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Isolation, characterization and antiplasmodial activity of steroidal alkaloids from *Funtumia elastica* (Preuss) Stapf

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Abstract—Bioassay-guided fractionation of the EtOH extract of the stem bark of *Funtumia elastica* resulted in the isolation of four steroidal alkaloids, holarrhetine (**1**), conessine (**2**), holarrhesine (**3**) and isoconessimine (**4**). Their structures were determined on the basis of 1D- and 2D-NMR techniques and mass spectrometry. Compounds **1**–**4** exhibited in vitro antiplasmodial activity against the chloroquine-resistant strain FcB1 of *Plasmodium falciparum* with IC_{50} values ranging from 0.97 to 3.39 μM. They showed weak cytotoxicity against a rat cell line L-6 with IC_{50} values ranging from 5.13 to 36.55 μM.

Parasitic infections caused by *Plasmodium* species are responsible for malaria, a severe disease causing 300 to 500 millions of cases and 1.1 million deaths per year in tropical zones worldwide according to World Health Organization estimations.[1] The most virulent protozoa, *P. falciparum* is the main cause of severe clinical malaria and death and shows an increasing prevalence of resistance to standard antimalarial drugs. The need of active antiplasmodial drugs with new mode of action becomes more and more urgent to replace ineffective drugs.

In many developing countries, medicinal plants have been used by traditional healers to treat malaria. Among them, three taxons of *Funtumia* (*F. latifolia*, *F. africana* and *F. elastica*) are commonly used in traditional medicine in West Africa for treatment of infectious diseases including malaria, bacterial and parasitic infections.[2]

Funtumia elastica (Preuss) Stapf (Apocynaceae), popular name "Ireh", is a forest tree growing in west and central Africa.[3] The stem latex of *F. elastica* is used for washing wounds, its leaves to treat haemorrhoids[4] and venereal diseases such as syphilis and gonorrhoea[4,5] and its bark powder in the treatment of respiratory ailments including asthma.

Previous phytochemical investigations of *F. elastica* led to the isolation of steroidal alkaloids of the conanine groups named irehdiamines A and B, irehamine, conkurchine or irehline and irehine from the leaves[3,6] and conamine, irehdiamine, conessine and *N*,*N*′-tetramethylhorrhimine from the seeds.[4] In addition the sterols, cyclofuntumienol and cycloeucalenol were both isolated from the leaves and the bark.[7]

As part of our search for novel antiplasmodial agents from higher plants used in folk medicine, an ethanolic extract of the stem bark of *F. elastica* was found to exhibit in vitro inhibitory activity on chloroquine-resistant strain FcB1 of *P. falciparum* growth (IC_{50} value of 3.3 μg/mL) with no significant toxic effect on human MRC-5 cell lines (IC_{50} value >50 μg/mL).[8] Samples of *F. elastica* were collected at Issia (Centre west part of Ivory Coast) in october 2003 and voucher specimen (L. AKE ASSI 13376) has been deposited at the Centre National de Floristique, Université Nationale de Cocody,

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Abidjan (Ivory Coast). Bioassay-guided fractionation of this extract led to the isolation of four steroidal alkaloids (**1**–**4**) as the active constituents responsible for the antiplasmodial activity. In this paper, we report the isolation, structure characterization and in vitro antiplasmodial activity of compounds **1**–**4**. In order to determine the selectivity of this activity, the in vitro cytotoxicity against the rat cell line L-6 was evaluated.

The EtOH crude extract was suspended in H_2O and partitioned successively with cyclohexane, EtOAc and *n*-BuOH to yield the corresponding soluble extracts. The activity was only found in the aqueous and *n*-BuOH extracts, which exhibited at 10 μg/mL, 98% and 89% inhibition, respectively. Cyclohexane and EtOAc extracts lacked antiplasmodial activity. The H_2O-soluble extract was first subjected to fractionation using a silica gel column eluted with CH_2Cl_2/MeOH/NH_4OH 90/10/1 which resulted into six fractions (F1–F6). Two fractions, F2 and F4 showed strong antiplasmodial activity with 98% inhibition at 10 μg/mL. F2 (0.4 g) was then subjected to further purification using a silica gel column (24 g) eluting with CH_2Cl_2/MeOH/NH_4OH 90/10/1 furnished pure compound **1** (50 mg) and a mixture of two compounds (54 mg) which were further separated by preparative TLC (CH_2Cl_2/MeOH/NH_4OH 85/15/3) affording compounds **2** (6 mg) and **3** (8 mg). Fraction F4 (100 mg) on column chromatography over silica gel using CH_2Cl_2/MeOH/NH_4OH 90/10/1 yielded pure compound **4** (13 mg).

Compound **1** isolated as an oil, showed a quasi-molecular ion at *m/z* 469.3742 $[M+H]^+$ (calcd for $C_{30}H_{49}N_2O_2$, 469.3794) in the ESI-TOF-MS spectrum which correspond to the molecular formula $C_{30}H_{48}N_2O_2$. The NMR ^1H spectrum showed characteristic singlets attributed to six methyl groups δ 0.91, 1.63, 1.73, 2.22, 2.27(x2) and a doublet at δ 0.96 ($J = 6.3$ Hz, H-21), two olefinic protons at δ 5.31 (H-6, H-3′), an oxymethine proton at δ 4.86 (dd, $J = 4.2$, 10.9 Hz, H-12), a doublet at δ 3.00 ($J = 7.3$ Hz, H-2′), a doublet at δ 2.83 ($J = 10.1$ Hz, H-20) and aliphatic protons between δ 1.0 and 2.4 typical for a steroidal skeleton. The ^{13}C NMR spectrum contained 29 signals including the C-1′ carbonyl resonance at δ 171.8. Extensive 2D NMR spectroscopic analysis (COSY, HSQC, HMBC and NOESY) allowed the assignment of all proton and carbon signals. The presence of a 4-methyl-3-pentenoyl side chain was deduced from both the two methyl signals at δ 1.63 (s) and 1.73 (s), the doublet at δ 3.00 ($J = 7.3$ Hz, H_2-2′) in its ^1H NMR spectrum and the carbon resonances at δ 18.0, 25.6, 34.5, 116.1, 135.3, 171.8 (Table 1). The measured optical rotation $[\alpha]_D^{20}$ −11.3 (*c* 0.32, $CHCl_3$) agreed with the literature data $[\alpha]_D^{20}$ −14.9 (*c* 1.12, $CHCl_3$).[9] From the above evidence, compound **1** was identified as 12β-*O*(4-methyl-3-pentenoyl)conessine known as holarrhetine.

Compound **2** had the molecular formula $C_{24}H_{40}N_2$ from its positive ESI-TOF-MS data (*m/z* 357.3214 $[M+H]^+$, calcd for $C_{24}H_{41}N_2$, 357.3269). Its NMR ^1H spectrum exhibited three singlets at δ 0.90, 2.25, 2.33 and a doublet at δ 1.07 characteristic of five methyl groups, an olefinic proton at δ 5.32, aliphatic protons between δ 0.92 and 2.44. From 2D-NMR data, the structure of compound **2** was deduced as conessine and its ^{13}C data were consistent with those of literature.[10] $[\alpha]_D^{20}$ +15 (*c* 0.31, EtOH) lit. $[\alpha]_D^{20}$ +26 (*c* 0.6, EtOH).

Compound **3** was obtained as colourless oil and its molecular formula $C_{29}H_{46}N_2O_2$ was established by ESI-TOF-MS (*m/z* 455.3585 $[M+H]^+$, calcd for $C_{29}H_{47}N_2O_2$ 455.3637). The NMR spectral data of **3** were very similar to those of **1**, except for the presence of only one methyl group signal at δ 2.43 (3H) instead of two at δ 2.27 (6H).[11] In the HMBC spectrum, the correlations of the methyl protons at δ 2.43 (H-23) with the carbon at δ 59.6 (C-3) and those of the methyl protons at δ 2.22 (H-22) with the carbons at δ 58.4 (C-18) and 64.1 (C-20), confirmed that the $NH(CH_3)$ group was anchored on C-3. Thus, the compound **3** was characterized as holarrhesine and its optical rotation $[\alpha]_D^{20}$ −9.2 (*c* 0.42, $CHCl_3$) correlated with the literature data $[\alpha]_D^{20}$ −19.1 (*c* 0.93, $CHCl_3$).[11]

The molecular formula $C_{23}H_{38}N_2$ for **4** was established by positive ESI-TOF-MS (*m/z* 343.3093 for $[M+H]^+$ calcd for $C_{23}H_{39}N_2$ *m/z* 343.3113). Its NMR spectral data closely resembled to those of **2**, except for the N-methyl singlets at δ 2.43 which corresponded to 3H instead of 6H in **2**. The long range heteronuclear correlation observed in the HMBC spectrum between the methyl protons H-22 and both C-18 and C-20 confirmed the linkage of a $NH(CH_3)$ group at C-3 as observed in compound **3**. The structure of compound **4** was elucidated to be isoconessimine.[12] Its optical rotation was $[\alpha]_D^{20}$ +3 (*c* 0.22, $CHCl_3$).

Compounds **1**–**4** determined to be holarrhetine (**1**), conessine (**2**), holarrhesine (**3**) and isoconessimine (**4**) had the same steroidal skeleton and differed between themselves by their substituents on the amino group at position 3 and by the substituent at 12 (Fig. 1). As ^{13}C NMR data are not available for compounds **1**, **3**, **4** and ^1H assignments of **1**–**4** are incomplete, they are given in Table 1.

Antiplasmodial activity of **1**–**4** was determined by inhibition of incorporation of [^3H]-hypoxanthine. The chloroquine-resistant FcB1/Colombia strain of *P. falciparum* (IC_{50} value for chloroquine of 0.13 μM) was maintained continuously in vitro on human erythrocytes in RPMI culture medium containing 7.5% of human serum according to Trager and Jensen.[13] The antiplasmodial activity was determined using a modification of the semi-automated microdilution technique.[14] The crude extract was dissolved in dimethylsulfoxide at a concentration of 10 mg/mL and serially diluted with culture medium in 96-well microplates. Asynchronous parasite cultures were added to each well (1% parasitemia and 1% final hematocrite) and the microplates maintained at 37 °C for 24 h under appropriate atmosphere. [^3H]hypoxanthine (1–5 Ci/mmol; Amersham, les Ulis, France) was then added to each well (0.5 μCi per well) and parasites maintained for further 24 h. The growth inhibition of the parasite by the crude extract, fractions

Table 1. ^{1}H and ^{13}C NMR data for compounds **1**–**4** in $CDCl_3$

Position	**1**		**2**		**3**		**4**	
	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)
1	38.2	1.06, m	38.2	1.03, m	38.8	1.03, dd (6.1, 9.8)	38.7	1.10, m
		1.78, m		1.83, m		1.75, m		1.80, m
2	24.3	1.30, m	24.8	1.40, m	28.0	1.26, m	28.8	1.35, m
		1.78, m		1.73, m		1.75, m		1.67, m
3	64.7	2.04, m	64.9	2.18, m	59.6	2.32, m	59.8	2.59, m
4	35.2	2.08, m	34.8	2.18, m	37.8	2.04, m	37.9	1.21, m
						2.27, m		1.42, m
5	141.5		141.4		140.8		141.3	
6	120.6	5.31, m	121.1	5.32, d (4.1)	120.9	5.28, m	120.8	5.40, d (3.9)
7	31.6	1.60, m	31.9	2.03, td	31.5	1.55, m	32.0	1.60, m
		2.04, m				2.04, m		2.02, m
8	32.2	1.30, m	33.4	1.31, m	32.3	1.26, m	33.2	1.21, m
9	48.9	1.06, m	49.9	0.92, m	48.9	1.04, m	50.0	0.94, m
10	36.8		36.9		37.1		37.1	
11	27.4	1.22, m	22.0	1.11, m	28.8	1.10, m	22.0	1.18, m
		1.60, m		1.56, m		1.75, m		1.67, m
12	79.0	4.86, dd (4.2, 10.9)	38.6	1.31, m	78.9	4.86, dd (3.9, 10.7)	39.3	1.32, m
				1.73, m				1.75, m
13	54.3		50.5		54.4		50.4	
14	54.7	1.18, m	55.8	1.11, m	54.7	1.10, m	55.9	1.21, m
15	24.3	1.30, m	24.3	1.31, m	24.3	1.26, m	24.5	1.23, m
		1.78, m		1.73, m		1.66, m		1.55, m
16	28.8	1.18, m	27.4	1.20, m	27.3	1.10, m	27.6	1.49, m
		1.78, m		1.56, m		1.55, m		1.70, m
17	51.8	2.04, m	53.5	1.83, m	51.8	2.04, m	53.6	1.97, m
18	64.0	2.33, d (10.1)	64.3	1.93, m	58.4	2.32, m	64.6	2.30, m
				3.03, d (10.7)		2.83, d (10.3)		2.79, d (10.2)
19	19.3	0.91, s	19.4	0.90, s	19.3	0.92, s	19.4	0.92, s
20	58.5	2.83, d (10.1)	63.4	2.44, m	64.1	2.32, m	63.2	2.32, m
21	15.1	0.96, d (6.3)	14.6	1.07, d (6.3)	14.9	0.96, d (6.3)	14.8	1.04, d (6.4)
22	40.6	2.22, s	41.1	2.25, s	40.6	2.22, s	41.1	2.20, s
23	41.7	2.27, s	41.3	2.33, s	32.9	2.43, s	33.4	2.43, s
24	41.7	2.27, s	41.3	2.33, s	—		—	
1′	171.8				171.8			
2′	34.5	3.00, d (7.3)			34.5	3.00, d (7.2)		
3′	116.1	5.31, m			116.1	5.28, m		
4′	135.3				135.3			
5′	18.0	1.63, s			18.0	1.63, s		
6′	25.6	1.73, s			25.6	1.72, s		

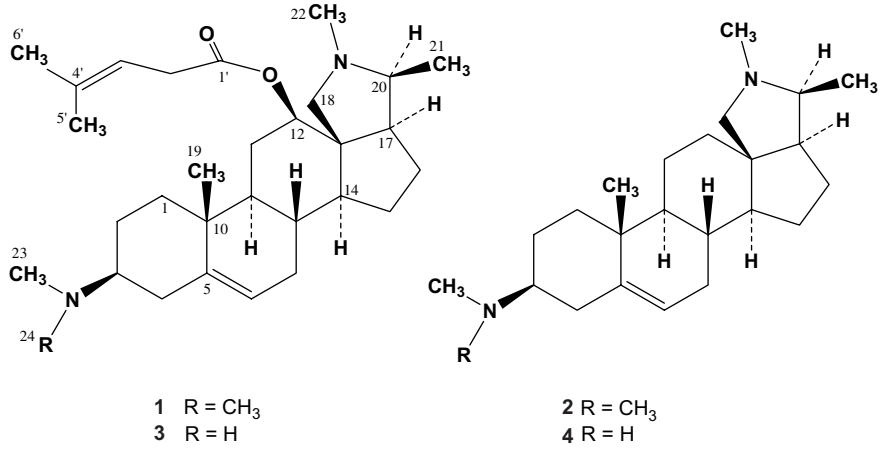

Figure 1. Steroidal alkaloids from *F. elastica*.

and pure isolated products was determined by comparison of the radioactivity incorporated into the treated culture with that in the control culture (without drug) maintained on the same plate. Concentrations causing 50% inhibition of parasite growth (IC_{50}) were determined from the dose–response curves. The results were

Table 2. Antiplasmodial activity and cytotoxicity of compounds (**1**–**4**)

Compounds	$IC_{50} \pm SD$ (μM)		Selectivity index
	P. falciparum FcB1	Rat L-6 cells	
Holarrhetine (**1**)	1.13 ± 0.32	5.13 ± 0.64	4.5
Conessine (**2**)	1.04 ± 0.14	14.60 ± 0.56	14.0
Holarrhesine (**3**)	0.97 ± 0.11	7.49 ± 0.66	7.7
Isoconessimine (**4**)	3.39 ± 0.79	36.55 ± 1.17	10.8
Chloroquine	0.13 ± 0.03	ND	

IC_{50} values are expressed as the mean micromolar concentrations ± standard deviations.
ND = not determined. All experiments were realized in triplicate. Selectivity Index are defined as the ratio of the IC_{50} value determined on L-6 cell line over the IC_{50} value on *P. falciparum*.

expressed as the mean ± the standard deviations determined from three independent experiments.

Cytotoxicity was evaluated in human diploid embryonic lung cell (MRC-5) and a rat myoblast-derived cell lines (L-6). Cell proliferation was followed by the colorimetric MTT test.[15]

Results of evaluation of toxicity against the chloroquine-resistant strain of *P. falciparum* and cytotoxicity against rat L-6 cell lines of compounds **1**–**4** are summarized in Table 2. Antiplasmodial activity of **1**–**4** was of interest because of the closely related structure of the four compounds. Each compound exhibited moderate cytotoxicity against rat L-6 cell line and showed significant inhibition of *P. falciparum* growth with IC_{50} values ranging from 0.97 to 3.39 μM. Holarrhetine (**1**) was the most cytotoxic. Elimination of an N-Me group (compound **3**) does not modify significantly the antiplasmodial activity, but decreases slightly the cytotoxicity, increasing thus the selectivity index which is defined as the ratio of cytotoxicity over the antiplasmodial activity. The same observation is made when the 4-methyl-3-pentenoic acid substituent was removed (compound **2**). Removal of the ester at C-12 and one of the N-methyl substituents, decrease both the antiplasmodial and the cytotoxic activities (compound **4**). The cytotoxicity assays revealed that **1** and **3** were more toxic than **2** and **4**, from which they differ by the presence of the ester substituent in position C-12. The selectivity was comprised between 4.5 and 14.0. Conessine (**2**) and isoconessimine (**4**) exhibited 10- to 14-fold higher activity against *P. falciparum* than against the rat L-6 cell line. They could be used as lead compounds for the synthesis of novel antiplasmodial agents with improved activity. Evaluation of in vivo antiplasmodial activity is being undertaken.

In conclusion, four steroidal alkaloids conessine, isoconessimine, holarrhetine and holarrhesine (**1**–**4**) were isolated from the stem bark of *Funtumia elastica* by means of bioassay-guided fractionation and combined silica gel chromatography. They were identified as the active constituents against the chloroquine-resistant strain FcB1 of *P. falciparum* and conessine showed the higher selectivity index.

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