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Karavilagenin C derivatives as antimalarials

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ABSTRACT

Karavilagenin C (**1**), a cucurbitane-type triterpenoid, previously isolated from the aerial parts of *Momordica balsamina*, was acylated with different alkanoyl, aroyl and cinnamoyl chlorides/anydrides, yielding ten new mono or diesters, karavoates F (**7**) and H-P (**8**–**16**). Furthermore, the new compound cucurbalsaminol C (**17**) was isolated from the same plant. Their structures were assigned by spectroscopic methods, including 2D NMR experiments. Compounds **1** and **17** and the acyl derivatives **8–16** along with other five esters (**2**–**6**, karavoates A–E), previously prepared from **1**, were evaluated for their in vitro antimalarial activity against the chloroquine-sensitive (3D7) and the chloroquine-resistant (Dd2) strains of *Plasmodium falciparum*. Compound **1** exhibited a moderate activity and **17** was inactive. However, a remarkable antiplasmodial activity was observed for most of karavilagenin C alkanoyl and monoaroyl/cynamoyl derivatives. Karavoates B, D, E, I, and M were the most active, displaying IC_{50} values similar to those found for chloroquine, particularly against the resistant strain (IC_{50} <0.6 μM). Structure–activity relationships (SAR) are discussed. Moreover, the preliminary toxicity toward human cells of compounds **1**–**17** was also evaluated in breast cancer cell line (MCF-7). Most of the esters showed no toxicity, displaying, in general, much higher selectivity index values than those obtained for the parent compound.

1. Introduction

In 2008, there were more than 200 million cases of malaria and nearly one million deaths, mostly among African children.[1] In spite of all efforts in drug development, malaria remains one of the most devastating tropical diseases. A major contributor to malaria mortality and morbidity is the increasing resistance of malarial parasites, particularly *Plasmodium falciparum*, the most dangerous species, to front-line drugs, like chloroquine, and antifolates.[2,3] Thus, there are a continuing need for new therapeutic agents, mainly with novel mechanisms of action and structurally unrelated to existing antimalarial agents.[4] Natural product-derived compounds have played a major role in drug discovery and development. In case of malaria, 11 drugs out of 15 included in the WHO therapeutic schemes for malaria treatment are natural products or related with natural products.[5] The great significance of plant-derived drugs for the treatment of the disease is highlighted by quinine, artemisinin and their derivatives, which are presently the mainstay of antimalarial therapy. *Momordica balsamina* L. (Cucurbitaceae), also referred as the balsam apple, or African pumpkin, is a vegetable extensively cultivated and used as food in many tropical and subtropical regions of the world.[6,7] It has also been widely used in traditional medicine in Africa to treat various diseases mostly diabetes, and malaria symptoms.[8–10]

Cucurbitane triterpenoids are the main constituents of Cucurbitaceae family.[11] They exhibit a broad range of potent biological activities namely hepatoprotective, cytotoxic, anti-inflammatory, cardiovascular, anti-diabetic and antiparasitic effects.[11,12]

In previous work, bioassay-guided fractionation of the methanol extract of the aerial parts of *M. balsamina* led to the isolation of several cucurbitane-type triterpenoids.[13–15] Most of the compounds displayed antimalarial activity,[13] and were found to be strong *P*-glycoprotein modulators in multidrug resistant cancer cells.[14] In order to find out some structure–activity relationships, the cucurbitane triterpene, karavilagenin C (**1**) (Fig. 1), isolated in large amount from the referred extract, was esterified with different acylating agents to afford the alkanoyl esters **2–6**, previously reported.[14] Further acylation of compound **1** yielded ten new ester derivatives (**7–16**). Compounds **1–16** along with a new cucurbitane (**17**) isolated from the same plant were evaluated for their antimalarial activity against the *P. falciparum* CQ-sensitive (3D7) and the CQ-resistant (Dd2) strains. Moreover, the cytotoxicity of all

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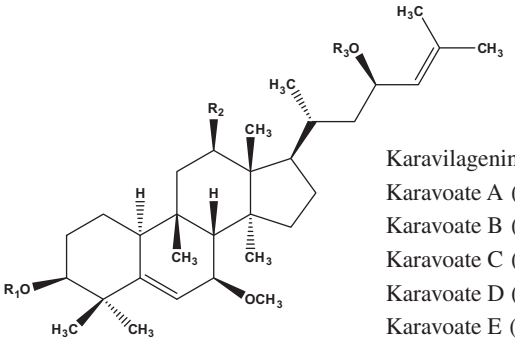


	R_1	R_2	R_3
Karavilagenin C (**1**)	H	H	H
Karavoate A (**2**)	H	H	$COCH_3$
Karavoate B (**3**)	$COCH_3$	H	$COCH_3$
Karavoate C (**4**)	H	H	$COCH_2CH_3$
Karavoate D (**5**)	$COCH_2CH_3$	H	$COCH_2CH_3$
Karavoate E (**6**)	H	H	$COCH_2CH_2CH_3$
Cucurbalsaminol C (**17**)	H	OH	H

Figure 1. Structures of compounds **2–6**, and cucurbalsaminol C (**17**).

compounds was evaluated on the human breast cancer (MCF-7) cell line, and a selectivity index was determined.

2. Results and discussion

2.1. Chemistry

Bioassay-guided fractionation of the methanol extract of the aerial parts of *M. balsamina* led to the isolation of several cucurbitane-type triterpenoids.[13–15] A large amount of karavilagenin C (**1**, 7β-methoxycucurbita-5,24-diene-3β,23(*R*)-diol) was isolated.[14] Acylation of its free hydroxyl groups at C-23 or C-3/C-23 with acetic, propionic, and butyric anhydrides yielded five mono or diacylated derivatives (**2–6**, karavoates A–E, Fig. 1).[14]

Compound **17**, named cucurbalsaminol C, was obtained as a white amorphous powder, with a molecular formula $C_{31}H_{52}O_4$ deduced by HR-EIMS, which showed a molecular ion $[M]^+$ at *m/z* 488.3867 (calcd for $C_{31}H_{52}O_4$: 488.3866). Its IR spectrum exhibited a characteristic absorption band for the hydroxyl function at 3443 cm^{-1}. The low resolution EIMS showed significant ions at *m/z* 470 $[M-H_2O]^+$, 452 $[M-2\times H_2O]^+$ and 434 $[M-3\times H_2O]^+$, due to the sequential loss of water molecules, suggesting the presence of three hydroxyl groups. The ^1H NMR spectrum showed resonances for seven tertiary methyl groups as singlets at δ_H 0.76, 0.93, 1.01, 1.02, 1.18, 1.67, and 1.70, one secondary methyl as a doublet at δ_H 1.07 (J = 6.4 Hz), and a methoxyl group at δ_H 3.33. Moreover, four oxygenated methine protons [δ_H 3.49 (br s, 2H), 3.86 (dd, J = 11.2, 4.8 Hz), 4.41 (td, J = 8.9, 3.6 Hz)], and protons of two trisubstituted double bonds [δ_H 5.16 (1H, d, J = 8.4 Hz), and 5.78 (1H, d, J = 4.8 Hz)] were also observed. The ^{13}C NMR spectrum of **17** revealed 31 carbon signals, which were assigned by a DEPT experiment as nine methyls, six methylenes, ten methines (including four oxygenated and two vinylic), and six quaternary carbons (two sp^2). All the above structural features were corroborated by two-dimensional NMR data (^1H–^1H COSY, HMQC and HMBC), which allowed clearly the assignment of all carbon signals (Table 1). In this way, ^1H–^1H COSY experiments revealed the structure of four spin-systems (Fig. 2), which were connected on the basis of key heteronuclear $^2J_{C-H}$ and $^3J_{C-H}$ correlations displayed in the HMBC spectrum that also allowed the location of the functional groups in the molecule (Fig. 2). Thus, the presence of an unusual OH group at C-12, indicated by the marked downfield shift of C-12 ($\Delta\delta_C \cong +40$ ppm, α-carbon),[15] was corroborated by the $^3J_{C-H}$ couplings between C-18 and H-12, and between C-10 and H-11, and the $^2J_{C-H}$ correlations between C-11, C-13 and H-12.

The relative configuration of the tetrahedral stereocenters of **17** was determined by a NOESY experiment (Fig. 3), taking into account cucurbitane-type triterpenoids biosynthesis,[16] and by comparison of the coupling constant pattern with that reported in literature for similar compounds. Significant NOE effects between H-12/H-10 (δ_H 2.33), H-12/Me-30 (δ_H 0.76), and H12/H-17, together with coupling constants, indicated an equatorial β-oriented OH group at C-12 also found in cucurbalsaminols A and B.[15] The configuration at C-23 was assigned as *R*, mainly by comparison of the ^{13}C NMR data of the side chain carbons of **17** with those reported for a lanostane-type triterpene, which stereochemistry was assigned by X-ray diffraction.[17] Therefore, the structure of compound **17** was established as 7β-methoxycucurbita-5,24-diene-3β,12β 23(*R*)-triol.

Further acylation of **1** with butyric anhydride gave rise to a new alkanoyl diester, named karavoate F (**7**). In the same way, acylation of karavilagenin C with four different aroyl chlorides, namely benzoyl, *p*-nitrobenzoyl, *p*-chlorobenzoyl, and *p*-methoxybenzoyl chlorides (Scheme 1), yielded seven new ester derivatives: three monoesters at C-23: karavoates I (**9**), K (**11**) and M (**13**), and four

Table 1
NMR data of cucurbalsaminol C (**17**), (MeOD, ^1H 400 MHz, ^{13}C 100.61 MHz; δ in ppm, J in Hz)

Position	^1H	^{13}C	DEPT
1	1.63 m; 1.70 m	22.5	CH_2
2	1.71 m; 1.98 m	30.1	CH_2
3	3.49 br s[a]	77.4	CH
4	–	42.4	C
5	–	149.1	C
6	5.78 d (4.8)	120.3	CH
7	3.49 br s[a]	78.4	CH
8	1.98 br s	48.8	CH
9	–	37.4	C
10	2.33 br d (10.0)	41.6	CH
11	1.38 m; 1.88 m	44.4	CH_2
12	3.86 dd (11.2, 4.8)	72.0	CH
13	–	52.4	C
14	–	51.1	C
15	1.30 m; 1.51 m	36.2	CH_2
16	1.65 m; 1.92 m	25.7	CH_2
17	1.88 m	52.6	CH
18	0.93 s	10.7	CH_3
19	1.01 s	29.3	CH_3
20	1.86 m	31.0	CH
21	1.07 d (6.4)	22.4	CH_3
22	0.98 m; 1.77 m	44.8	CH_2
23	4.41 td (8.9, 3.6)	67.8	CH
24	5.16 d (8.4)	130.6	CH
25	–	133.4	C
26	1.67 s	18.2	CH_3
27	1.70 s	25.9	CH_3
28	1.02 s	28.7	CH_3
29	1.18 s	26.0	CH_3
30	0.76 s	18.6	CH_3
7-OMe	3.33 s	56.4	CH_3

[a] Overlapped signals.

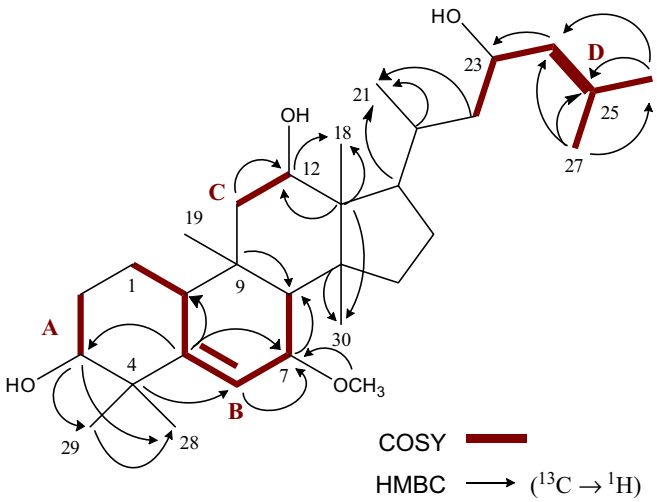


Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations of compound **17**.

diesters (at C-3 and C-23), karavoates H (**8**), J (**10**), L (**12**), and N (**14**). Two new cinnamoyl derivatives were also obtained, namely the monoester, karavoate O (**15**), and the diester, karavoate P (**16**).

The structure elucidation of these new compounds was based on a comparison of their spectroscopic data with those of karavilagenin C (**1**).[14,18] In this way, the main differences, between the NMR data of **1** and the esters **7–16**, were observed for carbon and proton signals of ring A and side chain. In the ^{1}H NMR spectra of the monoacylated derivatives (**9**, **11**, **13**, **15**) a significant paramagnetic effect was observed for the chemical shift of H-23, which appeared approximately, 1.4 ppm downfield. Similarly, deshielding effects at C-23 ($\Delta\delta_C \cong +5.9$ ppm, α-carbon), C-20, and C-25 ($\Delta\delta_C \cong +1.1$, and +2.7 ppm, γ-carbons, respectively), and shielding effects at C-22 and C-24 ($\Delta\delta_C \cong -1.7$, -3.9 ppm, β-carbons) were observed. In the diacyl derivatives (**8**, **10**, **12**, **14**, **16**), besides the difference in chemical shift of H-23, the signal of H-3 was also shifted downfield ($\Delta\delta_H \cong +1.24$ ppm). In the same way, in the ^{13}C NMR spectra, a paramagnetic effect at C-3 ($\Delta\delta_C \cong +3.3$ ppm, α-carbon), and diamagnetic effects at C-2 and C-4 ($\Delta\delta_C \cong -1.8$, -1.1 ppm, β-carbons) were also observed.

2.2. Antimalarial activity in vitro

Compounds (**1–17**) were evaluated for their in vitro antimalarial activity against chloroquine-sensitive (3D7) and resistant (Dd2) *P. falciparum* strains. A standardized SYBR Green I-based fluorescence assay was used in the experiment.[19–21] Chloroquine was used as a positive control. Moreover, in order to calculate the selectivity index (SI), defined as the ratio between cytotoxic (IC_{50}) and parasitic (IC_{50}) activities, the cytotoxic activity of all the compounds was determined on breast cancer cells (MCF-7). According to some authors, a promising lead in drug discovery should have a SI >10.[22] The effects of compounds (**1–17**) against both strains of *P. falciparum*, expressed as IC_{50} values in μM and μg/mL, are summarized in Table 2, as well as their cytotoxic activity, expressed as IC_{50} values in μM, and the selectivity index. It should be noted that a great diversity of criteria has been adopted for assessing in vitro antimalarial activity of pure compounds. Some authors consider compounds with IC_{50} values of 100 μM and higher as active,[23] while others use a stricter endpoint criteria, in which, compounds worthy of further studies should have IC_{50} values below 25 μM.[24] Taking into account these criteria, in this work the antimalarial activity of compounds was defined as: $IC_{50} \leqslant 1$ μM, excellent/potent activity; $1 < IC_{50} \leqslant 10$ μM, good activity; and $10 < IC_{50} \leqslant 30$ μM, moderate activity. A compound with an IC_{50} >30 μM, was considered inactive.

As can be observed in Table 2, most of the esters derivatives displayed antimalarial activity, having the parent compound (**1**) exhibited a moderate activity (IC_{50} = 10.4 and 11.2 μM for 3D7 and Dd2, respectively). Compound **17** was inactive. Interestingly, a remarkable activity was observed for most of the alkanoyl esters (**2–7**). The lowest IC_{50} values against both *P. falciparum* strains were displayed by the diacylated derivatives, karavoates B (**3**, IC_{50} = 0.5 and 0.5 μM, for 3D7 and Dd2, respectively) and D (**5**, IC_{50} = 1.5 and 0.4 μM, for 3D7 and Dd2, respectively). When compared with karavilagenin C (**1**), the former ester was approximately 20-fold more active against both strains. Importantly, it is interesting to point that compound **5** was much more active against the resistant strain (7 and 28-fold against 3D7 and Dd2, respectively). Similar results were also found for the monobutanoyl derivative, karavoate E (**6**) that showed a strong antiplasmodial activity, mainly against Dd2 strain (IC_{50} = 3.5 and 0.6 μM, for 3D7 and Dd2, respectively). According to some authors, the development of resistance to one agent, in drug-resistant cells (both prokaryotic and eukaryotic), can confer greater sensitivity to an alternate agent than observed in the parental (sensitive) cell line.[25] The phenomenon, named "collateral sensitivity", was first reported for *Escherichia coli*,[26] however its mechanistic basis is still unclear. Regarding the set of the aroyl derivatives of karavilagenin C (Table 2), karavoates I (**9**, IC_{50} = 2.6 and 0.5 μM, for 3D7 and Dd2, respectively) and M (**13**, IC_{50} = 1.3 and 0.6 μM, for 3D7 and Dd2, respectively), bearing a *p*-nitrobenzoyl and a *p*-methoxybenzoyl moiety at C-23, respectively, displayed the strongest activity, mainly against the Dd2 resistant strain. However, karavoate K (**11**), with a *p*-chlorobenzoyl residue at C-23, exhibited an activity lower (IC_{50} = 13.3 and 23.3 μM, for 3D7 and Dd2, respectively) than karavilagenin C, against both strains. A decrease of activity was still more evident when both positions, C-3 and C-23, bear an aroyl moiety, as shown in karavoates H (**8**), J (**10**), and L (**12**) (Table 2). In the same way, a large increase in IC_{50} values was also found

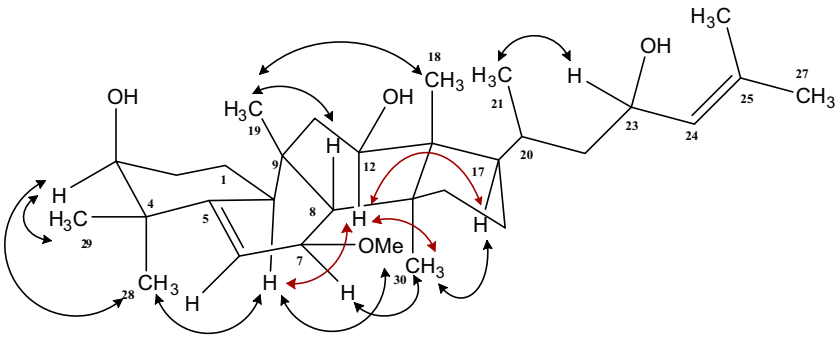
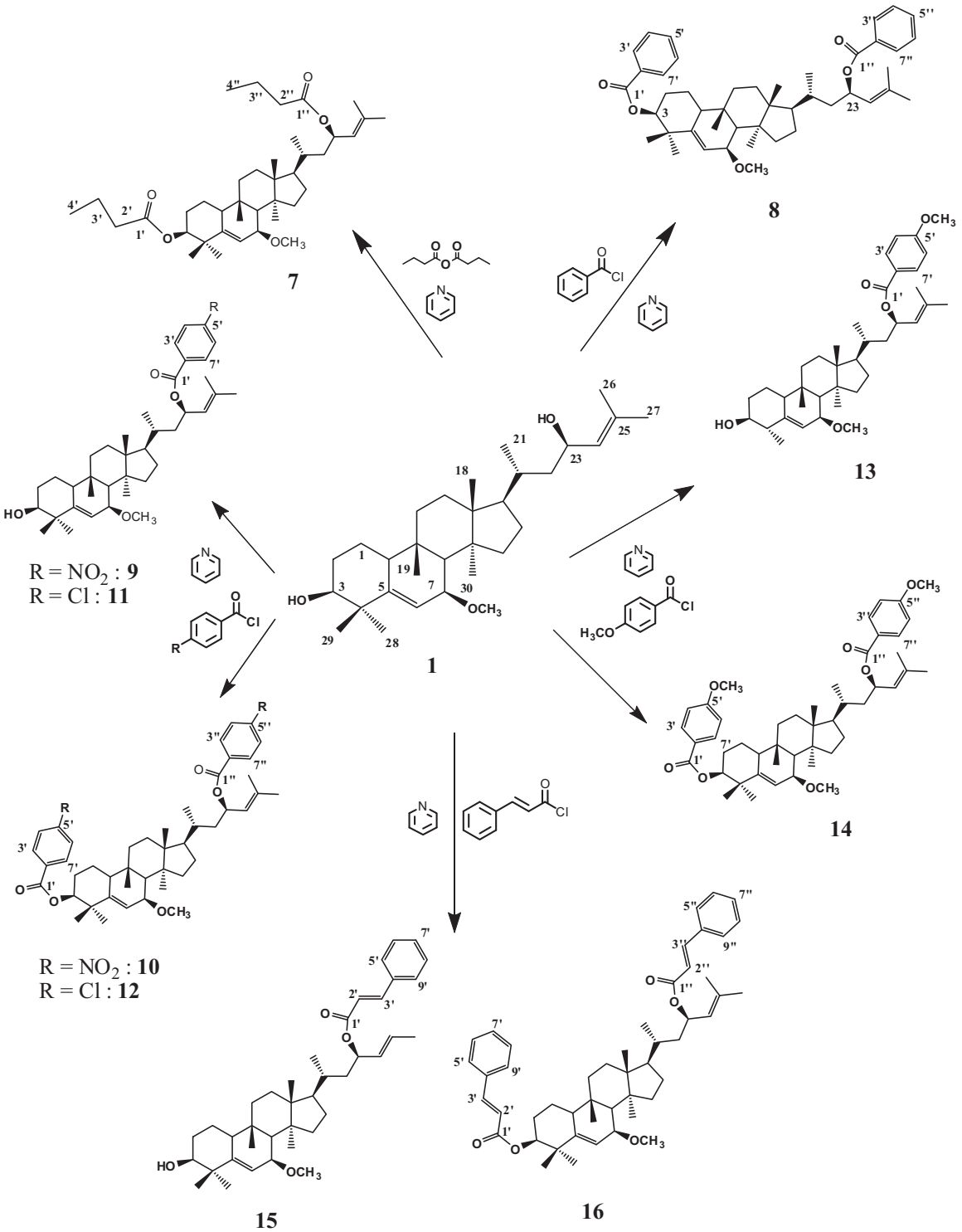


Figure 3. Key NOESY correlations of compound **17**.



Scheme 1. Preparation of karavilagenin C esters (**7**–**16**).

for the dicinnamoyl derivative (**16**, IC_{50} = 65.2 and 56.6 μM, for 3D7 and Dd2, respectively), showing the monocinnamoyl ester a good antimalarial activity (**15**, IC_{50} = 6.6 and 26.7 μM, for 3D7 and Dd2, respectively). Regarding cytotoxicity, no significant cytotoxic activity was found for karavilagenin C (**1**) esters. In fact, for the alkanoyl (**5** and **7**), aroyl (**8–14**) and cinnamoyl derivatives (**15** and **16**) of karavilagenin C an IC_{50} >133.3 μM was found. The remaining esters (**2–4** and **6**) were also inactive or showed a weak activity, displaying IC_{50} values ranging from 19.1 to 73.8 μM. More importantly, all derivatives (**2–16**) showed selectivity index values higher than those obtained for the original compound, karavilagenin C (**1**, SI = 1.6 and 1.4, for 3D7 and Dd2, respectively).

Compounds **1–17** represent a set of compounds that may allow considerations of structure–activity relationship. As illustrated above, most of the esters showed better activities than the parent compound, karavilagenin C (**1**). The lowest IC_{50} values were found when both positions C-3 and C-23 bear acetyl or propanoyl groups, as in karavoates B (**3**, IC_{50} = 0.5 and 0.5 μM, for 3D7 and Dd2,

Table 2
Antimalarial activity, cytotoxicity, and selectivity index of compounds **1–17**

Compounds	IC_{50} ± SD					Selectivity index[a]	
	P. falciparum 3D7		*P. falciparum* Dd2		MCF7 cells	MCF7/3D7	MCF7/Dd2
	μM	μg/mL	μM	μg/mL	μM		
Karavilagenin C (**1**)	10.4 ± 0.7	4.9 ± 0.3	11.2 ± 0.7	5.3 ± 0.3	16.7 ± 2.1	1.6	1.4
Karavoate A (**2**)	6.7 ± 1.1	3.5 ± 0.6	9.2 ± 0.6	4.7 ± 0.3	22.9 ± 1.24	3.4	2.5
Karavoate B (**3**)	0.5 ± 0.01	0.3 ± 0.01	0.5 ± 0.03	0.3 ± 0.01	68.1 ± 1.62	151.2	126.0
Karavoate C (**4**)	5.1 ± 0.02	2.7 ± 0.01	22.0 ± 2.3	11.6 ± 1.2	19.1 ± 0.76	3.7	0.9
Karavoate D (**5**)	1.5 ± 0.04	0.9 ± 0.02	0.4 ± 0.04	0.2 ± 0.02	>133.3	>89.0	>349.9
Karavoate E (**6**)	3.5 ± 0.02	1.9 ± 0.01	0.6 ± 0.2	0.3 ± 0.1	73.8 ± 2.13	20.8	116.3
Karavoate F (**7**)	6.9 ± 1.9	4.2 ± 1.2	8.4 ± 3.2	5.1 ± 1.9	>133.3	>19.4	>15.8
Karavoate H (**8**)	36.4 ± 7.4	24.8 ± 5.1	30.5 ± 3.9	20.8 ± 2.7	>133.3	>3.7	>4.4
Karavoate I (**9**)	2.6 ± 0.1	1.6 ± 0.05	0.5 ± 0.1	0.3 ± 0.08	>133.3	>51.4	>296.2
Karavoate J (**10**)	18.6 ± 2.5	14.3 ± 1.9	20.7 ± 4.1	16.0 ± 3.2	>133.3	>7.2	>6.43
Karavoate K (**11**)	13.3 ± 0.9	8.1 ± 0.5	23.3 ± 2.9	14.3 ± 1.8	>133.3	>10.0	>5.7
Karavoate L (**12**)	22.5 ± 0.5	16.8 ± 0.4	66.0 ± 2.2	49.4 ± 1.7	>133.3	>5.9	>2.0
Karavoate M (**13**)	1.3 ± 0.01	0.8 ± 0.01	0.6 ± 0.2	0.4 ± 0.1	>133.3	>43.6	>214.8
Karavoate N (**14**)	2.4 ± 0.1	1.8 ± 0.07	47.9 ± 12.7	35.5 ± 9.4	>133.3	>55.4	>2.8
Karavoate O (**15**)	6.6 ± 3.5	3.9 ± 2.1	26.7 ± 9.9	16.0 ± 6.0	>133.3	>20.3	>5.0
Karavoate P (**16**)	65.2 ± 5.3	47.8 ± 3.9	56.6 ± 1.2	41.5 ± 0.9	>133.3	>2.0	>2.4
Cucurbalsaminol C (**17**)	52.7 ± 2.4	25.7 ± 1.2	67.6 ± 7.4	33.0 ± 3.6	43.3 ± 3.7	0.8	0.6
CQ	0.016		0.2		b	b	b

[a] Selectivity index (SI) = cytotoxicity (IC_{50})/antiplasmodial activity (IC_{50}).
[b] IC_{50} >100 μM;[28,29] SI (MCF7/3D7) >6250; SI (MCF7/Dd2) >500.

respectively) and D (**5**, IC_{50} = 1.5 and 0.4 μM, for 3D7 and Dd2, respectively). Surprisingly, between butanoyl esters, the most active was the monoacylated derivative, karavoate E (**6**, IC_{50} = 3.5 and 0.6 μM, for 3D7 and Dd2, respectively). This compound displayed a very strong antimalarial activity against the Dd2 resistant strain, which was comparable to that found for CQ (IC_{50} = 0.016 μM and 0.20 μM for 3D7 and Dd2, respectively). In the same way, for the aroyl and cinnamoyl derivatives of karavilagenin C, the highest antimalarial activity was found for the monoesters. This is highlighted by the IC_{50} values obtained for karavoates I (**9**, IC_{50} = 2.6 and 0.5 μM, for 3D7 and Dd2, respectively), M (**13**, IC_{50} = 1.3 and 0.6 μM, for 3D7 and Dd2, respectively), and O (**15**, IC_{50} = 6.6 and 26.7 μM, for 3D7 and Dd2, respectively), which showed much better antiplasmodial activity than the corresponding diesters (**10**, **14**, and **16**, respectively). Moreover, these differences were more accentuated for the esters without any substituent at the aroyl/cinnamoyl moiety, karavoates H (**8**, IC_{50} = 36.4 and 30.5 μM, for 3D7 and Dd2, respectively) and P (**16**, IC_{50} = 65.2 and 56.6 μM, for 3D7 and Dd2, respectively). Therefore, when analyzing some physico-chemical properties of karavilagenin C and its esters (Table 3), the results suggest that their antimalarial activity might be influenced by molecular steric effects. In fact, compounds with molecular volumes between 536.1 and 618.4 showed an excellent/good activity, and those with values between 682.3 and 737.1 exhibited a weak effect or were inactive. Furthermore, one the highest IC_{50} values was found for compound **16** with the highest molecular volume.

In conclusion, this study suggests that karavilagenin C, a major constituent of the African plant medicinal plant *M. balsamina*, may be valuable as lead for the development of new antimalarials.

Table 3
Physico-chemical properties of compounds **1–17** (topological polar surface area, number of hydrogen bond acceptors and donors, molecular weight, octanol/water partition coefficient, and volume)[a]

Compound	TPSA	No. H		MW	log *P*	Volume
		acc.	Don.			
Karavilagenin C (**1**)	49.7	3	2	472	7.3	499.6
Karavoate A (**2**)	55.8	4	1	514	8.0	536.1
Karavoate B (**3**)	61.8	5	0	556	8.6	572.6
Karavoate C (**4**)	55.8	4	1	528	8.3	552.9
Karavoate D (**5**)	61.8	5	0	584	8.9	606.2
Karavoate E (**6**)	55.8	4	1	542	8.7	569.7
Karavoate F (**7**)	61.8	5	0	612	9.3	639.8
Karavoate H (**8**)	61.8	5	0	680	9.6	682.3
Karavoate I (**9**)	101.6	7	0	621	9.0	614.3
Karavoate J (**10**)	153.5	11	0	770	9.6	729.0
Karavoate K (**11**)	55.8	4	1	610	9.2	604.5
Karavoate L (**12**)	61.8	5	0	748	9.9	709.4
Karavoate M (**13**)	65.0	5	1	606	9.0	616.5
Karavoate N (**14**)	80.3	7	0	740	9.7	733.4
Karavoate O (**15**)	55.8	4	1	602	9.2	618.4
Karavoate P (**16**)	61.8	5	0	732	9.8	737.1
Cucurbalsaminol C (**17**)	69.9	4	3	488	6.4	507.6

[a] Physico-chemical parameters were determined by using the JME molecular editor (version April 2010, http://www.molinspiration.com/).

3. Experimental section

3.1. Chemistry

3.1.1. General experimental procedures

Optical rotations were obtained using a Perkin Elmer 241 polarimeter. IR spectra were determined on a FTIR Nicolet Impact 400, and NMR spectra recorded on a Bruker ARX-400 NMR spectrometer (^{1}H 400 MHz; ^{13}C 100.61 MHz), using MeOD, $CDCl_3$ and acetone as solvents. ESIMS were taken on a Micromass® Quattro micro™ API. EIMS, HR-EIMS were recorded on a Micromass Autospec spectrometer. TLC was performed on precoated SiO_2 F_{254} plates (Merck 5554 and 5744), with visualization under UV light and by spraying with sulfuric acid/methanol (1:1), followed by heating. Column chromatography (CC) was carried out on silica gel (Merck 9385). HPLC was carried out on a Merck–Hitachi instrument, with UV detection (210 and 220 nm), using a Merck LiChrospher 100 RP-18 (10 μm, 250 × 10 mm) column.

3.1.2. Extraction and isolation

Dried aerial parts of *M. balsamina* (1.2 kg) were powdered and exhaustively extracted with methanol (11 × 8 L) at room temperature, as previously described.[14] Briefly, the MeOH extract was evaporated to afford a residue (280 g), which was suspended in

H_2O (1 L) and extracted with EtOAc (9 × 0.5 L). The EtOAc residue (85 g) was suspended in MeOH/H_2O (9:1; 1 L), and extracted with *n*-hexane (5 × 0.5 L) for removal of waxy material that was not further studied. The remaining extract was evaporated under vacuum (40 °C), yielding a residue (45 g) that was chromatographed over silica gel (1 kg), using mixtures of *n*-hexane/EtOAc (1:0–0:1) and EtOAc/MeOH (19:1–0:1) as eluents to obtain six fractions (Fr 1–6), which were combined according to TLC analysis. Compound 17 (50 mg) was obtained from the crude fraction Fr 3, eluted with mixtures of *n*-hexane/EtOAc (9:11–1:19), after successive column chromatography, and further purification by HPLC (210 nm, MeOH/H_2O, 17:3, 5 mL/min, R_t = 9 min).

3.1.2.1. Cucurbalsaminol C, 7β-methoxycucurbita-5,24-diene-3β,12β 23(*R*)-triol (17). Amorphous white powder; $[\alpha]_D^{26}$ +117 (*c* 0.11, MeOH); IR (KBR) ν_{max} 3443, 1455, 1382, 1182, 1138, 1024, 978, 939 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; EIMS *m/z*: 488 $[M]^+$ (30), 470 $[M-H_2O]^+$ (43), 452 $[M-2\times H_2O]^+$ (16), 438 (8), 434 $[M-3\times H_2O]^+$ (12), 420 $[M-2\times H_2O-CH_3OH]^+$ (12), 402 (3), 388 (2), 344 (15), 312(13), 270 (7), 223 (23), 189 (19), 182 (18), 172 (17), 164 (25), 109 (100); HR-EIMS *m/z*: 488.3867 $[M]^+$ (calcd for $C_{31}H_{52}O_4$, 488.3866).

3.1.3. Preparation of karavilagenin C esters

3.1.3.1. Acylation with butyric anhydride. 31 mg of compound **1** were suspended in butyric anhydride (0.5 mL) and pyridine (0.5 mL), and the reaction mixture was stirred for 72 hours at 80 °C. The mixture was diluted with ethyl acetate and washed successively with Na_2CO_3 (5%) and HCl (1%) solutions, dried over Na_2SO_3, and filtered. The crude product was purified twice by preparative TLC, using *n*-hexane/EtOAc (13:7) and *n*-hexane/EtOAc (9:1), as eluents, to afford 26 mg of compound **7**.

*3.1.3.1.1. Karavoate F, 3β,23(R)-dibutanoyloxy-7β-methoxycucurbita-5,24-diene (**7**).* Compound **7** colorless oil; (KBr) ν_{max} 1730, 1243 cm^{-1}. ESIMS *m/z* (rel. int.): 635 $[M+Na]^+$ (62), 547 $[M+Na-CH_3CH_2CH_2COOH]^+$ (50), 517 (33), 454 (100). ^1H NMR (400 MHz, CD_3COCD_3): δ 5.79 (1H, d, *J* = 5.0 Hz, H-6), 5.67 (1H, td, *J* = 10.5, 3.0 Hz, H-23), 5.13 (1H, d, *J* = 8.9 Hz, H-24), 4.72 (1H, br s, H-3), 3.43 (1H, br d, *J* = 5.1 Hz, H-7), 3.30 (3H, s, 7-OMe), 2.42 (1H, br d, *J* = 10.6 Hz, H-10), 2.26 (2H, t, *J* = 7.3 Hz, H-2′), 2.24 (2H, t, *J* = 7.3 Hz, H-2″), 2.04 (1H, br s, H-8), 1.72 (3H, s, Me-26), 1.69 (3H, s, Me-27), 1.62 (4H, m, H-3′/H-3″), 1.12 (3H, s, Me-28), 1.11 (3H, s, Me-29), 0.98 (3H, d, *J* = 5.3 Hz, Me-21), 0.98 (3H, s, Me-19), 0.95 (3H, s, Me-18), 0.94 (3H, t, *J* = 7.3 Hz, Me-4′), 0.92 (3H, t, *J* = 7.4 Hz, Me-4″), 0.78 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 173.0 (C-1″), 172.9 (C-1′), 147.0 (C-5), 135.7 (C-25), 126.0 (C-24), 120.4 (C-6), 78.9 (C-3), 77.7 (C-7), 69.1 (C-23), 56.3 (7-OMe), 51.3 (C-17), 49.3 (C-8), 48.8 (C-14), 46.9 (C-13), 42.8 (C-22), 40.7 (C-4), 39.3 (C-10), 36.9 (C-2′/C-2″), 35.3 (C-15), 34.8 (C-9), 33.6 (C-11), 33.2 (C-20), 30.9 (C-12), 29.3 (C-19), 28.6 (C-16), 28.3 (C-28), 27.0 (C-2), 25.7 (C-27), 25.4 (C-29), 22.4 (C-1), 19.3 (C-3′/C-3″), 19.2 (C-21), 18.4 (C-26/C-30), 15.7 (C-18), 13.9 (C-4′/C-4″).

3.1.3.2. Acylation with benzoyl chloride. To compound **1** (30.0 mg) was added pyridine (1 mL) and benzoyl chloride (0.5 mL), and the mixture was stirred at room temperature for 1 hour. The reaction mixture was treated as above. The residue obtained was purified, sequentially, by column chromatography (*n*-hexane/CH_2Cl_2, 2:3 to *n*-hexane/CH_2Cl_2, 0:1) and preparative TLC (*n*-hexane/CH_2Cl_2, 1:9), to afford 15 mg of compound **8**.

*3.1.3.2.1. Karavoate H, 3β,23(R)-dibenzoyloxy-7β-methoxycucurbita-5,24-diene (**8**).* Compound **8** colorless oil; IR (KBr) ν_{max} 1716, 1520, 1455, 1270, 934, 712, 736 cm^{-1}. ESIMS *m/z* (rel. int.): 681 $[M+H]^+$ (18), 703 $[M+Na]^+$ (19), 581 $[M+Na-C_6H_5COOH]^+$ (28). ^1H NMR (400 MHz, $CDCl_3$): δ 8.04 (2H, d, *J* = 7.1 Hz, H-3″/H-7″), 8.02 (2H, d, *J* = 7.1 Hz, H-3′/H-7′), 7.56 (2H, m, H-5′/H-5″), 7.45 (4H, m, H-4′/H-4″/H-6′/H-6″), 5.89 (1H, d, *J* = 4.7 Hz, H-6), 5.87 (1H, td, *J* = 9.2, 2.8 Hz, H-23), 5.24 (1H, d, *J* = 8.9 Hz, H-24), 5.03 (1H, br s, H-3), 3.49 (1H, br d, *J* = 4.3 Hz, H-7), 3.40 (3H, s, 7-OMe), 2.40 (1H, dd, *J* = 11.2, 4.4 Hz, H-10), 2.08 (1H, br s, H-8), 1.84 (3H, s, Me-26), 1.74 (3H, s, Me-27), 1.22 (3H, s, Me-29), 1.17 (3H, s, Me-28), 1.04 (3H, s, Me-19), 1.03 (3H, d, *J* = 6.3 Hz, Me-21), 0.92 (3H, s, Me-18), 0.78 (3H, s, Me-30). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.1 (C-1″), 166.0 (C-1′), 146.8 (C-5), 135.9 (C-25), 132.8 (C-5′), 132.7 (C-5″), 130.9 (C-2″), 130.6 (C-2′), 129.7 (C-3′/C-7′), 129.5 (C-3″/C-7″), 128.4 (C-4′/C-6′), 128.3 (C-4″/C-6″), 124.6 (C-24), 119.5 (C-6), 79.3 (C-3), 77.4 (C-7), 70.3 (C-23), 56.3 (7-OMe), 50.5 (C-17), 48.2 (C-8), 48.0 (C-14), 46.2 (C-13), 42.1 (C-22), 40.3 (C-4), 38.6 (C-10), 34.7 (C-15), 34.1 (C-9), 33.0 (C-20), 32.2 (C-11), 30.1 (C-12), 28.5 (C-19), 28.1 (C-28), 28.0 (C-16), 26.5 (C-2), 25.8 (C-27), 25.2 (C-29), 21.7 (C-1), 19.1 (C-21), 18.4 (C-26), 18.0 (C-30), 15.4 (C-18).

3.1.3.3. Acylation with *p*-nitrobenzoyl chloride. To compound **1** (43.5 mg) was added pyridine (1 mL) and *p*-nitrobenzoyl chloride in excess (150 mg), and the mixture was stirred at room temperature for 24 hours. The excess of pyridine was removed with N_2 and the residue was purified by silica gel column chromatography (*n*-hexane/EtOAc, 0:1–1:1) and preparative TLC (*n*-hexane/EtOAc, 3:2) to afford 45 mg of compound **9** and 10 mg of **10**.

*3.1.3.3.1. Karavoate I, 23(R)-(p-nitrobenzoyloxy)-7β-methoxycucurbita-5,24-dien-3β-ol (**9**).* Compound **9** colorless oil; IR (KBr) ν_{max} 3447, 1716, 1606, 1529, 1457, 1273, 1085, 850, 758 cm^{-1}. ESIMS *m/z* (rel. int.): 644 $[M+Na]^+$ (68), 509 (100), 495 (25), 477 $[M+Na-C_7H_5O_4N]^+$ (21), 423 (45), 316 (29), 288 (83), 249 (22), 241 (13). ^1H NMR (400 MHz, CD_3COCD_3): δ 8.34 (2H, d, *J* = 7.8 Hz, H-4′/H-6′), 8.25 (2H, d, *J* = 7.9 Hz, H-3′/H-7′), 5.91 (1H, td, *J* = 8.8, 2.0 Hz, H-23), 5.73 (1H, d, *J* = 5.0 Hz, H-6), 5.27 (1H, d, *J* = 8.9 Hz, H-24), 3.46 (1H, br s, H-3), 3.36 (1H, br d, *J* = 5.2 Hz, H-7), 3.23 (3H, s, 7-OMe), 2.30 (1H, br d, *J* = 10.5 Hz, H-10), 1.98 (1H, br s, H-8), 1.79 (3H, s, Me-26), 1.70 (3H, s, Me-27), 1.15 (3H, s, Me-29), 1.02 (3H, d, *J* = 6.3 Hz, Me-21), 1.00 (3H, s, Me-28), 0.91 (3H, s, Me-19), 0.86 (3H, s, Me-18), 0.75 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 164.6 (C-1′), 151.5 (C-5′), 148.0 (C-5), 137.1 (C-2′), 137.0 (C-25), 131.4 (C-3′/C-7′), 125.1 (C-24), 124.5 (C-4′/C-6′), 120.1 (C-6), 77.8 (C-7), 76.5 (C-3), 71.7 (C-23), 56.2 (7-OMe), 51.2 (C-17), 49.1 (C-8), 48.8 (C-14), 46.9 (C-13), 42.7 (C-22), 42.1 (C-4), 39.6 (C-10), 35.4 (C-15), 34.7 (C-9), 33.8 (C-20), 33.3 (C-11), 31.0 (C-12), 30.0 (C-2), 29.2 (C-19), 28.7 (C-16), 28.5 (C-28), 26.0 (C-29), 25.8 (C-27), 21.9 (C-1), 19.4 (C-21), 18.6 (C-26), 18.5 (C-30), 15.7 (C-18).

*3.1.3.3.2. Karavoate J, 3β,23(R)-di-(p-nitrobenzoyloxy)-7β-methoxycucurbita-5,24-diene (**10**).* Compound **10** colorless oil; IR (KBr) ν_{max} 1716, 1605, 1530, 1455, 1383, 1276, 1102, 850 cm^{-1}. ^1H NMR (400 MHz, CD_3COCD_3): δ 8.39 (4H, dd, *J* = 8.9, 1.9 Hz, H4′/H4″/H-6′/H-6″), 8.30 (2H, d, *J* = 8.9 Hz, H-4′/H-6′), 8.23 (2H, d, *J* = 8.9 Hz, H-4″/H-6″), 5.96 (1H, td, *J* = 9.0, 3.0 Hz, H-23), 5.90 (1H, d, *J* = 4.7 Hz, H-6), 5.32 (1H, d, *J* = 9.0 Hz, H-24), 5.06 (1H, br s, H-3), 3.49 (1H, br d, *J* = 4.7 Hz, H-7), 3.34 (3H, s, 7-OMe), 2.55 (1H, br d, *J* = 10.0 Hz, H-10), 2.10 (1H, br s, H-8), 1.84 (3H, s, Me-26), 1.74 (3H, s, Me-27), 1.24 (3H, s, Me-28), 1.21 (3H, s, Me-29), 1.06 (3H, d, *J* = 6.4 Hz, Me-21), 1.03 (3H, s, Me-19), 0.93 (3H, s, Me-18), 0.84 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 164.7 (C-1″), 164.4 (C-1′), 151.6 (C-5′/C-5″), 146.6 (C-5), 137.1 (C-2′/C-2″), 137.0 (C-25), 131.4 (C-3′/C-3″/C-7′/C-7″), 125.1 (C-24), 124.6 (C-4′/C-4″/C-6′/C-6″), 121.0 (C-6), 81.3 (C-3), 77.6 (C-7), 71.8 (C-23), 56.3 (7-OMe), 51.3 (C-17), 49.1 (C-8), 48.8 (C-14), 47.0 (C-13), 42.7 (C-22), 41.0 (C-4), 39.1 (C-10), 35.4 (C-15), 34.8 (C-9), 33.8 (C-20), 33.1 (C-11), 30.9 (C-12), 29.2 (C-19), 28.7 (C-16), 28.1 (C-28), 26.9 (C-2), 25.8 (C-27), 25.6 (C-29), 22.6 (C-1), 19.4 (C-21), 18.5 (C-26/C-30), 15.7 (C-18).

3.1.3.4. Acylation with *p*-chlorobenzoyl chloride. To a solution of compound **1** (25 mg) in pyridine, 60 μL of *p*-chlorobenzoyl chloride were added. The mixture was stirred at room temperature, and after 15 min the excess of pyridine was removed with N_2. The crude product was purified by column chromatography (*n*-hexane/EtOAc, 1:0–7:3) and preparative TLC (*n*-hexane/EtOAc, 3:2), giving rise to 8 mg of compound **11** and 24 mg of **12**.

*3.1.3.4.1. Karavoate K, 23(R)-(p-chlorobenzoyloxy)-7β-methoxycucurbita-5,24-dien-3β-ol (**11**).* Compound **11** colorless oil; IR (KBr) ν_{max} 3431, 1716, 1594, 1455, 1381, 1272, 1088, 935, 851, 759 cm^{-1}. ESIMS *m/z* (rel. int.): 649 $[M+K]^+$ (29), 633 $[M+Na]^+$ (55.8), 477 $[M+Na–C_7H_5O_2Cl]^+$ (100). ^1H NMR (400 MHz, CD_3COCD_3): δ 8.00 (2H, d, *J* = 8.8 Hz, H-3′/H-7′), 7.54 (2H, d, *J* = 8.8 Hz, H-4′/H-6′), 5.88 (1H, td, *J* = 9.0, 3.3 Hz, H-23), 5.75 (1H, d, *J* = 5.0 Hz, H-6), 5.25 (1H, d, *J* = 8.8 Hz, H-24), 3.49 (1H, br s, H-3), 3.39 (1H, br d, *J* = 5.3 Hz, H-7), 3.26 (3H, s, 7-OMe), 2.33 (1H, br d, *J* = 10.3 Hz, H-10), 2.00 (1H, br s, H-8), 1.79 (3H, s, Me-26), 1.70 (3H, s, Me-27), 1.17 (3H, s, Me-29), 1.02 (3H, s, Me-28), 1.02 (3H, d, *J* = 7.7 Hz, Me-21), 0.93 (3H, s, Me-19), 0.88 (3H, s, Me-18), 0.77 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 165.3 (C-1′), 148.1 (C-5), 139.4 (C-5′), 136.6 (C-25), 131.8 (C-3′/C-7′), 130.5 (C-2′), 129.6 (C-4′/C6′), 125.4 (C-24), 120.1 (C-6), 77.8 (C-7), 76.5 (C-3), 70.9 (C-23), 56.2 (7-OMe), 51.2 (C-17), 49.2 (C-8), 48.8 (C-14), 46.9 (C-13), 42.7 (C-22), 42.1 (C-4), 39.6 (C-10), 35.3 (C-15), 34.7 (C-9), 33.8 (C-20), 33.3 (C-11), 30.9 (C-12), 30.1 (C-2), 29.2 (C-19), 28.7 (C-16), 28.5 (C-28), 26.0 (C-29), 25.7 (C-27), 21.9 (C-1), 19.4 (C-21), 18.5 (C-30), 18.4 (C-26), 15.6 (C-18).

*3.1.3.4.2. Karavoate L, 3β,23(R)-di-(p-chlorobenzoyloxy)-7β-methoxycucurbita-5,24-diene (**12**).* Compound **12** colorless oil; IR (KBr) ν_{max} 1717, 1559, 1455, 1381, 1272, 1088, 935, 850, 758 cm^{-1}. ESIMS *m/z* (rel. int.): 771 $[M+Na]^+$ (2). ^1H NMR (400 MHz, CD_3COCD_3): δ 8.04 (2H, d, *J* = 8.8 Hz, H-3″/H-7″), 7.98 (2H, d, *J* = 8.8 Hz, H-3′/H-7′), 7.56 (4H, m, H-4′/H-4″/H-6′/H-6″), 5.88 (1H, dt, *J* = 9.0, 3.3 Hz, H-23), 5.87 (1H, d, *J* = 4.7 Hz, H-6), 5.27 (1H, d, *J* = 8.8 Hz, H-24), 4.99 (1H, br s, H-3), 3.47 (1H, br d, *J* = 5.0 Hz, H-7), 3.31 (3H, s, 7-OMe), 2.52 (1H, br d, *J* = 10.0 Hz, H-10), 2.12 (1H, br s, H-8), 1.82 (3H, s, Me-26), 1.73 (3H, s, Me-27), 1.19 (3H, s, Me-29), 1.22 (3H, s, Me-28), 1.04 (3H, d, *J* = 6.0 Hz, Me-21), 1.03 (3H, s, Me-19), 0.93 (3H, s, Me-18), 0.84 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 165.4 (C-1″), 165.1 (C-1′), 146.8 (C-5), 139.5 (C-5′/C-5″), 136.6 (C-25), 131.8 (C-3′/C-3″/C-7′/C-7″), 130.5 (C-2′/C-2″), 129.7 (C-4′/C-4″/C6′/C-6″), 125.4 (C-24), 120.8 (C-6), 80.5 (C-3), 77.7 (C-7), 70.9 (C-23), 56.3 (7-OMe), 51.3 (C-17), 49.1 (C-8), 48.8 (C-14), 46.9 (C-13), 42.7 (C-22), 41.0 (C-4), 39.1 (C-10), 35.4 (C-15), 34.9 (C-9), 33.8 (C-20), 33.2 (C-11), 30.9 (C-12), 29.1 (C-19), 28.8 (C-16), 28.1 (C-28), 27.0 (C-2), 25.8 (C-27), 25.6 (C-29), 22.6 (C-1), 19.4 (C-21), 18.5 (C-30), 18.4 (C-26), 15.6 (C-18).

3.1.3.5. Acylation with *p*-methoxybenzoyl chloride. To a solution of compound **1** (32.7 mg) in pyridine (1 mL), 50 μL of *p*-methoxybenzoyl chloride were added. The mixture was stirred at room temperature. After 24 hours, the excess of pyridine was removed with N_2. The crude product was purified by column chromatography, (*n*-hexane/EtOAc, 1:0–4:1), and preparative TLC (*n*-hexane/EtOAc, 4:1), to afford 20 mg of compound **13** and 23 mg of compound **14**.

*3.1.3.5.1. Karavoate M, 23(R)-(p-methoxybenzoyloxy)-7β-methoxycucurbita-5,24-dien-3β-ol (**13**).* Compound **13** colorless oil; IR (KBr) ν_{max} 3446, 1716, 1593, 1455, 1271, 1171, 1093, 934, 851. 759 cm^{-1}. ESIMS *m/z* (rel. int.): 629 $[M+Na]^+$ (2), 454 $[M–C_8H_8O_3]^+$ (100). ^1H NMR (400 MHz, CD_3COCD_3): δ 7.97 (2H, d, *J* = 8.8 Hz, H-3′/H-7′), 7.02 (2H, d, *J* = 8.8 Hz, H-4′/H-6′), 5.86 (1H, td, *J* = 10.0, 3.2 Hz, H-23), 5.76 (1H, d, *J* = 5.0 Hz, H-6), 5.24 (1H, d, *J* = 8.8 Hz, H-24), 3.87 (3H, s, 5′-OMe), 3.49 (1H, br s, H-3), 3.39 (1H, br d, *J* = 5.3 Hz, H-7), 3.26 (3H, s, 7-OMe), 2.33 (1H, br d, *J* = 10.3 Hz, H-10), 2.01 (1H, br s, H-8), 1.79 (3H, s, Me-26), 1.69 (3H, s, Me-27), 1.18 (3H, s, Me-29), 1.03 (3H, s, Me-28), 1.02 (3H, d, *J* = 7.7 Hz, Me-21), 0.94 (3H, s, Me-19), 0.88 (3H, s, Me-18), 0.77 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 165.9 (C-1′), 164.3 (C-5′), 148.0 (C-5), 135.9 (C-25), 132.1 (C-3′/C-7′), 125.9 (C-24), 124.0 (C-2′), 120.1 (C-6), 114.5 (C-4′/C-6′), 77.8 (C-7), 76.5 (C-3), 69.9 (C-23), 56.2 (7-OMe), 55.8 (5′-OMe), 51.2 (C-17), 49.1 (C-8), 48.7 (C-14), 46.9 (C-13), 42.8 (C-22), 42.0 (C-4), 39.6 (C-10), 35.3 (C-15), 34.7 (C-9), 33.7 (C-20), 33.3 (C-11), 30.9 (C-12), 30.1 (C-2), 29.2 (C-19), 28.7 (C-16), 28.5 (C-28), 26.0 (C-29), 25.8 (C-27), 22.0 (C-1), 19.4 (C-21), 18.5 (C-30), 18.4 (C-26), 15.6 (C-18).

*3.1.3.5.2. Karavoate N, 3β,23(R)-di-(p -methoxybenzoyloxy)-7β-methoxycucurbita-5,24-diene (**14**).* Compound **14** colorless oil; IR (KBr) ν_{max} 1716, 1593, 1455, 1271, 1171, 1093, 934, 850, 759 cm^{-1}. ESIMS *m/z* (rel. int.): 763 $[M+Na]^+$ (67), 611 $[M+Na–C_8H_8O_3]^+$ (100), 589 (28), 561 (38), 517 (57), 501 (42), 473 (43), 457 (18). ^1H NMR (400 MHz, CD_3COCD_3): δ 7.96 (2H, d, *J* = 8.8 Hz, H-3″/H-7″), 7.93 (2H, d, *J* = 8.8 Hz, H-3′/H-7′), 7.02 (4H, m, H-4′/H-4″/H-6′/H-6″), 5.88 (1H, d, *J* = 5.0 Hz, H-6), 5.86 (1H, td, *J* = 10.0, 3.2 Hz, H-23), 5.26 (1H, d, *J* = 8.2 Hz, H-24), 4.95 (1H, br s, H-3), 3.88 (6H, s, 5′-OMe/5″-OMe), 3.47 (1H, br d, *J* = 4.7 Hz, H-7), 3.33 (3H, s, 7-OMe), 2.50 (1H, dd, *J* = 11.0, 3.0 Hz, H-10), 2.08 (1H, br s, H-8), 1.80 (3H, s, Me-26), 1.71 (3H, s, Me-27), 1.19 (3H, s, Me-29), 1.17 (3H, s, Me-28), 1.03 (3H, s, Me-19), 1.02 (3H, d, *J* = 5.8 Hz, Me-21), 0.91 (3H, s, Me-18), 0.82 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 165.9 (C-1″), 165.6 (C-1′), 164.4 (C-5″), 164.3 (C-5′), 147.0 (C-5), 136.0 (C-25), 132.1 (C-3″/C-7″), 132.0 (C-3′/C-7′), 125.9 (C-24), 124.0 (C-2″), 123.9 (C-2′), 120.6 (C-6), 114.6 (C-4′/C-4″/C-6′/C-6″), 79.5 (C-3), 77.7 (C-7), 69.9 (C-23), 56.3 (7-OMe), 55.9 (5′-OMe/5″-OMe), 51.2 (C-17), 49.1 (C-8), 48.8 (C-14), 46.9 (C-13), 42.8 (C-22), 41.0 (C-4), 39.1 (C-10), 35.3 (C-15), 34.8 (C-9), 33.7 (C-20), 33.1 (C-11), 30.9 (C-12), 29.1 (C-19), 28.7 (C-16), 28.1 (C-28), 27.0 (C-2), 25.8 (C-27), 25.6 (C-29), 22.5 (C-1), 19.4 (C-21), 18.5 (C-30), 18.4 (C-26), 15.7 (C-18).

3.1.3.6. Acylation with cinnamoyl chloride. Compound **1** (30.6 mg), dissolved in pyridine (1 mL), was added to cinnamoyl chloride (50 mg), and the reaction mixture was kept at room temperature for 48 hours. After removal of the excess of pyridine, the mixture was purified by column chromatography (*n*-hexane/CH_2Cl_2, 1:4–0:1, and CH_2Cl_2/Me_2CO, 0:1–9:1), and preparative TLC (*n*-hexane/EtOAc, 7:3) to give 12 mg of compound **15** and 8 mg of **16**.

*3.1.3.6.1. Karavoate O, 23(R)-cinnamoyloxy-7β-methoxycucurbita-5,24-dien-3β-ol (**15**).* Compound **15** colorless oil; IR (KBr) ν_{max} 3446, 1718, 1450, 1271, 1171, 759, 735 cm^{-1}. ESIMS *m/z* (rel. int.): 625 $[M+Na]^+$ (100), 477 $[M+Na–C_9H_8O_2]^+$ (49). ^1H NMR (400 MHz, CD_3COCD_3): δ 7.68–7.63 (2H, m, H-5′/H-9′), 7.67 (1H, d, *J* = 16.0 Hz, H-3′), 7.45 (3H, m, H-6′/H-8′/H-7′), 6.56 (1H, d, *J* = 16.0 Hz, H-2′), 5.80 (1H, td, J = 9.2, 2.8 Hz, H-23), 5.77 (1H, d, J = 5.2 Hz, H-6), 5.22 (1H, d, *J* = 8.9 Hz, H-24), 3.50 (1H, br s, H-3), 3.41 (1H, br d, *J* = 5.3 Hz, H-7), 3.28 (3H, s, 7-OMe), 2.36 (1H, br d, *J* = 11.2 Hz, H-10), 1.80 (3H, s, Me-26), 1.72 (3H, s, Me-27), 1.20 (3H, s, Me-29), 1.05 (3H, s, Me-28), 1.03 (3H, d, *J* = 6.2 Hz, Me-21), 0.97 (3H, s, Me-19), 0.94 (3H, s, Me-18), 0.79 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 166.5 (C-1′), 148.0 (C-5), 144.9 (C-3′), 136.0 (C-25), 135.4 (C-4′), 131.0 (C-6′/C-8′), 129.7 (C-7′), 128.9 (C-5′/C9′), 125.8 (C-24), 120.0 (C-6), 119.4 (C-2′), 77.7 (C-7), 76.4 (C-3), 69.5 (C-23), 56.3 (7-OMe), 51.2 (C-17), 49.1 (C-8), 48.7 (C-14), 46.8 (C-13), 42.7 (C-22), 42.0 (C-4), 39.6 (C-10), 35.2 (C-15), 34.6 (C-9), 33.6 (C-20), 33.2 (C-11), 30.7 (C-12), 29.9 (C-2), 29.1 (C-19), 28.5 (C-16), 28.4 (C-28), 25.9 (C-27), 25.6 (C-29), 21.8 (C-1), 19.3 (C-21), 18.3 (C-26/C-30), 15.6 (C-18).

*3.1.3.6.2. Karavoate P, 3β,23(R)-dicinnamoyloxy-7β-methoxycucurbita-5,24-diene (**16**).* Compound **16** colorless oil; IR (KBr) ν_{max}

1718, 1455, 1271, 1171, 759, 735 cm^{-1}. ESIMS *m/z* (rel. int.): 755 $[M+Na]^+$ (54), 607 $[M+Na-C_9H_8O_2]^+$ (100). ^1H NMR (400 MHz, CD_3COCD_3): δ 7.70–7.62 (4H, m, H-5′/H-5″/H-9′/H-9″), 7.68 (1H, d, J = 16.0 Hz, H-3″), 7.66 (1H, d, J = 16.0 Hz, H-3′), 7.47–7.40 (6H, m, H-6′/H-6″/H-7′/H-7″/H-8′/H-8″), 6.55 (1H, d, J = 16.0 Hz, H-2″), 6.54 (1H, d, J = 16.0 Hz, H-2′), 5.82 (1H, d, J = 5.6 Hz, H-6), 5.80 (1H, td, J = 9.6, 3.2 Hz, H-23), 5.20 (1H, d, J = 9.0 Hz, H-24), 4.85 (1H, br s, H-3), 3.44 (1H, br d, J = 4.6 Hz, H-7), 3.31 (3H, s, 7-OMe), 2.46 (1H, br d, J = 11.2 Hz, H-10), 1.76 (3H, s, Me-26), 1.70 (3H, s, Me-27), 1.16 (3H, s, Me-28), 1.14 (3H, s, Me-29), 1.01 (3H, d, J = 6.0 Hz, Me-21), 1.00 (3H, s, Me-19), 0.93 (3H, s, Me-18), 0.80 (3H, s, Me-30). ^{13}C NMR (101 MHz, CD_3COCD_3): δ 166.3 (C-1′/C-1″), 146.9 (C-5), 145.1 (C-3′), 144.9 (C-3″), 136.0 (C-25), 135.4 (C-4′/C-4″), 131.0 (C-6′/C-6″/C-8′/C-8″), 129.7 (C-7′/C-7″), 128.9 (C-5′/C-5″/C9′/C-9″), 125.8 (C-24), 120.5 (C-6), 119.4 (C-2′/C-2″), 79.2 (C-3), 77.6 (C-7), 69.6 (C-23), 56.2 (7-OMe), 51.2 (C-17), 49.0 (C-8), 48.7 (C-14), 46.8 (C-13), 42.7 (C-22), 40.8 (C-4), 39.1 (C-10), 35.2 (C-15), 34.7 (C-9), 33.6 (C-20), 33.1 (C-11), 30.8 (C-12), 29.1 (C-19), 28.5 (C-16), 28.1 (C-28), 26.9 (C-2), 25.7 (C-27), 25.4 (C-29), 22.4 (C-1), 19.3 (C-21), 18.4 (C-26), 18.3 (C-30), 15.6 (C-18).

3.2. Biological assays

3.2.1. Antimalarial assay

Human malaria parasites were cultured as previously described by Trager and Jensen (1976), with minor modifications.[27] Briefly, 3D7 and Dd2 *P. falciparum* strains were cultivated in recently collected erythrocytes as host cells in RPMI 1640 medium (Gibco) containing 25 mM HEPES (Sigma) and 6.8 mM hypoxanthine (Sigma) supplemented with 10% AlbuMAX II (Invitrogen). Cultures were maintained at 37 °C under an atmosphere of 5% O_2, 3–5% CO_2, and N_2. The antimalarial activity of the compounds was determined by a fluorometric method using SYBR Green I.[19,21] In brief, stock solutions of the samples were prepared in DMSO (10 mg/mL), and were diluted to give a series of concentrations ranging from 0.156 to 100 μg/mL. 50 μL of each testing concentration, together with 50 μL of a 1% red blood parasitized cell suspension with ring stages and 2% hematocrit were distributed in duplicate, into each of the 96-well plates. Plates were incubated for 48 h at 37 °C. After, 100 μL of lysis buffer with SYBR Green I(Tris 20 mM; pH 7.5, EDTA—5 mM, saponin—0.008%; wt/vol, Triton X-100—0.08%; vol/vol, and 0.2 μL of SYBR Green I/mL of lysis buffer) was added to each well. Plates were covered, mixed and incubated in the dark at room temperature for 1 h. Fluorescence intensity was measured on a fluorescence multiwell plate reader, Anthos venyth 3100 (Alfagene) excitation and emission wavelengths of 485 nm and 535 nm, respectively. Values were expressed in relative fluorescence units. Analysis of the results obtained and IC_{50} determination were performed with HN-NonLineV1.1 (H. Noedl, 2001) software.

3.2.2. In vitro cytotoxicity assay

Human breast cancer MCF-7 cell line was cultured in RPMI 1640 medium supplemented with 10% heat inactivated horse serum, L-glutamine (2 mM), and antibiotics, in a humidified atmosphere of 5% CO_2 at 37 °C. The effects of increasing concentrations of the compounds on cell growth were tested in 96-well flat-bottomed microtiter plates. The compounds were diluted in a volume of 50 μL medium. Then, 2×10^4 cells in 0.1 mL of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 24 h. At the end of the incubation period, 15 μL of MTT (thiazolyl blue, Sigma, St Louis, MO, USA) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37 °C for 4 h, 100 μL of sodium dodecyl sulfate (SDS) (Sigma, St Louis, MO, USA) solution (10%) was measured into each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100 - \left[\frac{OD_{sample} - OD_{medium\ control}}{OD_{cell\ control} - OD_{medium\ control}}\right] \times 100$$

where IC_{50} is defined as the inhibitory dose that reduces the growth of the compound-exposed cells by 50%. The IC_{50} values are expressed as means ± SD from three experiments.

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Supplementary data

Supplementary data (1D and 2D NMR spectra of compound **17**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.015. These data include MOL files and InChiKeys of the most important compounds described in this article.

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