on chromatogram were detected under UV light and by spraying with 5% H_2SO_4 in methanol. UV spectra were recorded in MeOH. Both 1D and 2D-NMR spectra were run in $CDCl_3$ (^1H: 500 MHz; ^{13}C: 125 MHz) on a Bruker AVANCE DRX-500 spectrometer. Accurate mass measurements were determined on a Kratos M525 RFA instrument. LC–MS (EI) were recorded using an Agilent 1100 series LC/MSD in the APCI mode.

4.2. Plant material

The roots of *D. assimilis* were collected at Vizianagram, India, in March 2002. The sample was authenticated by Dr. S. Hara Sreeramulu, Taxonomist, Botany Department, Dr. V.S. Krishna College, Visakhapatnam. A voucher specimen (SG/DAR/02/106) has been deposited at the Herbarium, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India (Herbarium Code = SKU).

4.3. Extraction and isolation

Shade dried roots (1.2 kg) were powdered in a Wiley mill and then extracted over 4 days with $CHCl_3$ (4 × 1.5 L) at room temperature. The combined extracts were concentrated under reduced pressure to yield 24 g of reddish brown gum. A portion of the chloroform extract (20 g) was chromatographed over silica gel and eluted, in succession, with petroleum ether (bp 40–60 °C), petroleum ether–chloroform mixtures, chloroform and chloroform–methanol mixtures (each 250 ml fractions).

Elution of the chromatogram with petroleum ether–chloroform (9:1) gave 0.023 mg of colourless needles that were identified as lupeol (mp 212–214 °C). Fractions eluted with petroleum ether–chloroform (7:3) afforded 60 mg of colourless needles identified as β-sitosterol (mp 136-137 °C), while the later fractions eluted with petroleum ether–chloroform (1:1) gave yellow needles (**1**, 24 mg). Elution with petroleum ether– chloroform (3:7) gave an orange colour fraction that revealed two compounds on TLC. This fraction on preparative silica gel TLC using petroleum ether–chloroform (1:4) afforded yellow needles (**2**, 25 mg) and red needles (**3**, 30 mg). On eluting the column with pure chloroform, a dark red fraction was obtained that revealed two compounds on TLC. This fraction was also purified by prep. TLC on silica gel eluting with $CHCl_3$-MeOH (99:1) to afford, orange-red prisms (**4**, 40 mg) and dark red needles (**5**, 70 mg). Further elution of the column with chloroform–methanol (95:5) gave red needles (**6**, 20 mg), while the mother liquor deposited 0.035 mg of colourless needles (mp 185–187 °C) identified as betulinaldehyde.

Compounds **1–3** gave a positive reaction to Tollen's reagent, confirming the presence of an aldehyde group and fluoresced yellow on TLC when exposed to UV light. Compounds **4–6** gave a purple colour with alkali indicating the presence of a *peri*-hydroxy naphthoquinones. Both **4** and **5** appeared orange-brown in colour in visible light but under UV light, **4** emerged orange-red, while **5** was dark brown.

4-Hydroxy-3,5-dimethoxy-2-naphthaldehyde **1**; Yellow needles from MeOH; mp 151–152 °C; UV λ_{max} nm (log

ε): 231 (4.95), 260 (4.84), 291 (4.22), 390 (3.88); HR-EIMS *m/z* 232.0733 [M^+], calcd. for $C_{13}H_{12}O_4$, 232.0736; ^1H and ^{13}C NMR ($CDCl_3$) spectra, see Table 1.

4-Hydroxy-5-methoxy-2-naphthaldehyde **2**; Yellow needles from MeOH; mp 85–90 °C; UV λ_{max} nm (log ε): 221 (4.42), 254 (4.49) 379 (3.76); HR-EIMS *m/z* 202.0627 [M^+], calcd. for $C_{12}H_{10}O_3$, 202.0630; ^1H and ^{13}C NMR ($CDCl_3$) spectra, see Table 1.

5-Hydroxy-4-methoxy-2-naphthaldehyde **3**; Orange-red needles from MeOH; mp 103-104 °C; (lit. 103–104 °C, Harper et al., 1970); LC–MS $(M+1)^+$ 203; ^1H and ^{13}C NMR ($CDCl_3$) spectra, see Table 1.

Diospyrin (5,5′-dihydroxy-7,7′-dimethyl-2,6′-binaphthalene-1, 4,1′,4′-tetrone) **4;** Orange-red prisms from MeOH; mp 252–254 °C (lit. 256–258 °C); LC–MS $(M+1)^+$ 375; ^1H and ^{13}C NMR (Yoshida and Mori, 2000).

8′-Hydroxyisodiospyrin (5,5′,8′-trihydroxy-7,2′-dimethyl-8, 3′-binaphthalene-1,4,1′,4′-tetrone) **5**; Red needles from MeOH; mp 275 °C (lit. 274 °C); LC–MS $(M+1)^+$ 375; ^1H and ^{13}C NMR (Baker et al., 1998).

Plumbagin, 5-Hydroxy-2-methyl-1,4-naphthoquinone **6**; Orange needles from MeOH; mp 78–79 °C (lit. 75 °C); LC–MS $(M+1)^+$ 169; ^1H and ^{13}C NMR (Fallas and Thomson, 1968).

4.4. Bioassays

4.4.1. Antiprotozoal activity

4.4.1.1. In vitro assay for Trypanosoma brucei rhodesiense. *T. brucei rhodesiense* STIB 900 strain and the standard drug melarsoprol (Arsobal) were used for the assay. Minimum Essential Medium (50 μL) supplemented (Baltz et al., 1985) with 2-mercaptoethanol and 15% heat-activated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 μg/mL and then added to the wells. Then 10^4 bloodstream forms of *Trypanosoma b. rhodesiense* STIB 900 in 50 μL were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Ten microlitres of resazurin solution (Orhan et al., 2006; Weniger et al., 2006) (12.5 mg resazurin dissolved in 100 mL distilled water) was then added to each well and incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Raz et al., 1997) Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) which calculated IC_{50} values.

4.4.1.2. In vitro assay for Trypanosoma cruzi. Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 μL RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 hours the medium was removed and replaced by 100 μL per well containing 5000 trypomastigote forms of *T.cruzi* (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene) (Weniger et al., 2006). Forty-eight hours later the medium was removed from the wells and replaced by 100 μL fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 90 μg/mL to 0.123 μg/mL. Each drug was tested in duplicate. After 96 hours of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/ Nonidet (50 μL) was added to all wells. A colour reaction developed within 2–6 hours and could be read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Benznidazole was the standard drug used.

4.4.1.3. In vitro assay for Leishmania donovani (axenic amastigote assay). Fifty microlitres of culture medium, a 1:1 mixture of SM medium (Cunningham, 1977) and SDM-79 medium (Brun and Schonenberger, 1979) at pH 5.4 supplemented with 10% heat-inactivated FBS, was added to each well of a 96-well microtiter plate (Costar, USA). Serial drug dilutions in duplicates were prepared covering a range from 30 to 0.041μg/mL. Then 10^5 axenically grown *Leishmania donovani* amastigotes (strain MHOM-ET/67/L82) in 50 μL medium were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Ten microlitres of resazurin solution (Weniger et al., 2006) (12.5 mg resazurin dissolved in 100 mL distilled water) were added to each well and incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Raz et al., 1997) Fluorescence development was measured and expressed as percentage of the control. Data were transferred into the graphic programme Softmax Pro (Molecular Devices) and IC_{50} values calculated. Miltefosin (Zentaris GmbH, Germany) was used as a positive reference.

4.4.1.4. In vitro assay for Plasmodium falciparum. Antiplasmodial activity was determined using the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^3H]-hypoxanthine incorporation assay was used (Matile and Pink, 1990). Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax II were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 0.5 μCi ^3H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate TM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration

and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated. The IC_{50}values are the means of four values of two independent assays carried out in duplicate.

4.4.1.5. Cytotoxicity. The cytotoxicity assay of the tested naphthalene derivatives was done following the method of Page (Page et al., 1993) with the modification of Ahmed (Ahmed et al., 1994). Cell line L-6 (rat skeletal muscle myoblasts) were seeded in 96-well Costar microtiter plates at 2×103 cells/100 ml, 50 ml per well in MEM supplemented with 10% heat inactivated FBS. A three-fold serial dilution ranging from 90 to 0.13 mg/ml of compounds in test medium was added. Plates with a final volume of 100 ml per well were incubated at 37 °C for 72 h in a humidified incubator containing 5% CO_2. Resazurin was added as viability indicator according to Ahmed et al. (1994). After an additional 2 h of incubation, the plate was measured with a fluorescence scanner using an excitation wavelength of 536 nm and an emission wavelength of 588 nm (SpectraMax GeminiXS, Molecular Devices). Podophyllotoxin (Polysciences Inc., USA) was used as a positive reference.

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