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Anti-protozoal and plasmodial FabI enzyme inhibiting metabolites of *Scrophularia lepidota* roots ☆

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Abstract

The ethanolic root extract of *Scrophularia lepidota*, an endemic plant of the Turkish flora, has been investigated for its anti-protozoal and inhibitory effect towards plasmodial enoyl-ACP reductase (FabI), a key enzyme of fatty acid biosynthesis in *Plasmodium falciparum*. Chromatographic separation of the extract yielded 10 iridoids (**1**–**10**), two of which are new, and a known phenylethanoid glycoside (**11**). The structures of the new compounds were determined as 3,4-dihydro-methylcatalpol (**8**) and 6-*O*-[4″-*O*-*trans*-(3,4-dimethoxycinnamoyl)-α-L-rhamnopyranosyl]aucubin (scrolepidoside, **9**) by spectroscopic means. The remaining metabolites were characterized as catalpol (**1**), 6-*O*-methylcatalpol (**2**), aucubin (**3**), 6-*O*-α-L-rhamnopyranosyl-aucubin (sinuatol, **4**), 6-*O*-β-D-xylopyranosylaucubin (**5**), ajugol (**6**), ajugoside (**7**), an iridoid-related aglycone (**10**) and angoroside C (**11**). Nine isolates were active against *Leishmania donovani*, with the new compound **9** being most potent (IC_{50} 6.1 μg/ml). Except for **4**, all pure compounds revealed some trypanocidal potential against *Trypanosoma brucei rhodesiense* (IC_{50} values 29.3–73.0 μg/ml). Only compound **10** showed moderate anti-plasmodial (IC_{50} 40.6 μg/ml) and FabI enzyme inhibitory activity (IC_{50} 100 μg/ml). **10** is the second natural product inhibiting the fatty acid biosynthesis of *Plasmodium falciparum*.

Keywords: *Scrophularia lepidota*; Scrophulariaceae; Iridoid; Phenylethanoid glycoside; *Plasmodium*; *Trypanosoma*; *Leishmania*; Enoyl-acyl carrier protein reductase (FabI)

1. Introduction

Malaria, trypanosomiasis and leishmaniasis belong to the most widespread and poorly controlled parasitic diseases in the world. Approximately 40% of the world's population is at risk of malaria with more than 300 million new cases and 1 million deaths annually (WHO, 2000). Trypanosomiasis, caused by both *Trypanosoma brucei* and *T. cruzi,* threatens millions of people living in tropical regions. Visceral leishmaniasis, caused by *Leishmania donovani*, is endemic in many parts of the world and affects an estimated 15 million people worldwide (Ashford et al., 1992). Anti-protozoal drugs, on the other hand, are inadequate due to their toxicity, lack of efficacy and inability to eliminate all life cycles stages of these parasites from the host. Furthermore, there is an

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escalating problem of widespread resistance to commonly used chemotherapeutic agents. Thus, new anti-protozoal agents with novel targets are urgently needed.

Fatty acids (FAs) are crucial for all living organisms and the enzymes involved in their biosynthesis are organized in two distinct ways. Higher eukaryotes and fungi accomplish FA biosynthesis by a large, multifunctional protein, which is classified as type I fatty acid synthase (FAS-I) (Smith, 1994), while plants and most prokaryotes perform the same enzymatic steps using separate enzymes (type II fatty acid synthase, FAS-II) (Harwood, 1996). It has been shown that a number of obligate endoparasites, including *P. falciparum* and *T. brucei* synthesize their own FAs using a type II pathway (Waller et al., 1998; Gardner et al., 1998; Morita et al., 2000). As type II FAS is absent in humans, this pathway shows great potential as a target for the development of anti-protozoal agents. The enoyl-ACP reductase enzyme (also known as FabI) is one of the most important enzymes in FA biosynthesis of *P. falciparum* and commits the final reduction step in chain elongation (Waller et al., 1998). Therefore, it is an excellent target for anti-malarial drug discovery.

In the course of our search for new anti-protozoal lead compounds from endemic Turkish plants, we investigated the roots of *Scrophularia lepidota* Boiss. (Scrophulariaceae), which showed trypanocidal, leishmanicidal, anti-malarial and plasmodial FabI enzyme inhibiting properties in initial screenings. Chromatographic separation of the crude extract afforded 9 iridoid glycosides, two of which (**8** and **9**) are new to the literature, an iridoid-related aglycone (**10**) and a known phenylethanoid glycoside, angoroside C (**11**). The current study presents the isolation, structure elucidation and the biological profile of compounds **1**–**11**.

2. Results and discussion

In this study, the ethanolic root extract of *S. lepidota*, on which no chemical or biological study had been performed before, has been investigated. In preliminary screening (Table 2), the crude extract was inactive against *T. cruzi*, but it showed activity against *T. brucei rhodesiense* (IC_{50} 38.4 μg/ml), *L. donovani* (IC_{50} 26 μg/ml) and multidrug-resistant (K1) strain of *P. falciparum* (IC_{50} 17.5 μg/ml). The latter activity prompted us to investigate the ability of the crude extract to inhibit the purified *P. falciparum* FabI enzyme (IC_{50} 80 μg/ml). By employing a combination of C18-VLC, C18-MPLC and SiO_2 column chromatography (CC), eleven compounds were isolated. The detailed procedure for purification of the compounds **1**–**11** is elaborated in Section 4.

Based on the spectroscopic data, compounds **1** and **2** were readily recognized as catalpol (**1**) and 6-*O*-methylcatalpol (**2**), very common iridoid glucosides of the family Scrophulariaceae (El-Naggar and Beal, 1980; Boros and Stermitz, 1990). Again from the 1D and 2D NMR, MS and $[\alpha]_D$ data, compounds **3**–**7** were identified as known iridoid glycosides, aucubin (**3**), 6-*O*-α-L-rhamnopyranosyl-aucubin (sinuatol, **4**), 6-*O*-β-D-xylopyranosylaucubin (**5**), ajugol (**6**), ajugoside (**7**) (El-Naggar and Beal, 1980; Boros and Stermitz, 1990) and angoroside C (**11**), a phenylethanoid glycoside previously reported from several *Scrophularia* species (Çalış et al., 1988; de Santos et al., 2000).

Compound **8** was isolated as an amorphous powder, which was conclusively identified as 3,4-dihydro-methylcatalpol on the basis of extensive 1D and 2D NMR experiments (HSQC, DQF-COSY, HMBC and ROESY). It was assigned the molecular formula $C_{16}H_{26}O_{10}$, determined by ESIMS and HR-MALDIMS. The ^{13}C NMR spectrum contained 16 signals, six of which belonged to a β-glucose unit (Table 1). The remaining 10 signals were sorted out as a methoxyl, three methylenes, three oxymethines, two methines and a fully substituted C atom. Comparison of the 1D NMR data of **8** with those of 6-*O*-methylcatalpol (**2**) suggested many similarities. The most striking exception was the absence of the typical olefinic signals ($\Delta^{3,4}$) of the cyclopentane-pyran iridoid aglycone in **8**. Instead, these protons were replaced by two pairs of non-equivalent methylene signals; one pair of aliphatic (H-4a δ 1.56, H-4b δ 1.77) and one pair of oxygenated (H-3a δ 3.53; H-3b δ 3.89). In the HSQC spectrum, these CH_2 carbons resonated at δ 24.2 (C-4, *t*) and δ 63.0 (C-3, *t*), suggesting that the olefinic bond ($\Delta^{3,4}$) was saturated. These observations were in good agreement with the molecular formula, UV and the IR data. Further proof for the 3,4-dihydro-iridoid structure came from the DQF-COSY and the HMBC data (Table 1). The relative stereochemistry of **8** was established by a ROESY experiment. The ROE cross peaks between H-6/H-7, H-6/H-1, H-7/H-1, H-5/H-9 and H-1/H-1′ indicated the relative configuration of **8** to be the same as that of **2**. Hence, **8** is 3,4-dihydro-methylcatalpol.

Compound **9**, named as scrolepidoside, was also obtained as pale yellow amorphous powder. The ESIMS and HRMALDI-MS revealed the molecular formula $C_{32}H_{42}O_{16}$, requiring 12 double bond equivalents (DBE). The UV spectrum of **9** exhibited absorption bands at λ_{max} 214, 240, 280(sh) and 316 nm, characteristic for an iridoid enol–ether system and a cinnamoyl chromophore. The IR displayed intense absorption bands due to hydroxyl (3374 cm^{-1}), conjugated carbonyl (1705 cm^{-1}), conjugated double bond (1632 cm^{-1}) and aromatic ring (1599, 1514, 1453 cm^{-1}). The ^1H NMR spectrum of **9** was very similar to that of **4**, except for the presence of additional signals due to a *trans* -3,4-dimethoxycinnamic acid moiety [δ 6.45 (*d*, H-α) and δ 7.67 (*d*, H-β); δ 6.97 (*d*, H-5‴); δ 7.19 (*dd*,

Table 1
1D NMR data of **8**,**9** and HMBC correlations observed for **8** (CD_3OD, 600 MHz for ^1H; 150 MHz for ^{13}C, δ in ppm, J in Hz)*

Atom	**8**			**9**	
	^1H	**^{13}C**	**HMBCs (C to H)**	**^1H**	**^{13}C**
1	4.77 (*d*, 9.2)	97.8 *d*	H_2-3, H-5, H-9, H-1′	4.93 (*d*, 7.3)	98.1 *d*
3a	3.53 (*ddd*, 2.5, 12.8, 15.0)	63.0 *t*	H-1, H-4, H-5	6.34 (*dd*, 1.8, 6.1)	142.1 *d*
3b	3.89[a]				
4a	1.56 (*br. d*, 14.3)	24.2 *t*	H_2-3, H-6	5.13 (*dd*, 3.9, 6.1)	105.6 *d*
4b	1.77 *m*				
5	2.10 (*q*, 7.7)	36.6 *d*	H-1, H_2-4, H-6, H-7, H-9	2.82 *m*	44.4 *d*
6	3.86[a]	82.6 *d*	H_2-4, H-5, H-7, H-9, H_3-OMe	4.48 (*dd*, 1.7, 3.5)	89.4 *d*
7	3.68 *s*	58.3 *d*	H-6, H-9, H-10	5.89 (*br. s*)	127.3 *d*
8		66.3 *s*	H-1, H-7, H-9, H-10		149.8 *s*
9	2.26 (*dd*, 7.7, 9.2)	43.2 *d*	H-1, H_2-4, H-5, H-10	2.92 *m*	48.3 *d*
10a	4.05 (*d*, 13.1)	61.2 *t*	H-7, H-8	4.19 (*d*, 16.1)	61.6 *t*
10b	3.79 (*d*, 13.1)			4.38 (*d*, 16.1)	
1′	4.69 (*d*, 7.9)	99.4 *d*	H-1, H-2′	4.69 (*d*, 8.0)	100.1 *d*
2′	3.22[a]	74.9 *d*	H-1′, H-3′	3.22 (*dd*, 8.0, 9.2)	75.1 *d*
3′	3.37[a]	77.9 *d*	H-2′, H-4′	3.39 (*t*, 9.0)	78.0 *d*
4′	3.26[a]	71.8 *d*	H-3′, H-5′	3.29[a]	71.7 *d*
5′	3.27[a]	78.6 *d*	H-1′, H-4′, H_2-6′	3.28[a]	78.4 *d*
6′a	3.63 (*dd*, 6.2, 11.7)	63.0 *t*	H-4′, H-5′	3.64 (*dd*, 5.4, 12.0)	62.8 *t*
6′b	3.90 (*dd*, 1.8, 11.7)			3.85[a]	
1″				4.86 (*d*, 1.2)	101.4 *d*
2″				3.83[a]	70.5 *d*
3″				3.89[a]	72.8 *d*
4″				5.08 (*t-like dd*, 9.7)	75.7 *d*
5″				3.92[a]	68.5 *d*
6″				1.19 (*d*, 6.3)	18.1 *q*
α				6.45 (*d*, 15.9)	116.6 *d*
β				7.67 (*d*, 15.9)	146.9 *d*
1‴					128.9 *s*
2‴				7.22 (*d*, 1.9)	111.8 *d*
3‴					150.9 *s*
4‴					153.0 *s*
5‴				6.97 (*d*, 8.4)	112.8 *d*
6‴				7.19 (*dd*, 1.9, 8.4)	124.2 *d*
OMe	3.49 *s*	57.9 *s*	H-6	3.86 *s*	56.6 *q*
OMe				3.87 *s*	56.7 *q*
C=O					168.8 *s*

[a] Signal multiplicity is unclear due to overlapping.
* All assignments are based on 2D NMR experiments (HSQC, HMBC, DQF-COSY and ROESY).

Table 2
Anti-protozoal activity of the crude extract of *Scrophularia lepidota* and its constituents

Compound	*Trypanosoma b. rhodesiense*	*Trypanosoma cruzi*	*Leishmania donovani*	*Plasmodium falciparum*	L-6 cells
Standard	0.0033[a]	0.70[b]	0.32[c]	0.002[d]	0.0075[e]
Crude extract	38.4	>90	26.0	17.5	>90
1	54.8	>90	10.4	>50	>90
2	32.5	>90	8.3	>50	>90
3	51.1	>90	10.9	>50	>90
4	>100	>90	>100	>50	>90
5	38.0	>90	8.5	>50	>90
6	31.8	>90	7.2	>50	>90
7	56.4	>90	8.5	>50	>90
8	73.0	>90	12.7	>50	>90
9	33.3	>90	6.1	>50	>90
10	58.5	>90	>100	40.6	>90
11	29.3	>90	8.0	>50	>90

IC_{50} in μg/ml, [a] melarsoprol, [b] benznidazole, [c] miltefosin, [d] artemisinin, [e] phodophyllotoxin.

H-6‴), δ 7.22 (*d*, H-2‴); δ 3.86 (*s*, OMe) and δ 3.87 (*s*, OMe)] (Çalıs et al., 1992). All these data were almost identical with those of unduloside II (6-*O*-[3″-*O*-*trans*-(3,4-dimethoxycinnamoyl)-α-L-rhamnopyranosyl]aucubin) reported from *Verbascum undulatum* (Magiatis et al., 2000), except for some differences in the rhamnosyl parts. A comparison showed that the H-3″ signal of unduloside II was seen at low field (δ 5.07 *dd*, $J = 9.5$ and 3.5 Hz), while in **9** the H-4″ signal (δ 5.08 *t-like dd*, $J = 9.7$ Hz) appeared at low field, demonstrating the 4″ position in **9** to be the site of acylation. Also a cross peak between the acyl carbonyl (δ 168.8) and the H-4″ signal supported this assumption. The ROESY spectrum confirmed the relative stereochemistry of the chiral centers within **9** as shown. Thus, scrolepidoside is 6-*O*-[4″-*O* *trans*-(3,4-dimethoxycinnamoyl)-α-L-rhamnopyranosyl] aucubin.

The iridoid aglycone (**10**) was isolated as a minor component. Its molecular formula was deduced to be $C_9H_{14}O_3$ (ESIMIS, HR-MALDIMS), which required three DBE. Due to the absence of sugar signals, the NMR spectra of **10** were rather simple. Both ^1H and ^{13}C NMR data (in MeOD) of **10**, assigned on the basis of 2D NMR experiments (DQF-COSY, HSQC, HMBC, ROESY), were found to be identical with those of ningpogenin, an iridoid-related aglycone isolated from the roots of *Scrophularia ningpoensis* (Qian et al., 1992). This compound was previously reported from the same plant by Kajimoto et al. (1989) and its most plausible structure was proposed as 1-dehydroxy-3,4-dihydroaucubigenin (**12**). Based on extensive 1D- and 2D NMR analyses, Qian et al. (1992) revised the structure of this metabolite as shown (**10**). However, there is a considerable difference in the specific rotation values of these compounds [**12**: $[\alpha]_D$ +181° (MeOH, Kajimoto et al., 1989), ningpogenin: $[\alpha]_D$ +16° (MeOH, Qian et al., 1992)]. On the other hand, our compound **10** displays a weak negative optical rotation ($[\alpha]_D$ −6°, MeOH). Since the ROESY spectrum of **10** supports the same relative stereochemistry for H-1, H-5 and H-6 as assigned for ningpogenin, we conclude that **10** might be the enantiomer of (+)-ningpogenin. However, since the optical rotation values are highly dependent on the purity of the individual compounds, a decisive conclusion concerning the absolute configuration of **10** still remains open. Ningpogenin and its 6-epimer have been semi-synthetically obtained from aucubin (Bonini et al., 1985; Carnevale et al., 1988), but their specific rotation values have not been reported. Very recently, Gouda et al. (2003) briefly mentioned the isolation of **12** from *Kigelia pinnata* (Bignoniaceae). However, as their report does not contain any data, a reliable comparison with (+)-ningpogenin, **12** or **10** is not possible. Thus, we enclose the full NMR data and other spectroscopic and physical data here (see Section 4).

Anti-protozoal activity of purified compounds against the same broad panel of parasites is shown in Table 2. Eight iridoids and the phenylethanoid glycoside (**11**) showed appreciable activity against the amastigote forms of *L. donovani*, with the new compound **9** being the most potent (IC_{50} 6.1 μg/ml). None of the isolates showed trypanocidal activity against *T. cruzi*, but all compounds, except for **4**, exhibited some potential against *T. brucei rhodesiense* with IC_{50} values ranging from 29.3 to 73.0 μg/ml. Although the crude extract exhibited growth inhibitory activity (IC_{50} 17.5 μg/ml) against chloroquine- and pyrimethamine-resistant (K1) strain of *P. falciparum*, the single isolates were inactive or not as potent as anticipated from the extract. Only the minor compound **10** exhibited moderate anti-plasmodial activity with IC_{50} value of 40.6 μg/ml. When screened for FabI inhibitory potential, again only **10** showed weak enzyme inhibitory potential (IC_{50} 100 μg/ml), retaining the activity of the crude EtOH extract (IC_{50} 80 μg/ml). As shown in Table 2, none of the single components exerts cytotoxicity towards the rat skeletal myoblasts (L6 cells).

3. Conclusions

Iridoids have been shown to possess a number of biological activities (Mandal et al., 1998). Only a few iridoid glycosides, such as arbortristosides A, B, C and 6β-hydroxy-loganin (Tandon et al., 1991) or iridoid mixtures, such as picroliv from *Picrorrhiza kurroa* (Puri et al., 1992; Mittal et al., 1998) have been reported to have protective or growth inhibitory activity against *L. donovani*. To our knowledge, this is the first report of the iridoid compounds and the phenylethanoid glycoside (angoroside C) isolated in this study to exhibit anti-leishmanial effects. Almost all compounds were also active against *T. brucei rhodesiense*, however the trypanocidal potential was much weaker. Fortunately, none of the compounds were cytotoxic towards the host (L6) cells. In a previous paper, we reported anti-protozoal, including appreciable anti-leishmanial, activity of a number of phenylethanoid glycosides with no significant cytotoxicity (Kırmızıbekmez et al., 2004). Hence, iridoids and phenylethanoid glycosides may be considered as safe, potential leishmanicidal agents.

The current evidence suggests that the enoyl-ACP reductase (FabI), a key regulatory and rate-limiting enzyme of type II FAS pathway in *P. falciparum*, is a good anti-malarial target (McLeod et al., 2001; Surolia and Surolia, 2001). To date, the synthetic antibacterial agent triclosan is the most potent inhibitor of plasmodial FabI (IC_{50} 14 ng/ml in this study). Triclosan shows in vitro anti-malarial activity (IC_{50} sub μM level) and is also active in vivo in *P. berghei* mouse model (Surolia and

Surolia, 2001). Very recently, we reported the first FabI-inhibiting anti-malarial natural product from another endemic Turkish plant, *Phlomis brunneogaleata* (Kırmızıbekmez et al., 2004). Despite its moderate anti-malarial and FabI inhibitory activity, compound **10** still deserves to be mentioned as it is the second natural product with plasmodial FabI inhibitory potential. Further efforts to find novel anti-malarial agents selectively inhibiting the FabI enzyme of *P. falciparum* are in progress in our laboratory.

4. Experimental

4.1. General

The 1D and 2D NMR spectra were obtained on a Bruker DRX 600 MHz spectrometer operating at 600 (^{1}H NMR) and 150 (^{13}C NMR) MHz. The chemical shift values are reported as parts per million (ppm) units relative to tetramethylsilane (TMS). ESI-mass spectra were taken on a Bruker Esquire-LC-MS (ESI mode) spectrometer. Positive mode HR-MALDIMS were recorded on a Ionspec-Ultima-FTMS spectrometer, using 2,5-dihydroxybenzoic acid (DHB) as matrix. UV spectra (MeOH) were measured on a Hewlett–Packard 8452 Diode Array spectrophotometer. A Perkin–Elmer 241 MC polarimeter was employed to record optical rotations (Na lamp, 589 nm). SiO_2 (70–230 mesh, Merck) was used for gravity-driven CC, while VLC separation was carried out over RP-18 HL (40–63 μm, Chemie Uetikon). MPLC separations were performed on a Labomatic glass column (2.6 × 46 cm and 3 × 24 cm, i.d.), packed with LiChroprep C_{18}, using a Büchi 681 pump. TLC was carried out on precoated silica gel 60 F_{254} (Merck) TLC plates using $CHCl_3$–MeOH–H_2O mixtures (80:20:2, 70:30:3 and 61:32:7, v/v) as developing systems. Compounds were detected by spraying with 1% vanillin in conc. H_2SO_4 followed by heating at 105 °C for 5 min.

4.2. Plant material

Scrophularia lepidota Boiss. (Scrophulariaceae) was collected from Sivas, Divrigi, Akmese village in June 2001 and identified by one of us (A.A.D.). Voucher specimens (AAD 9606) have been deposited at the Herbarium of the Biology Department, Faculty of Science (HUB), Hacettepe University, Ankara.

4.3. Extraction and isolation

The powdered roots of *Scrophularia lepidota* (90 g) were extracted with EtOH (4 × 500 ml) at room temperature for 8 h and filtered. The filtrates were combined and evaporated to dryness in vacuo to yield 9.28 g crude extract. This extract was fractionated over a C-18 VLC column. Step-gradient elution with H_2O–MeOH mixtures (100% H_2O to 100% MeOH) gave 11 main fractions (1–11). Fr. 4 and 5 were combined (1.04 g) and applied to a RP-18 MPLC column, employing gradient amounts of MeOH in water (5–50%) to afford 4 fractions (A–D). Catalpol (**1**, 10 mg) was isolated after subjection of Fr. A (148 mg) to silica CC (solvent system $CHCl_3$-MeOH, 85:15). Repeated CC of fr. B (194 mg) over silica ($CHCl_3$–MeOH–H_2O, 70:30:3) yielded **8** (4 mg) and **3** (13.5 mg). Compounds **10** (1.5 mg) and **5** (8 mg) were purified from fr. C (77 mg) by silica CC in a similar fashion. Fr D. (219 mg) was applied to a column packed with SiO_2 and eluted with the mixtures of $CHCl_3$–MeOH–H_2O (80:20:2 to 61:32:7) to yield **2** (9.7 mg), **6** (8.8 mg) and **4** (2 mg). Combined VLC frs 6-9 (188 mg) were separated by a combination of RP-18 MPLC (0–30% MeOH) and silica CC ($CHCl_3$–MeOH, 9:1) to give **7** (11 mg). Initial VLC fr. 10 (883 mg) was chromatographed on RP-18 MPLC (0% to 50% MeOH), followed by silica CC employing $CHCl_3$–MeOH (85:15) as mobile phase, to yield 10 mg of **11**. Finally, fr. 11 (218 mg) was fractionated over a RP-18 MPLC column using MeOH–H_2O gradient (20–55% MeOH). The final purification of the major fraction (120 mg) from this column by silica CC ($CHCl_3$–MeOH, 9:1) afforded **9** (33 mg).

4.3.1. 3,4-Dihydro-methylcatalpol (*8*)

Amorphous powder; $[\alpha]_D^{22}$ −77° (*c* 0.3, MeOH); IR (film): ν_{max} 3374, 2925, 1598, 1374, 1077, 1036 cm^{-1} ; UV (MeOH): λ_{max} 206 nm; ESI-MS *m/z* (rel. int.) 401 (100) $[M + Na]^+$; HR-MALDIMS *m/z* 401.1416 $[M + Na]^+$, calcd for $C_{16}H_{26}O_{10}Na$ 401.1420; ^{1}H NMR (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR (CD_3OD, 150 MHz), see Table 1.

4.3.2. Scrolepidoside (*9*) (6-O-[4″-O-trans-(3,4-dimethoxycinnamoyl)-α-L-rhamnopyranosyl]aucubin)

Amorphous powder; $[\alpha]_D^{22}$ −140° (*c* 0.23, MeOH); IR (film): ν_{max} 3374, 2922, 1705, 1632, 1599, 1514, 1453, 1260 cm^{-1} ; UV (MeOH): λ_{max} 214, 240, 280(sh), 316 nm; ESI-MS *m/z* (rel. int.) 705 (100) $[M + Na]^+$, 413 (35); HRMALDI-MS *m/z* 705.2350 $[M + Na]^+$, calcd for $C_{32}H_{42}O_{16}Na$ 705.2354; ^{1}H NMR (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR (CD_3OD, 150 MHz), see Table 1.

4.3.3. Compound *10*

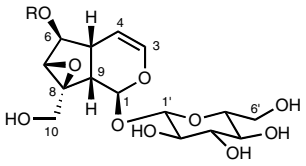
Colorless oil; $[\alpha]_D^{22}$ −6° (*c* 0.1, MeOH); IR (film): ν_{max} 3376, 2922, 1715, 1580, 1046 cm^{-1} ; UV (MeOH): λ_{max} 208, 224 nm; ESI-MS *m/z* (rel. int.) 193 (100) $[M + Na]^+$; HRMALDI-MS *m/z* 193.0833 $[M + Na]^+$, calcd for $C_9H_{14}O_3Na$ 193.0836; ^{1}H NMR (CD_3OD, 600 MHz) δ 5.01 (H-1, *dd*, *J* = 1.5, 7.5 Hz), 3.64 (H-3_{ax}, *ddd*, *J* = 6, 9, 12.5 Hz), 3.72 (H-3_{eq}, *ddd*, *J* = 6, 7,

12.5 Hz), 1.83 (H-4_{ax}, *dddd*, J = 6, 7, 9, 12.5 Hz), 1.92 (H-4_{eq}, *td*, J = 6, 12.5 Hz), 3.07 (H-5, *dddd*, J = 6, 7, 7.5, 8 Hz), 2.94 (H-6, *qq*-like, J = 1.5, 6, 8, 11.0 Hz), 5.60 (H-8, *quint*, J = 1.5 Hz), 3.77 (H-9a, A of ABX, *dd*, J = 6, 11 Hz), 3.66 (H-9b, B of ABX, *q*-like, J = 11 Hz), 4.14 (H-10a, AB*q*, J = 1.5, 14.5 Hz) ; 4.11 (H-10b, AB*q*, J = 1.5, 14.5 Hz), ^{13}C NMR (CD_3OD, 150 MHz) δ 88.3 (C-1, *d*), 68.4 (C-3, *t*), 28.9 (C-4, *t*), 44.3 (C-5, *d*), 49.8 (C-6, *d*), 150.2 (C-7, *s*), 127.0 (C-8, *d*), 62.3 (C-9, *t*), 61.0 (C-10, *t*).

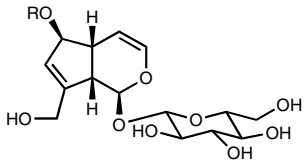
4.4. Biological assays

4.4.1. In vitro anti-protozoal and cytotoxic activity

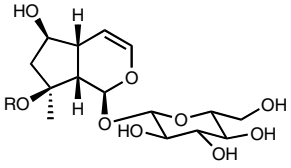
Anti-parasitic assays for *P. falciparum*, *T. cruzi* and *T. b. rhodesiense* as well as the cytotoxicity against rat skeletal myoblasts (L6 cells) were performed as previously described (Sperandeo and Brun, 2003). The assay for *L. donovani* was done using the Alamar Blue assay as described for *T. b. rhodesiense*. Briefly, axenic amastigotes were grown in SM medium (Cunningham, 1977) at pH



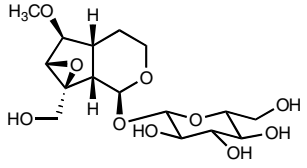
R = H **1**
R = CH_3 **2**



R = H **3**
R = α-L-rhamnose **4**
R = β-D-xylose **5**



R = H **6**
R = $COCH_3$ **7**



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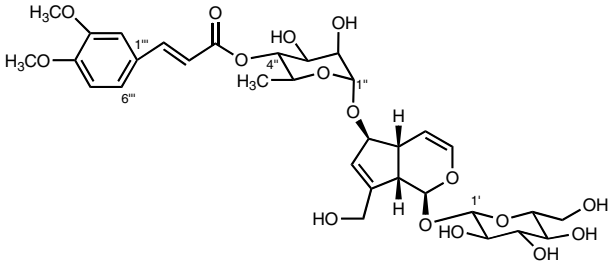

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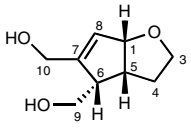

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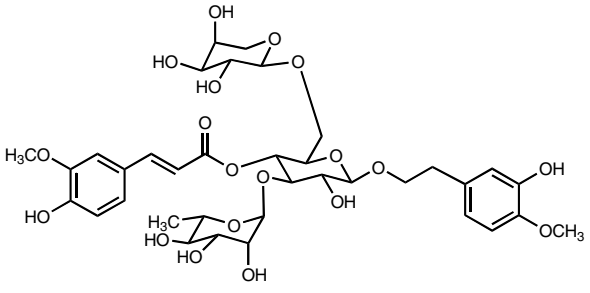

11

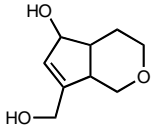

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Chart 1. Secondary metabolites isolated from *Scrophularia lepidota* (**1**–**11**) and **12**.

5.4 supplemented with 10% foetal bovine serum. 100 μl of the culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtiter plates. After 72 h of incubation 10 μl of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) were then added to each well and the plates incubated for another 2 h. Then the plates were read with a microplate fluorometer as previously described (Sperandeo and Brun, 2003). Artemisinin, benznidazole, melarsoprol, miltefosin and podophyllotoxin were used as positive controls. The IC_{50} values of reference compounds are shown in Table 2.

4.4.2. *P. falciparum enoyl-ACP reductase (FabI) inhibition assay*

The FabI protein was purified as described (Perozzo et al., 2002). All measurements were performed on a Uvikon 941 Plus spectrophotometer (Kontron Instruments) in 1 ml of 20 mM Tris/HCl pH 7.4 and 150 mM NaCl. The compounds were dissolved in MeOH and tested at 10–100 μg/ml in the presence of 1 μg (20 nM) enzyme and 200 μM NADH. The reaction was started by addition of 50 μM crotonoyl-CoA (substrate). The reaction mixture was read spectrophotometrically for 1 min by following the oxidation of NADH to NAD^+ at 340 nm ($\varepsilon = 6.3\ mM^{-1}\ cm^{-1}$). IC_{50} values were estimated from graphically plotted dose–response curves. Triclosan was used as a positive control (IC_{50} 50 nM = 14 ng/ml) (see Chart 1).

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