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Isolation of caryatin as an Antiplasmodial component of *Symphyopappus casarettoi* (Asteraceae)

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Abstract
Malaria remains a serious public health problem, being lethal to near 400,000 people every year, mostly in Africa. Due to the resistance of *Plasmodium falciparum* to available antimalarials, including artemisinin derivatives, and *P. vivax* to chloroquine, new therapies are necessary, since drug treatment remains as the main method to control the disease. The medicinal plants are an important source of malaria treatment and remains as an important strategy in the field of drug discovery. This study aimed to identification the antimalarials bioactive compounds isolated from *Symphyopappus casarettoi* inflorescences. The extract was investigated using a bioassay-guided fractionation protocol based on the assay with *P. falciparum*, and the cytotoxicity using monkey kidney cells. A fraction derived from the crude extract was active against *P. falciparum* and showed low cytotoxicity. From this fraction we isolated the flavonoid caryatin, a compound with activity against *P. falciparum in vitro*. It was, however, not active in the experiments with animals infected with *P. berghei*. The present work is the first to report the presence of caryatin in the family Asteraceae and its antiplasmodial activity. Another finding was the isolation of a new labdane type diterpene with a rare hydroxylation pattern.

Keywords: *Plasmodium falciparum, Symphyopappus casarettoi*; ethnopharmacology; medicinal plant; chemotherapy

1. Introduction
Despite efforts from research groups all around the world and the availability of effective drugs for prevention and treatment, malaria remain a serious health treat in most tropical and sub-tropical regions of the globe. Asteraceae is one of the largest family of plant kingdom, comprising more the 24000-species distributed among nearly 1100 genera. Many Asteraceae species are used in popular medicine and are known to produce important bioactive compounds, including the potent antimalarial compound artemisinin [1]. The genus *Symphyopappus* comprises 13 species occurring exclusively in Brazil and another growing in Peru [2].

Previous biological investigation of species from this genus disclosed antimicrobial [3], antioxidant [4] and anticancer activities [5]. Studies focusing on the chemistry of this genus showed the presence of flavonoids, guaianolides, luteolin, 30-hydroxybetulinic acid and thirteen kolavane-type diterpenes [6, 8].

The species *Symphyopappus casarettoi* B. L. Rob. (syn. *Eupatorium casarettoi* B.L. Rob), a shrub that grows in the coastal regions in the south of Brazil, showed interesting activity *in vitro* against *Plasmodium falciparum* and in mice infected with *P. berghei* [9]. The present work aimed at the isolation and identification of the active compounds of this species using a bioassay-guided fractionation protocol based on the *in vitro* assay with *P. falciparum*. The identified active component, a flavonoid, was synthesized and tested *in vivo* in mice infected with *P. berghei*. We also report the identification of a new labdane diterpene from *S. casarettoi*.

2. Materials and Methods
2.1. Plant Material and Extract Preparation
The specimens of *S. casarettoi* (B. L. Rob), syn. *Eupatorium casarettoi* (B. L. Rob) Steyern Asteraceae was collected at Estrada do Mar, Arroio do Sal – RS, Brazil in March 2005; a voucher was deposited in the Herbarium of the Universidade Luterana do Brasil, Canoas, RS, with the code Bordignon *et al*., 2396. The inflorescences (100 g) were macerated with ethanol (3 x 2 L, 24 h) to yield, after solvent removal, 2.1 g of the crude extract. The plant collection and access to genetic resources was registered in the National System for the management of Genetic Heritage and Associated Traditional Knowledge (SisGen, Process N°A38FA26).

2.2. Extract Fractionation

The crude extract (2 g) was suspended in hexane (200 ml) and placed in an ultrasonic bath (100W) for 2 h, after which it was centrifuged and the phases separated to yield, after solvent removal, 1.44 g of a dark gum (FrA) and 0.56 g of yellowish oil (FrB). An aliquot of FrA (200 mg) was dissolved in methanol and incorporated in 700 mg of RP-4 silica gel. The free-flowing material was transferred to a small (1 x 3 cm) column, which was used to inject the sample in a chromatograph equipped with a semipreparative 250 x 20 mm Shimpak ODS separation column (5 µm particle diameter). The sample was chromatographed using mixtures of 0.1% aq. TFA (solvent A) and ACN containing 0.1% TFA (solvent B) as eluent. The HPLC pump was set at a flow rate of 9 ml/min and a low-pressure mixing valve set to generate the following gradient: 5% solvent A was pumped during 10 min and then a linear ramp from 5 to 55% solvent B in 80 min was run. Forty fractions (A1-A40) were collected based on the ELSD detector response. The solvents were removed overnight in a vacuum centrifuge at 45 °C before being used in the assay with *P. falciparum*. The crude extract and all its fractions FrA, FrB and A1-A40, were tested to identify the active fractions.

2.3. *In vitro* Assay with *Plasmodium falciparum* Blood Stage Parasites

The activity of the extract and its fractions was evaluated against *P. falciparum* chloroquine-resistant and mefloquine sensitive W2 clone blood parasites, cultured as previously described [10]. Briefly, the parasites were kept at 37 °C in human erythrocytes (A^+, AB^+ or O^+) collected from healthy volunteers (approved by Ethics Committee, Centro de Pesquisas René Rachou-FIOCRUZ, CAAE 67011617.8.0000.5091 at 06/27/2017) in complete medium (RPMI 1640 supplemented with albumax II 1%, 2 mM L-glutamine, 25 mM Hepes, 11 mM glucose, 0.37 mM hypoxanthine, 23.8 mM sodium bicarbonate and gentamicin sulfate 40 mg/ml). The ring stages were synchronized in sorbitol and immediately incubated with various concentrations of samples previously dissolved in 0.05% DMSO (v/v). Each test was performed in triplicate, and the results compared with control cultures in the absence of drugs. Chloroquine was also used in each experiment as a positive control. The anti-*P. falciparum* effects of the compounds were measured using two different methodologies: (i) the immunoenzymatic test with commercially available specific monoclonal antibodies (MPFM ICLLAB-55A® and MPFG55P ICLLAB®, USA) to a parasite protein histidine- and alanine-rich (HRPII), performed as described [10], and, (ii) the SYBR test as previously described [11]. The anti-HRPII test was performed with 0.05% parasitemia and 1.5% hematocrit; binding of the HRPII antibodies was quantified at 450 nm using a spectrophotometer (SpectraMax340PC384, Molecular Devices). The SYBR test was performed using 0.5% parasitemia and 2% haematocrit, and the fluorescence from viable parasites read in a fluorometer (Synergy H4 Hibrid Reader, BioteK) with excitation at 485 nm and emission of 535 mm. The half-maximal drug inhibitory response (IC_{50}) was estimated by curve fitting, using software from the OriginLab Corporation (Northampton, MA, USA). The results were then compared to parasite growth in the drug-free controls.

2.4. Cytotoxicity Tests

The cytotoxicity assay was performed using a monkey kidney cell line (BGM) (ATCC, Manassas, VA, USA) cultured in 75-cm^2 sterile flasks containing RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum and 40 mg/L gentamicine) under a 5% CO_2 atmosphere, at 37 °C. When confluent, the cell monolayer was washed with culture medium, trypsinized, distributed in a flat-bottomed 96-well plate (2.5×10^5 cells/ml), and incubated for 18 h at 37 °C for cell adherence as described [10]. The compounds (20 µl) at various concentrations (≤1000 µg/ml) were added to the cell plates and incubated for 24 h under a 5% CO_2 atmosphere at 37 °C. The toxicity was determined using the neutral red incorporation assay as described before. To each well was added 0.2 ml medium containing 50 µg/ml. The plate was returned to the incubator for another 3 h at 37 °C to allow for the uptake of the vital dye into the lysosomes of viable uninjured cells. Thereafter the medium was removed, the cells were added of 200 µl of a mixture of 1% formaldehyde-l% $CaCl_2$, and incubated by 5 min. The supernatant was removed and 100 µl of a solution of 1% acetic acid-50% ethanol were added to each well to extract the dye. After a brief agitation on a microtitre-plate shaker, the optical density of each well of the plate was measured using a 540 nm wavelength (SpectraMax340PC384, Molecular Devices). This absorbance has shown a linear relationship with the number of surviving cells. In the neutral red uptake method, it is possible to evaluate the lysosomal integrity and distinguishes live cells from dead by its ability of incorporate the dye. Cell viability was expressed as the percentage of the control absorbance in the untreated cells after subtracting the appropriate background. The minimum lethal dose for 50% of the cells (MLD_{50}) was determined as previously described [12]. The ratio between drug cytotoxicity (MLD_{50} in µg/ml) and activity (IC_{50} in µg/ml) was used to estimate the selective index (SI), as shown before [13], where SI ≤ 10 was indicative of toxicity.

2.5. Antimalarial tests against *Plasmodium berghei* in mice

Peters suppressive test of parasite growth with *P. berghei*, NK65 strain in mice, was performed as described [10]. Briefly, adult Swiss outbred adult female mice (20 ± 2 g weigh) inoculated with 1×10^5 red blood cells infected with *P. berghei*, by intraperitoneal route, were maintained together for at least 2 h, divided randomly in groups of 5 animals per cage, then treated with 50, 100 and 200 mg/kg of each compound diluted in dimethyl sulfoxide (DMSO) (Sigma Aldrich) 3% (v/v) given daily by gavage, for three consecutive days. Two control groups were used in parallel, one treated with CQ (15 mg/kg) and one with the vehicle (not treated control). Blood smears were prepared on days 5 and 7 post-infection, methanol-fixed, stained with Giemsa and examined microscopically. Parasitaemia was evaluated in coded smears and the percent inhibition of parasite growth calculated in relation to the untreated control group (considered 100% growth). The compounds reducing parasitaemia by 40% or more were considered active, those reducing 30-40% as partially active and reductions of less than 30% as inactive. The protocol for animal use was approved by the Ethics Committee at FIOCRUZ (CEUA LW-6/18 at 06/28/2018).

2.6. Analytical Data

Melting points were determined on an Electrothermal apparatus Model 9100 and were uncorrected. IR spectra of the samples in KBr disks were obtained on a Shimadzu FTIR model 8400. The optical rotations were measured with an

Anton Paar polarimeter model MCT300. One and two-dimensional NMR experiments were run on a Bruker DRX-500 spectrometer, using TMS as an internal standard and standard pulse programs. The ROESY experiment was run in perdeuterated DMSO. High resolution mass spectra were recorded on a Bruker ETD-maXis qTOF spectrometer under the following conditions: end plate offset, -500 voltage (V); capillary V, 4500 V; nebulizer pressure, 0.4 bar; dry gas (nitrogen) flow rate, 4.0 L/min; dry temperature, 1800C; collision-induced dissociation energy, 25 eV; collision energy, 7 eV; ion cooler radio-frequency, 25 V; transfer time, 45 µs.

Compound 1: crystalized from 50% aqueous acetonitrile as fine, pale yellow needles, showing mp 263-268 °C dec ([13] mp 263-264). IR (KBr, cm-1): 3429br; 1605, 1216; 1035; 1003. ^{1}H NMR (500 MHz, DMSO-d6) δ ppm 3.70 (s, 3 H, H11) 3.80 (s, 3 H, H12) 6.35 (d, *J*=1.77 Hz, 1 H, H6) 6.43 (d, *J*=1.77 Hz, 1 H, H8) 6.88 (d, *J*=8.59 Hz, 1 H, H5') 7.38 (dd, *J*=8.59, 1.89 Hz, 1 H, H6') 7.50 (d, *J*=1.89 Hz, 1 H, H2') 9.31 (br s, 1 H, OH) 9.59 (br s, 1 H, OH) 10.70 (br s, 1 H, OH). ^{13}C NMR (125 MHz, DMSO-d6) δ ppm 55.9 (C12) 59.2 (C11) 94.7 (C8) 96.1 (C6) 107.4 (C10) 115.1 (C2') 115.7 (C5') 120.0 (C6') 121.3 (C1') 139.8 (C3) 145.1 (C3') 147.9 (C4') 151.9 (C2) 158.0 (C9) 160.7 (C5) 162.4 (C7) 172.1 (C4).

Compound **2:** crystalized from 50% acetonitrile as fine, white needles, mp 207.5-208.5 °C, Optical activity: $[\alpha]_D^{23}$ = -15.2 (*c* 0.57, MeOH). IR (KBr, cm^{-1}): 3350 (O-H); 2923 (C-H); 1712 (C=O); 1045 (C-O). HRMS-ESI (*m/z*) $[2M+H]^+$ calcd for $C_{40}H_{73}O_8$, 681.5294; found, 681.5300. $[2M+Na]^+$ calcd for $C_{40}H_{72}NaO_8$, 703.5119; found, 703.5111. HRMS^2 of $[2M+H]^+$ cluster generated $[M+H-2H_2O]^+$ calcd for $C_{20}H_{33}O_2$, 305.2475; found, 305.2475. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.02 (1H, br s, H-2), 2.43 (1H, dd, *J* = 14.7 Hz, *J* = 6.5 Hz, H-14'), 2.11 (1H, br dd, *J* = 14.7 Hz, *J* = 7.8 Hz, H-14"), 1.91 (1H, m, H-13), 1.72 (1H, s, H-7'), 1.69 (1H, s, H-6), 1.65 (1H, s, H1'), 1.64 (1H, s, H-3'), 1.49 (1H, s, H-11), 1.45 (1H, s, H-3"), 1.48 (1H, s, H-1"), 1.42 (1H, s, H-7"), 1.24 - 1.30 (1H, m, H-12), 1.22 (3H, s, H-20), 1.11 (3H, s, H-17), 1.00 (3H, s, H-19), 1.00 (3H, s, H-16), 0.98 (1H, s, H-5), 0.94 (3H, s, H-18), 0.80 (1H, d, *J* = 3.4 Hz, H-9). 13C NMR (125 MHz, $CDCl_3$) δ = 177.4 (C-15), 73.9 (C-8), 67.7 (C-2), 60.9 (C-9), 53.9 (C-5), 47.0 (C-3), 47.1 (C-1), 43.0 (C-7), 42.6 (C-14), 42.1 (C-12), 40.9 (C-10), 33.8 (C-18), 33.0 (C-4), 32.5 (C-13), 30.8 (C-17), 25.4 (C-19), 24.1 (C-11), 19.0 (C-16), 19.7 (C-20), 20.0 (C-6).

Semi-synthesis of compound 1. This compound was produced from rutin following the procedure described before [14]. Briefly, dry rutin (5 g, 8 mmol) was reacted with benzyl chloride in presence of sodium carbonate to afford the tri-benzylated intermediate (1.6 g, 1.7 mmol, 21% yield). The sugar moiety was then removed by acidic hydrolysis to yield the tri-benzylated aglycon (0.9 g, 1.6 mmol, 94% yield). The free hydroxyl groups at C-3 and C-5 were methylated with excess methyl iodide to afford the permethylated compound (0.45 g, 0.7 mmol, 44%), after which the benzyl groups were removed by Pd/C catalyzed hydrogenolysis to give the final product (0.15 g, 0.5 mmol, 71% yield). The overall yield from starting material was 7%. The resulting semi-synthetic compound showed identical chromatographic behavior and analytical data with that of the isolated natural compound and the mixed melting point was unchanged, confirming their identity as caryatin (3,5-dimethylquercetin).

3. Results and Discussion

The maceration of *S. casarettoi* inflorescences (100 g) in ethanol yielded, after solvent removal, 2.1 g (2.1%) of a gummy, dark green crude extract. This extract (2 g) was treated with hexane to afford two fractions: FrA, insoluble in hexane and containing the more polar components (1.44 g), and FrB, soluble in hexane, enriched with low polarity compounds (0.56 g). The crude extract, along with FrA and FrB were tested *in vitro* with the intra erythrocytic forms of *P. falciparum*, showing that the activity was higher in FrA (Table 1). The chloroquine was used as a positive control in all assays.

Table 1: Cytotoxicity (MLD_{50}, minimum lethal dose that kills 50% of the cells) of *Symphyopappus casarettoi* samples to monkey kidney cells (BGM), activity (IC_{50}, compound concentration causing 50% parasite growth inhibition) *in vitro* against blood forms of *Plasmodium falciparum* (W2 clone, chloroquine-resistant) and selectivity indexes (SI), a ratio between cytotoxicity and activity.

Sample	MLD_{50} [a] (µg/ml)	IC_{50} (µg/ml) [b]	Selectivity Index (SI) [c]	Activity
Crude Extract	36.1 ± 6.8	4.8 ± 2.1	8	Toxic
FrA	55.4 ± 0.8	2.5 ± 1.2	22	Yes
FrB	177.5 ± 0.7	26 ± 10	NA	No
BP-181-6	≥250	7.2 [d]	≥35 [d]	Yes
Caryatin (BP204)	≥1000	3.5 ± 1.3	≥286	Yes
Chloroquine	457.0 ± 22.0	0.146 ± 0.023	3130	NA

[a] Cytotoxicity evaluated by the incorporation of neutral red uptake assay by BGM cell line. [b] Antiplasmodial activity using SYBR test. IC_{50} ≤10 µg/ml = active; 10-20 µg/ml partially active; >20 µg/ml inactive. [c] MLD_{50}/ IC_{50} [10]. [d] Value resulting from a single test. NT = not tested; NA = not applicable.

FrA (250 mg) was chromatographed on a semi-prep (2x25 cm) reversed-phase C18 HPLC column to yield 40 fractions. After removal of the solvents, all fractions, FrA and the crude extract were tested against *P. falciparum* at 10 µg/ml.

The fraction BP181-6, that presented the higher parasite growth inhibition at 10 µg/ml (90%) and IC_{50} of 7.2 µg/ml when tested against blood forms of *P. falciparum* (Table 1), was selected for further fractionation using a Shimpak ODS column (250x20 mm, 5 µm particle diameter), and one of its fractions presented 99% of growth inhibition (BP183-15) at 20 µg/ml. Figure 1 shows the ELSD chromatogram (line) and the antiplasmodial activity of the fractions (dots) obtained from BP181-6. The fraction eluting at 39 min afforded 1.2 mg of fine pale-yellow needles of compound **1**.

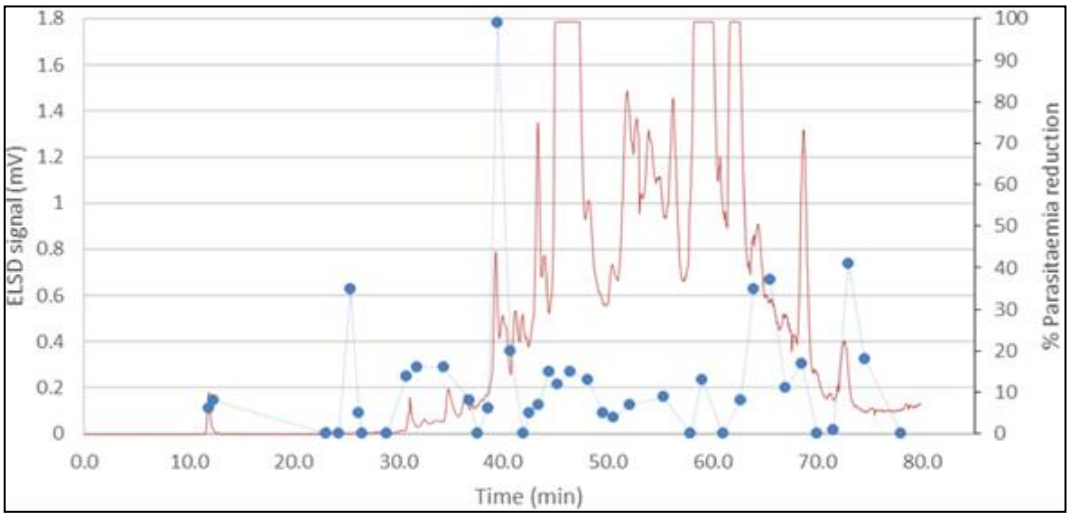

Fig 1: ELSD Chromatogram of FrA (line) and the results of the *in vitro* antiplasmodial activity of the fractions against *P. falciparum* using the anti-HRPII (histidine- and alanine-rich protein II) assay (connected dots). The peak at 39 min correspond to compound **1**.

All other fractions showed weak or no antiplasmodial activity or were complex mixtures of compounds. The major compound of the fraction BP181-6, as inferred by the ELSD signal, eluted at 59 min and afforded compound **2**, which was inactive in the assay with *P. falciparum*.

HRMS analysis of **1** indicated the presence of a compound with predicted molecular formula $C_{17}H_{14}O_7$. Its UV spectra showed λ_{max} at 251 and 357 nm. Losses of water, methyl and CO were observed in the MS^2. Analysis of 1D and 2D NMR spectra of **1** in perdeuterated dimethylsulfoxide (DMSO) and comparison with literature data [14] allowed us to identify it as the flavonoid 3,5-dimethoxy quercetin (caryatin, Fig. 2)

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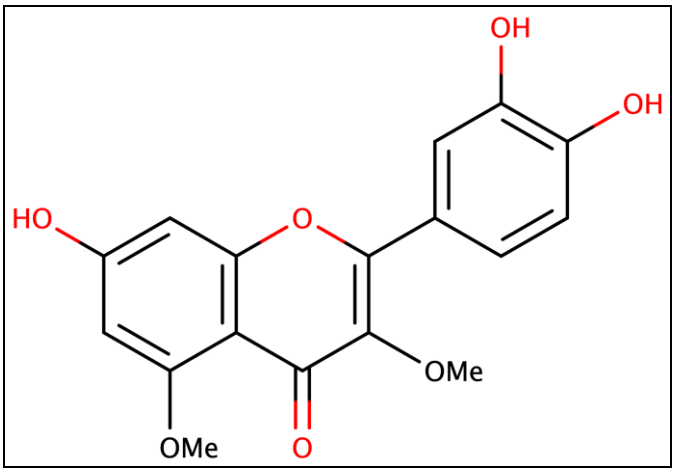

Fig 2: Structure of compound **1** (caryatin)

Caryatin was previously described in few species of the families Ericaceae [15], Juglandaceae [16, 17], Graminae [18], Dioscoreaceae [19], Polygonaceae [20], Thymelaeaceae [21], and Cunoniaceae [22, 23].

To the best of our knowledge, the present work is the first to report the presence of caryatin in the family Asteraceae. Previous works on the bioactivity of caryatin or plants containing it reported pharmacological tests for thrombin inhibition [14], hypoglycemic and aldose reductase inhibitory effect [17], hepatoprotection [18], antioxidant [20], anti-tumor promoting [24] and mutagenic activity in *Salmonella typhimurium* [25].

Caryatin presented no toxicity at the highest dose tested against the BGM cells (MDL_{50} ≥1000 µg/ml) (Table 1) and presented a good activity *in vitro* against the blood forms of the *P. falciparum* with an IC_{50} value of 3.5 µg/ml (10.5 µM) by the SYBR green assay.

The selectivity index (SI), which is the ratio between *in vitro* cytotoxicity and activity, was of ≥286 indicating that caryatin is more toxic to the parasite than to the normal BGM cell line.

The present work is the first describing the antiplasmodial activity of caryatin *in vitro*.

As a previous work with the *S. casarettoi* crude extract showed some antimalarial activity in mice infected with *P. berghei* [9], it was decided to prepare caryatin in enough amounts to evaluate its activity in this model. For this purpose, a published synthetic route using rutin as the starting material [14] was adopted. The reactions were not optimized and the overall yield (7%) was well below the 77% described in the original paper.

The low yield was probably due to the use of the benzyl chloride instead of benzyl bromide in the first step. The natural and semi-synthetic compounds showed identical retention factors in TLC, using different solvent systems as well unchanged mixed melting point, and identical analytical data. The IC_{50} value of the synthetic compound was 11 µM in the *in vitro* assay with *P. falciparum* blood forms.

Caryatin was tested *in vivo* against *P. berghei* at 50, 100 or 200 mg/Kg (data not shown) resulting in no reduction of parasitemia or increase of animal's survival ($P > 0.05$). Thus, the anti-inflammatory [26] and antiplasmodial activities [27] described for other terpenoids [28] that could be related to the control of the inflammatory activity previously demonstrated in malaria [29], was not observed for caryatin in our experiments.

Compound 2 (Table 2), although inactive against *P. falciparum*, is included in the present paper because it is a new natural product and presents a rare hydroxylation pattern of the labdane skeleton.

Its HRMS showed peaks corresponding to $[2M+H]^+$ (*m/z* 681.5294) and $[2M+Na]^+$ (*m/z* 703.5111), compatible with the molecular formula $C_{20}H_{36}O_4$. $HRMS^2$ of the $[2M+H]^+$ cluster generated a $[M+H - 2\ H_2O]^+$ fragment with *m/z* 305.2476, corresponding to the molecular formula $C_{20}H_{32}O_2$, probably due to the presence of two hydroxyl groups.

Table 2: NMR data of compound 2

No.	δC, type	δ mult. (J in Hz)	Cosy	HMBC (H→C)
1'	47.1, CH_2	1.48 s		C2
1"		1.65 s	H2	
2	67.7, CH	4.02 br s	H1, H3	
3'	47.0, CH_2	1.45 s	H2, H3b	C1, C2, C5, C18
3"		1.64 s	H2, H3a	
4	33.0, C			
5	53.9, CH	0.98 s		
6	20.0, CH_2	1.69 s	H7	C5, C8
7'	43.0, CH_2	1.72 s	H6	C6
7"		1.42 s	H6	
8	73.9, C			
9	60.9, CH	0.80 d 3.4	H11	C1, C9, C10
10	40.9, C			
11	24.1, CH_2	1.49 s	H9, H12	C9
12	42.1, CH_2	1.27 m	H11, H13	
13	32.5, CH	1.91 m	H12, H14, H16	C11
14'	42.6, CH_2	2.43 dd 14.7, 6.5	H13	C13, C15
14"		2.11 br dd 14.7, 7.8		
15	177.4, C			
16	19.0, CH_3	1.00 s		
17	30.8, CH_3	1.11 s		C7
18	33.3, CH_3	0.94 s	H3	
19	25.4, CH_3	1.00 s	H3	C5

Analysis of the high resolution 1D and 2D NMR spectra indicated the presence of five methyl groups, seven methylene, four methine and four quaternary carbon atoms, one of them being a COOH group (Table 2). These groups account for the formula $C_{20}H_{34}O_2$ and, together with the two OH groups detected by MS, the molecular formula $C_{20}H_{36}O_4$ is obtained. This molecular formula indicates three degrees of unsaturation and, as the NMR spectra show only the presence of a COOH group and no other signal of multiple bonds, the structure must contain two carbocycles. These features together with observed COSY and HMBC correlations (Fig. 3) allowed us to propose a labdane diterpene identified as 2,8-dihydroxy-labdan-15-oic acid.

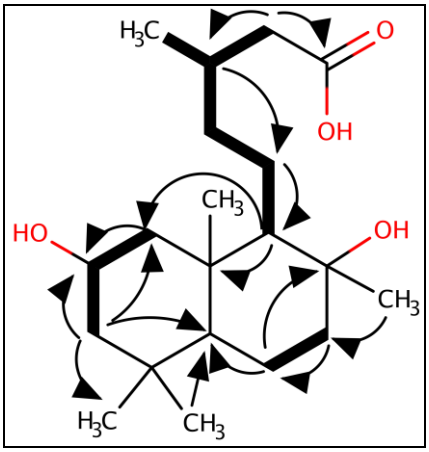

Fig 3: Relevant COSY (bold bonds) and HMBC (curved arrows) correlations observed in 2D NMR experiments at 500 MHz in $CDCl_3$.

The presence of a hydroxyl group at C-2 was inferred by COSY correlations between the carbinol group at δ3.84 (m, 1H) with two flanking methylene groups at C-1 and C-3. This spin-system was corroborated by HMBC correlations (Fig. 3, Table 2). The C-2 oxygenation is very rare among the labdane class of natural products and was previously described only in a related compound, 2α-iso-valeroyloxy eperuic acid, described in *Eupatorium petiolare* [30]. Analysis of the ^1H-^1H-ROESY showed correlations (Fig. 4A) compatible with a tridimensional structure in which the hydroxyl groups and the side chain are on the same side of the *trans* decalin system. The correlation between the methyl at 0.83 and hydroxymethine at 3.84 indicates that the left cyclohexyl ring can flip between chair and twisted boat conformations in which these groups would be 1,3-diaxial (Fig. 4B).

The configuration of C-13 could not be deduced due to the flexibility of the side chain. Based on the available analytical data, the substance was identified as (-)-*rel*-2,8b-dihydroxy-5a,9a,13ξ-labdan-15-oic acid (Fig. 4C).

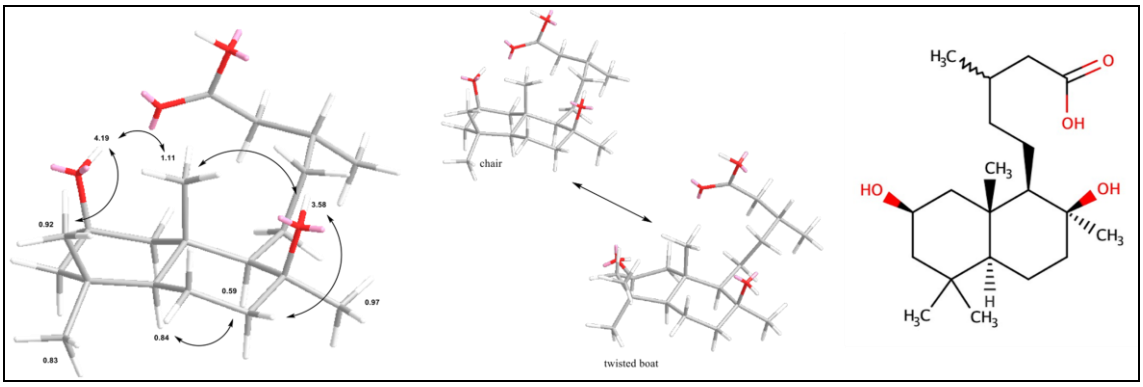

Fig 4: (**A**) Relevant ROESY observed in a 2D NMR experiment at 500 MHz in perdeuterated DMSO; (B) 3D structure showing the twisted boat conformation that explain the ROESY correlation between the methyl group at d 0.83 and the hydroxymethine at d 3.84; (C) structure of (-)-*rel*-2,8b-dihydroxy-5a,9a,13ξ-labdan-15-oic acid.

4. Conclusions

This work shows that caryatin is one of the compounds responsible for the observed *in vitro* antiplasmodial activity of the *E. casarettoi* extract. However, as caryatin was ineffective in the *P. berghei* model while the crude extract showed a moderate antimalarial activity, we speculate that another compound or a mixture of compounds, not identified in the present work, must account for this activity and, consequently further work is needed to identify them. In parallel, we identified a new labdane diterpene with a rare hydroxylation pattern.

5. Acknowledgments

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7. Author Contributions: CLZ, TMAA and ARC performed the fractionation, semi-synthesis and chemical structure identification; MRBN and GS carried out the extraction of the *S. casarettoi* inflorescences; AUK was the project leader; IPC performed the biological *in vitro* and *in vivo* assays, statistical analysis, and conceived and led the study. All authors read and approved the final manuscript.

8. References

1. Luo XD, Shen CC. The Chemistry, Pharmacology, and Clinical-Applications of Qinghaosu (Artemisinin) and its Derivatives. Medicinal Research Reviews. 2003; 7(1):29-52.
2. Hattori EKO. Filogenia molecular da subtribo Disynaphiinae (Eupatoriae: Asteraceae), tratamento taxonômico e sinopse de *Symphyopappus*, e anatomia floral do clado *Grazielia/Symphyopappus.* PhD Thesis. Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, 2013. http://www.bibliotecadigital.ufmg.br/dspace/handle/1843/BUOS-9ATJFT?show=full.
3. Apel MA, Lima MEL, Souza A, Cordeiro I, Young MCM, Sobral MEG, *et al*. Screening of the biological activity from essential oils of native species from the Atlantic rain forest (São Paulo - Brazil). Pharmacology Online. 2006; 3:376-383.
4. Netto-Benetti MR, Rudnicki M, Zanotto-Filho A, De Oliveira MR, Kurek AG, Coitinho A, *et al*. Evaluation of antioxidant effect of extracts of *Symphyopappus casarettoi*. Fitoterapia. 2007; 78:232-234.
5. Ribeiro-Varandas E, Ressurreição F, Viegas W, Delgado M. Cytotoxicity of *Eupatorium cannabinum* L. ethanolic extract against colon cancer cells and interactions with Bisphenol A and Doxorubicin. BMC Complementary Medicine and Therapies. 2014; 14:264.
6. Bohlmann F, Zdero C, King RM, Robinson H. Thirteen kolavane derivatives from *Symphyopappus* species. Phytochemistry. 1981; 20:1657-1663.
7. Bohlmann F, Trinks C, Jakupovic J, King RM, Robinson H. Isolation of a guaianolide from *Symphyopappus compressus*. Planta Medica. 1984; 50(3):276-277.
8. Mesquita AAL, Correa DB, Padua AP, Guedes MLO, Gottlieb OR. Flavonoids from four Compositae species. Phytochemistry. 1986; 25:1255-1256.
9. Netto-Benetti MR. Estudos sobre atividades biológicas de *Symphyopappus casarettoi* Robinson (Asteraceae): evidências preliminares quanto à presença de efeitos antitumoral, antimalárico e antioxidante, PhD Thesis. Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, 2007. http://www.dominiopublico.gov.br/pesquisa/DetalheObraForm.do?select_action=&co_obra=94475.
10. Ceravolo IP, Zani CL, Figueiredo FJB, Kohlhoff M, Santana AEG, Krettli AU, *et al*. *Aspidosperma pyrifolium*, a medicinal plant from the Brazilian caatinga, displays a high antiplasmodial activity and low cytotoxicity. Malaria Journal. 2018;17:436.
11. Siqueira EP, Ceravolo IP, Kohlhoff M, Krettli AU, Zani CL. Synthesis and Antiplasmodial Activity of 2-Methyl-3-Carboxyl-Naphtho [2,3-*b*] Furan Quinone Derivatives. Journal of Medicinal Chemistry and Drug Design. 2018; 1:1-8.
12. Do Céu de Madureira M, Paula Martins A, Gomes M, Paiva J, Proença da Cunha A, Rosário V, *et al*. Antimalarial activity of medicinal plants used in traditional medicine in S. Tomé and Príncipe islands. Journal of Ethnopharmacology. 2002;81:23-29.
13. Bézivin C, Tomasi S, Lohézic-Le Dévéhat F, Boustie J. Cytotoxic activity of some lichen extracts on murine and human cancer cell lines. Phytomedicine. 2003;10(6-

7):499-503.
14. Shi Z-H, Li N-G, Tang Y-P, Li W, Yin L, Yang J-P, *et al*. Metabolism-based synthesis, biologic evaluation and SARs analysis of O-methylated analogs of quercetin as thrombin inhibitors. Europen Journal of Medicinal Chemistry. 2012;54:210-222.
15. Harborne JB. Flavonoid patterns and phytogeography: the genus *Rhododendron* section Vireya. Phytochemistry. 1986; 25:1641-1643.
16. Sasak T. Studies on the components of pecan (*Carya pecan* Engl. & Graebn). II. On a new flavonol "caryatin" isolated from the bark of pecan, and its structure. Yakugaku Zasshi. 1964;84:47-51.
17. Abdallah HM, Salama MM, Abd-elrahman EH, El-Maraghy SA. Antidiabetic activity of phenolic compounds from Pecan bark in streptozotocin-induced diabetic rats. Phytochemistry Letters. 2011;4:337-341.
18. Mohamed GA, Adbel-Lateff A, Fouad MA, Ibrahim SRM, Elkhayat ES, Okino T, *et al*. Chemical composition and hepato-protective activity of *Imperata cylindrica* Beauv. Pharmacognosy Magazine. 2009;5:28-36.
19. Q He, L Liu, Q Shi, Y Cheng, W Shui, Z Ge, *et al*. Effective constituent of *Dioscorea bulbifera*, its preparation method and application in antitumor medicinal formulation. CN. Patent CN101347566A; c2009.
20. Ulubelen A, Tan N, Tuzlaci E. Constituents of Daphne mucronata. Fitoterapia. 1990;61:281.
21. Bate-Smith EC, Harborne JB, Davenport SM. In Occurrence of azaleatin and caryatin in Eucryphia. Nature. 1966;212:1065-1066.
22. Bate-Smith EC, Davenport SM, Harborne JB. Comparative biochemistry of flavonoids. III. A Correlation between chemistry and plant geography in the genus *Eucryphia*. Phytochemistry. 1967;6:1407-1413.
23. Gao H, Kuroyanagi M, Wu L, Kawahara N, Yasuno T, Nakamura Y, *et al*. Antitumor-promoting constituents from *Dioscorea bulbifera* L. in JB6 mouse epidermal cells. Biological Pharmaceutical Bulletin. 2002;25(9):1241-1243.
24. MacGregor JT, Jurd L. Mutagenicity of plant flavonoids: structural requirements for mutagenic activity in *Salmonella typhimurium*. Mutation Research/Environmental Mutagenesis and Related Subjects. 1978;54:297-309.
25. Clavin M, Gorzalczany S, Macho A, Muñoz E, Ferraro G, Acevedo C, *et al*. Anti-inflammatory activity of flavonoids from *Eupatorium arnottianum*. Journal of. Ethnopharmacology. 2007;112(3):585-589.
26. Soré H, Sanon S, Hiolu A. Antiplasmodial properties of plants isolated flavonoids and their derivatives. International Journal of Herbal Medicine. 2018;6:43-56.
27. Demetzos C, Dimas KS. In *Labdane-type diterpenes: chemistry and biological activity*; Attaur-Rahman Ed.; Elsevier: New York, NY, USA. 2001;25:235.
28. Johansen DA. Plant Micro techniques. 1st edition, (McGraw-Hill Book Company, New York and London; c1940. p. 182-203.
29. Higgins SJ, Kain KC, Liles WC. Immunopathogenesis of falciparum malaria: implications for adjunctive therapy in the management of severe and cerebral malaria. Expert Review of Anti-infective Therapy. 2011;9(9):803-819.
30. Calderon JS, Quijano L, Garduno M, Gomez F, Rios T. 2α-iso-Valeroyloxyeperuic acid, a diterpene from *Eupatorium petiolare*. Phytochemistry. 1983;22:2617-2619.