FIRST BIOGUIDED PHYTOCHEMICAL APPROACH TO *CAVANILLESIA AFF. HYLOGEITON*

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Key Word Index: *Cavanillesia aff. hylogeiton*; Bombacaceae; stem-bark; sterols; sterone; glycoside; fatty acids; glycerol derivatives; *Plasmodium falciparum*

RESUMEN

En nuestro programa de estudios de especies vegetales bolivianas reportadas por su uso etnofarmacológico contra síntomas de enfermedades tropicales ampliamente expandidas en Bolivia como el paludismo, la leishmaniasis o la enfermedad de Chagas, hemos recolectado plantas expresamente descritas contra tales enfermedades. También de las mismas farmacopeas se colectaron plantas descritas para otros usos como *Cavanillesia aff. hylogeiton*; Bombacaceae, usada por los Tacanas para lograr el aumento de peso en humanos y animales y usada también como contraceptivo. El estudio de la planta permitió la descripción de compuestos grasos comunes que no manifestaron sin embargo ninguna actividad antipalúdica. Los compuestos fueron identificados principalmente mediante el uso de técnicas de RMN. Este constituye el primer reporte químico de la especie.

ABSTRACT

Among the minor bolivian ethnic groups that we have studied for their tropical illnesses pharmacopoeia, like malaria, leishmaniasis or Chagas' disease, we harvested medicinal plants reported for their antiparasitic activity as well as other species reported for other pharmacological applications. *Cavanillesia aff. hylogeiton*; Bombacaceae, is a plant reported by the Tacanas ethnic group for its gaining weight induction properties in animals and humans as well as for its contraceptive use.[1] The bioguided separation of the extract afforded fatty common compounds like fatty acids and triglyceride mixtures, a triterpen, steroids and a steroid glycoside. None of these substances manifested any antimalarial activity. This constitutes the first chemical survey of the species and also a first evaluation of its biological activities. Compounds were structurally described mainly by the use of NMR.

INTRODUCTION

The tropical genus *Cavanillesia*[2] is composed of three species. *Cavanillesia aff. hylogeiton*[2] is a 25 m high tree, natural from ecological stages from 250 to 850 m over sea level of the amazonic rain forests. The phytochemical study of genus *Cavanillesia*, has not been undertaken profoundly enough yet. The chemistry of the family Bombacaceae[3,4,5] is actually described in many genus and it can be characterized by the presence of fatty products, mainly fatty acids like palmitic, oleic and linoleic acid that have been found in *Ceiba pentandra* and *Bombax sessile*. As another example we can also mention sterculic and malvalic acid extracted from *Adansonia digitata*, *Bombax oleagineum* and *Ceiba pentandra* and *C. Acuminata* as well.

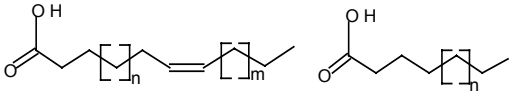
Sterculic and malvalic acids

Glycolipids and polyphenols like quercetin, kaempferol and caffeic acid have been reported in *Ceiba pentandra*. Quinones and naphtoquinones are present in *Bombax malabaricum*.[3]

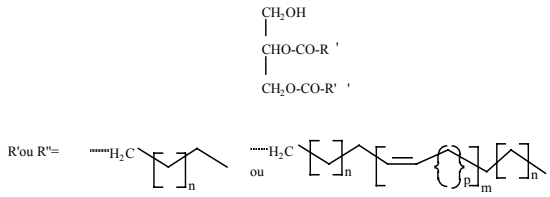
RESULTS AND DISCUSSION

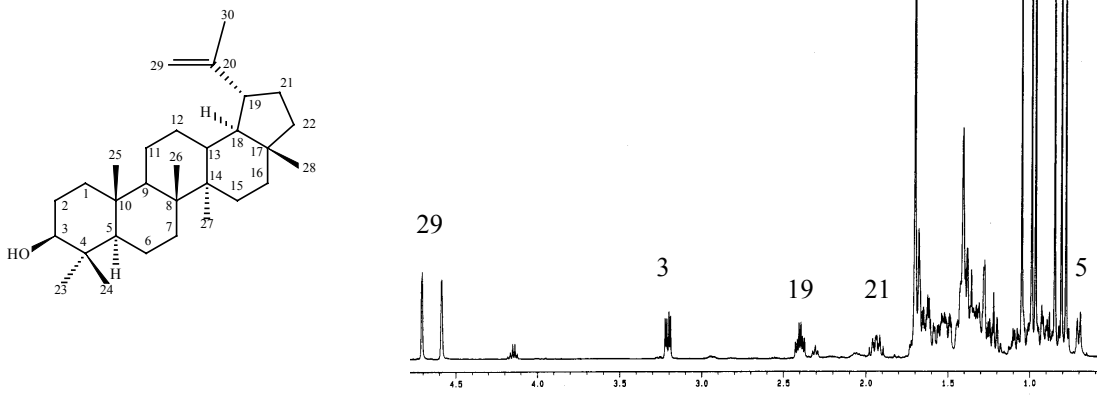
The quest of the antimalarial responsible active principles in the initial active extract and subsequent chromatographic fractions from *C. aff. Hylogeiton*, guided us to the isolation and identification of a terpenoid and three sterols besides the obtention of mixtures of lipid substances. The dried and then pulverized stem bark of *C. aff hylogeiton* was macerated in a 30% hydroethanolic mixture. After elimination of alcohol, the extract was tested against chloroquine- resistant and sensitive strains of *Plasmodium falciparum* with an activity index of an IC_{50} of 1 μg/ml. The extract was then diluted in water and mixed with methylene chloride. The organic layer after solvent elimination demonstrated to maintain the same activity index. The methylene

chloride active extract was divided in a VLC column with silica gel with a CH_2Cl_2-MeOH elution system of increasing eluting power. From the 25 fractions obtained, *fractions 3* and *16* showed a complete inhibition of parasitaemia of *P. falciparum* at less than 1 μg/ml. *Fraction 3* contained compound **1** identified after NMR and EIMS analysis as the lupene derivative lupeol.[6] *Fraction 16* and other similar composition neighboring fractions were regrouped and divided into fractions in a liquid chromatography open column on silica gel. From the 68 collected fractions, fraction 1 showed from its ^{1}HNMR spectrum, to be a mixture of lipid compounds of partial structures as follows.



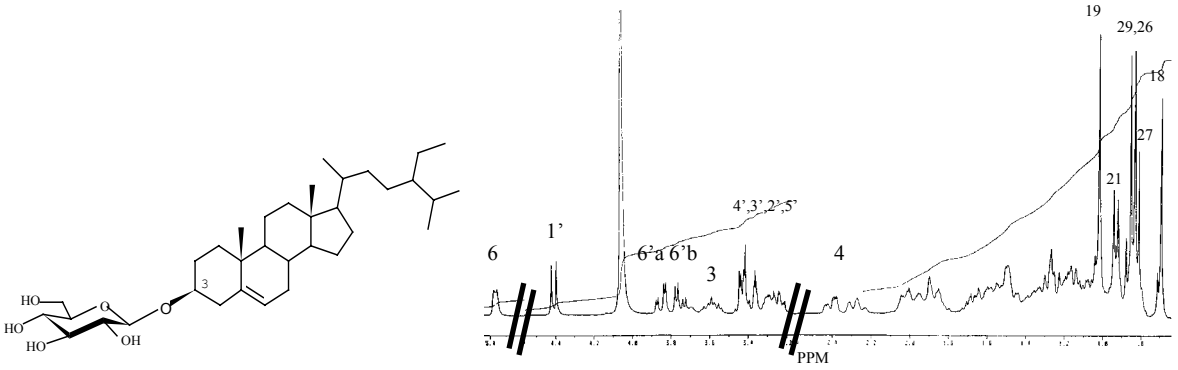
Fractions 22-27 from the column made with *fraction 16* were purified by rinsing with methanol to afford the major compound **2**, identified as O-*β*-glucopyranosyl *β*-sitosterol. Inactive *fractions 7-9* were separated in a LC open silica gel column into 135 fractions. From these, fractions 16-18 and 27-30 were separately treated in preparative TLC to isolate compounds **3**, *β* sitosterol and **4**, *β*-sitostenone besides lupeol (**1**). *Fractions 10-11* were chromatographically divided into 109 fractions. From these, fractions 61-69 and 70-82 were treated by preparative TLC to afford compound **2** and sterified glycerol derivatives whose structures were not definitely elucidated.





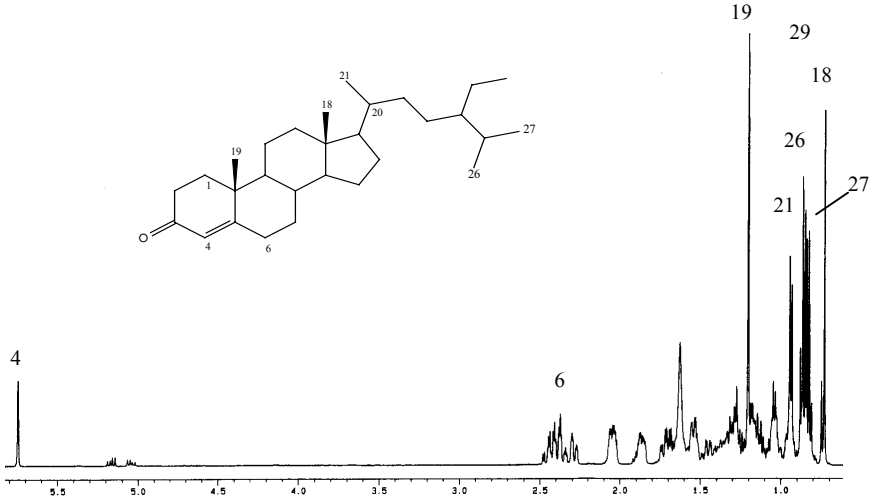
*Compound **1** : lupeol, ^{1}HNMR, $CDCl_3$, 500 MHz*

The ^{1}HNMR spectrum of **1** revealed the presence of a disubstituted exocyclic double bond in the two fine doublets with a coupling constant of $J = 2$ Hz for two gem methylenic *ene* protons shifted to 4.59 and 4.71 ppm. A secondary alcohol proton appears at δ 3.20 as a double doublet (J = 10.6 and 4.8 Hz), witnessing for an axial position of H-3. The allylic proton H-19 is distinguishable as a triple doublet (J = 16.4 and 5.3 Hz) deshielded at δ 2.40, cause of the neighboring π electronic system. A long singlet shifted to δ 1.69, correspond to a methyl group placed over a sp^2 quaternary carbon (H-30). Six intense and fine singlets corresponding to six angular methyls were assigned after correlations obtained from the XHCORR experiment, these are H-26 (δ 1.04), H-23 (δ 0.99), H-27 (δ 0.97), H-25 (δ 0.85), H-28 (δ 0.81) and H-24 (δ 0.79). The ^{13}CNMR DEPT experiment shows four quaternary carbons at cycle junction positions C-10 (δ 37.1), C-8 (δ 40.8), C-14 (δ 42.8) and C-17 (δ 43.0), additional quaternary carbon signals were assigned to C-4 (δ 38.7) and to sp^2 C-20 (δ 150.9) from the side chain. The lup-20(29)-en-3-*β*-ol structure was confirmed by the mass spectrum showing a molecular peak at *m/z* 426 analyzed for $C_{30}H_{50}O$. [7]



*Compound **2** : O-β-glucopyranosyl β-sitosterol, ^{1}HNMR, $CD_3OD+CDCl_3$, 300 MHz*

We can find the outstanding signals corresponding to *β*-sitosterol[8] in the ^{1}H NMR spectrum of **2** like δ 5.37 (*dl*, *J* = 5.0 Hz, H-6), 3.59 (*m*, H-3), 1.01 (3H, *s*, H-19), 0.94 (3H, *d*, *J* = 7.0 Hz, H-21), 0.85 (3H, *t*, *J* = 7.0 Hz, H-29), 0.84 (3H, *d*, *J* = 7.0 Hz, H-26), 0.82 (3H, *d*, *J* = 7.0 Hz, H-27), 0.69 (3H, *s*, H-18). Many protons in α of hydroxyl resonate between 3.2 and 4.4 ppm letting imaging the existence of a sugar. The anomeric proton is present as a doublet at δ 4.41 (*d*, *J* = 7.9 Hz), the AB part of an ABX system is at δ 3.84 (1H, *dd*, *J* = 10.2 and 5.0 Hz) and at δ 3.74 (1H, *dd*, *J* = 10.2 and 3.7 Hz) for a secondary alcohol grouping CH-$\underline{CH_2}$OH. Homonuclear correlations observed in the COSY experiment from the deshielded signal of the anomeric proton at δ 4.41 and throughout the rest of the sugar signals conducted to the definition of a glucose. The ^{1}HNMR spectrum of **2** was identical to that of an authentic sample of the hemisynthetic 3-O-*β*-D-glucopyranosyl *β*-sitostérol.[9] The ^{13}CNMR spectrum of **2**, exhibits 29 peaks of the steroidal skeleton and six osidic carbons of glucose. The chemical shift values observed confirm the *β*-sitosterol moiety from the values reported in the literature for the *β*-sitostérol glucoside[10] and from other *β*-sitosterol glycosides,[11, 12] as well as the identity of the *β*-D-glucose moiety.[10]



*Compound **4** : β-sitostenone, ^{1}HNMR, $CDCl_3$, 500 MHz*

Compound **4** was identified as *β*-sitostenone or (24*R*)-stigmast-4-en-3-one after comparison of its spectral data to that already published.[13] Regarding sitosterol (**3**), the proton spectrum of **4** lacks the signal corresponding to H-3. The doublet at 5.37 ppm (H-6 in **3**) appears in **4** deshielded in Δ +0,42 ppm at δ 5.75 (*bs*, H-4), chemical shift corresponding to an *ene* proton in α of carbonyl of an α,β-unsaturated ketone, data corroborated by the presence of the carbonyl at δ 199,7 (C-3). The mass spectrum of 4, presents its molecular peak at *m/z* 412 ($C_{29}H_{48}O$), fragmentation stepwise pattern correspond to that already referred in the literature.[14, 15]

*NMR data for compounds **1**, **2** and **4**,*

Atom	**1** ^{13}C	**2** ^{13}C	**4** ^{13}C	**1** ^{1}H*	**2** ^{1}H*	**4** ^{1}H
1	38,8	37,0	35,6	1,7 0,9	1,86; 1,08	
2	27,4	29,3	33,9	1,73	1,91	
3	79,0	78,8	199,7	3,20 *dd* (10,6, 4,8)	3,59 *m*	-
4	38,7	39,5	123,7	-	2,41; 2,27	5,75
5	55,3	140,0	171,7	0,7	-	-
6	18,3	121,8	32,9	1,64; 1,4	5,37 *d* (5)	
7	34,3	31,6	32,0	1,4	1,96; 1,98	
8	40,8	31,6	35,6	-		
9	50,4	49,9	53,8	1,28	0,92	
10	37,1	36,4	38,6	-	-	
11	20,9	20,8	21,0	1,4; 1,28	1,52; 0,84	
12	25,1	38,4	39,5	1,68; 1,05	2,02	
13	38,0	42,0	42,3	1,7	-	
14	42,8	56,5	55,9	-		
15	27,4	24,0	24,1	1,58	1,58	
16	35,6	27,9	28,1	1,49 1,43		
17	43,0	55,8	55,8	-	1,23	
18	48,3	11,5	11,9	1,36	0,69 *m*	0,74
19	48,0	18,9	17,3	2,4 *dd* (16,4, 5,3)	1,01	1,21
20	150,9	35,8	36,1	-		
21	29,8	18,4	18,7	1,94; 1,35	0,94 *d* (7)	0,94
22	40,0	33,6	33,8	1,42; 1,22		
23	28,0	25,7	26,0	0,99	1,18	
24	15,4	45,6	45,8	0,79	0,92	
25	16,1	28,8	29,1	0,85	1,66	
26	16,0	19,4	19,8	1,04	0,84 *d* (7)	
27	14,5	18,6	19,0	0,97	0,82 *d* (7)	
28	18,0	22,8	23,0	0,81	1,27	
29	109,3	11,6	11,9	4,71; 4,59	0,85 *t* (7)	
30	19,3			1,69		
1'		100,8			4.41 *d* (7)	
2'		75,6			3.29	
3'		76,2			3.43	
4'		69,9			3.43	
5'		73,3			3.26	
6'		61,5			3.84 *dd* (10.2, 5.0) 3.74 *dd* (10.2, 3.7)	

*signal assignment through HMQC and COSY

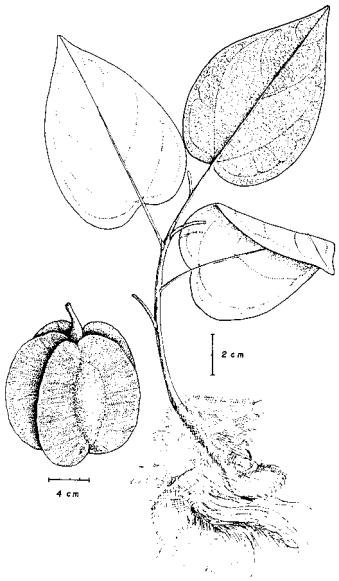
The latest biological activity tests results of the isolated compounds and fatty mixtures do not agree with the initial antimalarial indexes obtained from the departure extracts as well as for compound **1** (CI_{50} de 1 μg/ml) . For *fraction 16*, initially also detected as very active against *P. falciparum*, its components like compound **2** and the fatty mixtures found did not demonstrated any antimalarial activity either. These facts talk to us about the possibility of the lost of active principles during the separation and purification processes or the possibility of initial activity indexes provoked by the presence of polluting agents like solvent traces during biological assays.

Antimalarial tests results with extracts, fractions and pure compounds from Cavanillesia aff. hylogeiton

Fraction	CI_{50} (μg/ml) ¶	CI_{50} (μg/ml)†
Initial assays		
Hydroethanol extract	< 1	< 1
Methylene chloride extract	< 1	< 1
Fraction 3 (**1**)	< 1	< 1
Fraction 16	< 1	< 1
Latest assays		
1	> 20	> 20
2	> 20	> 20
4	> 20	> 20
Fatty acids mixt	> 20	> 20
Glycerol esters mixt.	> 20	> 20

¶chloroquine sensitive strain; †chloroquine resistant strain

EXPERIMENTAL



Cavanillesia aff. hylogeiton Ulbr. (Bombacaceae)**.**
Illustration by C. Maldonado, LPB.

General- 2D NMR experiments were run with BRUKER microprograms on AC 300 and DRX 500 spectrometers. Mass spectra acquired under electronic impact at 70 eV, Optical activity measurements were measured on a PERKIN ELMER 241. VLC Silica gel : 60H MERCK (5-40 μm). LC open column silicagel :MERCK 60, 0.063-0.200 mm (70-230 mesh ASTM). TLC plates: WHATMAN (250 and 500 μm, PK 6F, gel 60A).

Plant material-The stem-bark of *Cavanillesia aff. hylogeiton* was collected in the Tacana ethnic group territories, Apolo province of the La Paz department at 600 meters over sea level. A voucher specimen is deposited at the National Herbarium of Bolivia under SN 04.

Extraction and isolation- Dried and powdered stem-bark (352 g) were percolated with EtOH-H_2O (70:30) for 18 days, renewing solvent (1.2 L) every three days. The hydroethanolic solution was concentrated and the residue (10 g) was dissolved in H_2O and partitioned with CH_2Cl_2. The CH_2Cl_2 phase was evaporated and afforded the methylene chloride extract (6 g, 1.7%).

- **VLC system**: 3 g of the CH_2Cl_2 extract was submitted to a VLC separation method, under a CH_2Cl_2-MeOH increasing eluting power mixtures system. 25 fractions (volume of 250 mL) were collected: frs. 1-2 (CH_2Cl_2-MeOH, 100:0), fr. 3 containing pure lupeol (**1**), 0.1088 g, 0.03%; frs. 4-6 (CH_2Cl_2-MeOH, 99:1); frs. 7-8 (CH_2Cl_2-MeOH, 98:2); frs 9-11 (CH_2Cl_2-MeOH, 97:3); frs. 12-17 (CH_2Cl_2-MeOH, 96:4); frs. 18-20 (CH_2Cl_2-MeOH, 95:5); fr 21 (CH_2Cl_2-MeOH, 90:10); fr. 22 (CH_2Cl_2-MeOH, 80:20); frs. 23-25 (CH_2Cl_2-MeOH, 0:100).
- **Purification of VLC fractions**:

Fractions 7-8 (592.8 mg) were chromatographed on silica gel liquid column (LC) chromatography to give 135 fractions (volume of 40 mL): frs. 1-13 ($CHCl_3$-Hex, 50:50); frs. 14-15 ($CHCl_3$-Hex, 50:50) representing pure lupeol (**1**), 0.002 g, 0.0006%; frs. 16-18 ($CHCl_3$-Hex, 50:50) comprising a mixture of **3**, **4** and fatty acids; frs. 19-23 ($CHCl_3$-Hex, 50:50) being pure sitosterol (**3**) (0.0447 g, 0.0127%); frs. 24-26 ($CHCl_3$-Hex, 50:50) containing a mixture of **1**, **3**, **4** and mixed fatty acids; frs. 27-30 ($CHCl_3$-Hex, 50:50) containing a mixture of **3** and mixed fatty acids; fr. 31 ($CHCl_3$-Hex, 50:50); frs. 32-35 ($CHCl_3$-Hex, 50:50); frs. 36-39 ($CHCl_3$-Hex, 50:50) containing mixed fatty acids; frs. 40-116 ($CHCl_3$-Hex, 50:50); frs. 117-121 ($CHCl_3$); frs.122-124 ($CHCl_3$-MeOH, 100:0, 99:1); frs.125-127 ($CHCl_3$-MeOH, 99:1, 98:2); frs. 128-133 ($CHCl_3$-MeOH, 98:2, 97:3, 96:4); fr. 134 ($CHCl_3$-MeOH, 96:4); fr. 135 (MeOH).

Obtention of pure compounds: the fractions (16-18) were submitted to a prep. TLC [(250 μm)x2, $CHCl_3$-MeOH (99,3:0,7)] twice eluted, to isolate **4** (0.003 g, 0.0009%, R_f 0.65), **1** (0.004 g, 0.002%, R_f 0.57), **3** (0.005 g, 0.002%, R_f 0.46) and fatty acids mixture of type CH_3CH_2-$(CH_2)_n$-CH=CH-$(CH_2)_n$-$CH_2CH_2CH_2$-COOH, 0.006 g, 0.002%, R_f 0.28. The fractions (27-30) were submitted to a prep. TLC [(250 μm)x2, $CHCl_3$-MeOH (99,3:0,7)] twice eluted, to isolate **3**, 0.005 g, 0.002%, R_f 0.46 and fatty acid mixture of type CH_3CH_2-$(CH_2)_n$-CH=CH-$(CH_2)_n$-$CH_2CH_2CH_2$-COOH.

Fractions 10-11 (229 mg) were treated in LC chromatography. 109 fractions (volume of 15 mL) were obtained; frs. 1-5 (Hex-AcOEt, 100:0, 90:10); frs. 6-9 (Hex-AcOEt, 90:10); frs. 10-13 (Hex-AcOEt, 90:10, 80:20); frs. 14-18 (Hex-AcOEt, 80:20) comprising mixed fatty acids; frs. 19-35 (Hex-AcOEt, 80:20) being a mixture of glycerol ester derivatives; frs. 36-60 (Hex-AcOEt, 80:20); frs. 61-82 (Hex-AcOEt, 80:20) representing a mixture of **2** and mixed glycerol ester derivatives; frs. 83-104 (Hex-AcOEt, 80:20); frs. 105-108 (Hex-AcOEt, 50:50); fr. 109 (MeOH).

Fractions 14-22 (213 mg) were chromatographed on a silica gel column to give 68 fractions (volume of 20 mL): frs. 1-20 (Et_2O-MeOH, 100:0) representing a mixture of fatty acids; frs. 21-49 (Et_2O-MeOH, 95:5); frs. 50-53 (Et_2O-MeOH, 94:6); frs. 54-57 (Et_2O-MeOH, 92:8); frs. 58-62 (Et_2O-MeOH, 90:10); frs. 63-67 (Et_2O-MeOH, 70:30); fr. 68 (100 mL, MeOH).

Fractions 22-27 yielded pure crystals of 3-O-*β*-glucopyranosyl *β*-sitosterol (**2**) from MeOH (16.3 mg, 0.0046%);

Compound ***1,*** *lupeol*. $C_{30}H_{50}O$; EIMS, 70 eV: *m/z* (rel. int.): 426 $[M]^+$ (60), 411 (22), 408 (4), 393 (7), 315 (17), 299 (5), 272 (8), 257 (13), 247 (11), 234 (17), 218 (48), 207 (77), 203 (45), 190 (44), 189 (87), 176 (18), 175 (36), 161 (41), 149 (43), 147 (50), 135 (98), 133 (47), 121 (96), 119 (68), 109 (100), 107 (96), 105, (59); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): see table.

Compound ***2****, 3-O-β-glucopyranosyl β-sitosterol*. $C_{35}H_{60}O_6$; $[\alpha]_D$ -15,4° ($CHCl_3$-MeOH, 1,0:0,2 *c* 0,28); ^{1}H NMR ($CDCl_3$-CD_3OD, 300 MHz) and ^{13}C NMR ($CDCl_3$-CD_3OD, 75 MHz): see table.

Compound ***3****, β-sitosterol.* $C_{29}H_{50}O$; EIMS 70 eV: *m/z* (rel. int.): 414 $[M]^+$ (65), 396 (25), 381 (16), 329 (20), 303 (30), 274 (100), 259 (54), 248 (25), 245 (10), 231 (17), 219 (23), 213 (28), 205 (37), 189 (18), 173 (34), 159 (37) 149 (52), 134 (50), 119 (66), 107 (85); ^{1}HNMR ($CDCl_3$, 250 MHz): 0.69 (*s*, H-18), 0.81 (*d*, 6 Hz, H-27), 0.84 (*d*, 6 Hz, H-27), 0.85 (*t*, 6 Hz, H-29), 0.93 (*m*, H-24), 0.94 (*d*, 6 Hz, H-21), 0.95 (*m*, H-9), 0.98 (*m*, H-14), 1.01 (*s*, H-19), 1.03 (*m*. H-22), 1.08 (m, H-15'), 1.09 (*m*, H-17), 1.10 (*m*, H-1'), 1.15 (*m*, H-23), 1.19 (*m*, H-12'), 1.25 (*m*, H-28), 1.29 (*m*, H-16'), 1.30 (*m*, H-22'), 1.32 (*s*, H-20), 1.48 (H-11), 1.53 (*m*, H-7), 1.54 (*m*, H-2'), 1.58 (*m*, H-15), 1.68 (*m*, H-25), 1.85 (*m*, H-1), 1.85 (*m*, H-2), 1.86 (*m*, H-16), 1.93 (*m*, H-8), 1.98 (m, H-7), 1.99 (*m*, H-12), 2.27 (*m*, H-4), 3.53 (*tt*, 11, 4 Hz, H-3), 5.36 (*dl*, 5 Hz, H-6); ^{13}C NMR ($CDCl_3$, 62,9 MHz): 11,9 (C-18), 12.0 (C-29), 18.8 (C-21), 19.0 (C-27), 19.4 (C-19), 19.8 (C-26), 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.2 (H-16), 29.2 (C-25), 31.7 (C-2), 31.8 (C-8), 31.9 (C-7), 34.0 (C-22), 36.5 (C-10), 37.3 (C-1), 39.8 (C-12), 42.3 (C-13), 42.3 (C-4), 45.9 (C-24), 50.1 (C- 9), 56.1 (C-17), 56.8 (C-14), 71.8 (C-1), 121.7 (C-6), 140.8 (C-5), 121.7 (C-6)

Compound ***4****, β-sitostenone.* $C_{29}H_{48}O$; EIMS 70 eV: *m/z* (rel. int.): 412 $[M]^+$ (50), 397 (10), 384 (3), 370 (22), 355 (6), 327 (9), 298 (6), 289 (22), 271 (23), 257 (6), 245 (11), 229 (50), 215 (5), 213 (28), 203 (7), 189 (8), 175 (12), 161 (14) 149 (29), 147 (34), 135 (32), 124 (110), 107 (36); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz): see table.

Antimalarial in vitro assays. The culture of chloroquine resistant (Indo) and chloroquine sensitive (F32-Tanzania) *Plasmodium falciparum* strains is realized according to Trager and Jensen[16] in a RPMI 1640 medium enriched by glucose and completed by a 10% of human serum at 37ºC. 50 µl of DMSO are added to samples of vegetal extracts solved in RPMI 1640 medium using an ultrasound box. Final concentration of DMSO never exceeds 0.1%. 150 µl of culture medium containing the dilute extract and a human hematies ("O" + group, 5% of hematocrite) with 1% of parasitaemia, all placed in 96-receptacle-plates for micro titration. All test were executes by triplicate. After a 24-hours-incubation period at 37°C in an incubator containing a candle, the medium is renewed and incubation continues during still 48 more hours. At the third day a sample of blood is extracted from each receptacle to measure the parasitaemia. Each assay is correlated to a solvent untreated witness and chloroquine positive witness. The parasitaemia of each receptacle as well as the inhibition percentage for each extract concentration are calculated regarding the untreated witness. The values of the IC_{50} were determined graphically taking into account the concentration versus the percentage.

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