Malaria Group, University of Antioquia, Medellín, Colombia

Antimalarial activity of neurolenin B and derivates of *Eupatorium inulaefolium* (Asteraceae)

S. Blair, J. Mesa, A. Correa, J. Carmona-Fonseca, H. Granados and J. Sáez

Dried stems and leaves of *Eupatorium inulaefolium (Austroeupatorium inulaefolium)* (Asteraceae) were used to obtain four crude extracts (hexane, dichloromethane, methanol and ethanol). Two fractions were obtained from the hexane extract (S1 and S2) and three compounds (neurolenin B, lobatin A and lobatin B) from the dichloromethane extract. The ethanol, hexane, dichloromethane and methanol extracts, two fractions from the hexane extract (S1 and S2), and neurolenin B were evaluated *in vitro* against *Plasmodium falciparum*, FCB-2 strain. Two extracts (dichloromethane and methanol), the S2 fraction and neurolenin B showed statistically significant antiplasmodial activity.

1. Introduction

Malaria is a serious public health problem in Colombia and around the world. Medicinal plants have been used for the treatment of malaria during centuries. Alkaloids, triterpenoids, iridoids and sesquiterpenoids compounds isolated from plants have shown antimalarial activity [1]. Similarly, *in vitro* antimalarial activity has been demonstrated in several species of plants of the Asteraceae family, i.e. *B. pilosa*, *A. maritima*, *R. lacinata, E. rufencens, S. selloi and E. squalidum* [2–5]. Several of these compounds have a sesquiterpene lactone chemical structure and some of them had confirmed cytotoxic activity [6, 7]. This study intended to evaluate the *in vitro* activity of extracts and fractions of *Eupatorium inulaefolium* H.B.K (Asteraceae) against *Plasmodium falciparum*, and to isolate and identify a pure compound from extracts and fractions of the plant. Various plant extracts (dichloromethane, hexane, methanol) lack mutagenic activity when assayed by the Ames test [8].

2. Investigations and results

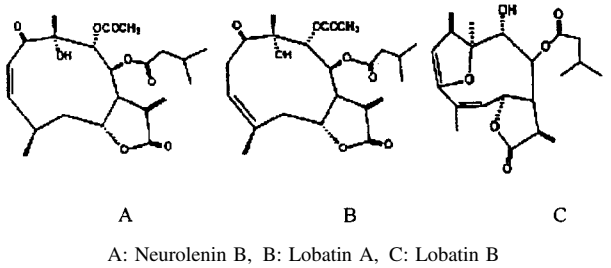
Fractioning of the dichloromethane extract allowed the isolation, purification and identification of three sesquiterpenlactones: neurolenin B, lobatin B, and lobatin A. Structural elucidation of these compounds was performed by ^{1}H NMR, ^{13}C NMR (Table 1), COSY ^{1}H-^{1}H, H-^{13}C and mass analysis, which confirmed previously reported data [6, 9].

TLC analysis of fraction S2 obtained from the hexane extract showed a mixture of several components, neurolenin B and lobatin A, among other. Neurolenin B is the major compound of the plant *E. inulaefolium*.

Parasitaemia of negative controls was between 1.103% and 3.064% (mean: 1.799). Parasitaemia in all wells treated with chloroquine, but not in hexane extract wells, was higher than in the wells cultured with the extract/fraction. Table 2 shows the mean percentage of parasitised red blood cells in wells treated with the extract/fraction. Parasitaemia was between 0.448% and 1.041%. Except in experiments 4 (ethanol extract) and 6 (fraction S1), when parasitaemia oscillated between 1.388% and 1.508%. In all the experiments, except in 6 (fraction S1), the mean parasitaemia was significantly lower ($p < 0.00001$) that in the negative control. Experiment 6 is omitted from the subsequent analysis.

Parasitaemia was lower in the cultures treated with neurolenin B, which showed antimalarial activity up to dilution 10 (0.057 ppm). Fraction S2 exhibited antiplasmodial activity up to dilution 7 (2.5 ppm). In both experiments, chloroquine was effective against the parasite up to dilution 7 (0.156 ppm). Therefore, neurolenine B had a better parasiticide effect than chloroquine (Table 3).

The extracts/fractions inhibited parasitaemia by 16 and 74%. However, the methanol and dichloromethane extracts, fraction S2 and neurolenine B, showed inhibition rates ranging from 67 to 74% (Table 2). The Pearson correlation coefficient oscillated between −0.545 (fraction



A: Neurolenin B, B: Lobatin A, C: Lobatin B

Sesquiterpenlactones of *Eupatorium inulaefolium*

Table 1: ^{13}C NMR data of compounds 1, 2 and 3

C	Neurolenin B (1)	Lobatin B (2)	Lobatin A (3)
1	204.4	205.0	210.7
2	125.2	104.2	36.1
3	147.9	186.0	121.4
4	28.0	131.3	136.7
5	40.0	134.3	42.8
6	76.2	72.6	72.5
7	41.0	43.3	42.1
8	73.8	78.1	76.5
9	73.7	75.3	76.5
10	79.2	90.7	80.5
11	134.7	139.6	134.3
12	168.6	168.6	168.0
13	126.3	124.6	124.3
14	23.7	19.6	25.4
15	19.5	17.7	22.4
1′	170.9	171.5	170.3
2′	42.4	42.7	42.1
3′	24.8	25.3	25.4
4′	22.1	22.0	22.3
5′	22.1	22.1	22.2
1″	170.0	–	170.8
2″	20.4	–	20.5

Table 2: Parasitaemia and percentage inhibition of parasitaemia according to treatment with *Eupatorium inulaefolium*[1]

Experiment	Treatment	Wells[2]	Mean	SD	r[3]	% Inhibition[4]
4	Ethanol extract	120	1.388	1.182	−0.820	23
1	Methanol extract	120	0.518	0.371	−0.694	71
2 + 3	Dichlorometane ext.	240	0.468	0.558	−0.659	74
8	Neurolenine B	120	0.554	0.374	−0.728	69
5	Hexane extract	120	1.041	0.488	−0.878	42
6	Fraction S1 of hexane	120	1.508	0.270	N.E.[5]	16
7	Fraction S2 of hexane	120	0.598	0.648	−0.545	67
1–8	Controls[6]	704	1.799	0.380	–	0

[1] In all the experiments, but in #6, the mean parasitaemia observed in samples cultured with *A. inulaefolium* was lower than the negative control. With neurolenine B and fraction S2, the mean parasitaemia was lower than the positive chloroquine control.
[2] Number of wells plated.
[3] r: linear correlation coefficient.
[4] parasitaemia percentage inhibition compared to control.
[5] Not estimated since no parasiticide action was observed with Fraction S1.
[6] 88 control wells in each experiment. This summarizes results of all wells ($8 \times 88 = 704$)

Table 3: Initial concentration of the extracts, fractions and compounds of the plant *Eupatorium inulaefolium*

Extract, fraction or compound				Positive control (chloroquine)		
Substance	Initial	Antimalarial activity up to		Initial	Antimalarial activity up to	
	ppm	Dilution	ppm	ppm	Dilution	ppm
Ethanol extract	3520	8	55	10	–	–
Hexane extract	80	3	20	10	8	0.078
Fraction S1	160	Not active	–	10	9	0.039
Fraction S2	160	7	2.5	10	7	0.156
Dichlorometane extract	1000	7	7.8	10	7	0.156
Methanol extract	3027	9	11	10	–	–
B Neurolenine	29.54	10	0.057	20	7	0.3125

S2) and −0.878 (hexane extract). The r value was always negative, indicating an inverse dose-response association: while the dose of the treatment increases, the number of parasites decreases (Table 2).

In summary, methanol and dichloromethane extracts, fraction S2 and neurolenin B (from dichloromethane extract) obtained from the plant *Eupatorium inulaefolium* exhibited a significant parasiticide action against *Plasmodium falciparum*. Moreover, the antiplasmodial effect of neurolenin B, dichloromethane extract, as well as the fraction S2, was significantly better than chloroquine.

3. Discussion

The most important finding of our study is the high antimalarial activity of neurolenin B at concentrations significantly lower (at least 0.057 ppm) than chloroquine (0.156 ppm) (Table 3). The compound neurolenin B has been previously reported by Guido et al. [3], who obtained it from a dichloromethane extract of the plant *Neurolaena lobata* (Asteraceae) and was shown to have antimalarial activity with an IC_{50} of 0.236 ppm. Here we report for the first time that extracts, fractions and compounds obtained from *E. inulaefolium*, have antimalarial properties.

Several other species of the Asteraceae family are effective against the parasite [4, 10, 11] and some others have cytotoxic activity [12]. Characteristically, plants of this family contain high amounts of sesquiterpenlactones, which apparently have cytotoxic activity due to the proximity of the double link near the keto of the carbon 1. Sesquiterpenlactones such as artemisinine isolated from *Artemisa annua,* have resulted in the production of highly effective antimalarials drugs.

We made experiments to measure the mutagenic activity of the plant *E. inulaefolium* by the Ames test, on TA-98 and TA-100 strains of *Salmonella tiphimurium* [13, 14]. We evaluated the extracts at concentrations of 3.0 (ethanol), 1.0 (dichloromethane), and 0.08 (hexanic) mg/plate, and found no mutagenic effect [8]. This opens exciting perspectives for the search of new antimalarial compounds from *Eupatorium inulaefolium*.

4. Experimental

4.1. General experimental procedures

UV spectra were obtained using a UV/VIS Genesis 2 spectrophotometer; IR spectra were run in a RX-FTIR spectrophotometer; NMR spectra were obtained in a Bruker AC 250–300 MHz for ^1H and 100 MHz for ^{13}C. For NMR, all the samples were diluted in deuterated chloroform. Cromatography fractioning was performed in G-60 silica gel eluted with hexane-ethyl acetate 7 : 3 v/v.

4.2. Plant processing and extract or fraction preparation

Eupatorium inulaefolium was collected in El Bagre (Antioquia province, Colombia) and Tumaco (Nariño province, Colombia), one sample was deposited at the herbarium of University of Antioquia (Medellín, Colombia) (number 83377).

For preparation of the extracts, 1800 g of dried and powdered stems and leaves were successively percolated with hexane, dichloromethane, methanol and ethanol, to produce 107.35 g, 84.45 g, 191.0 g and 20.2g of each extract.

For S1 and S2 fractions preparation, 46.8 g of the n-hexane extract were subjected to CC analysis and eluted with hexane-ethyl acetate (7 : 3 v/v); 43 fractions were obtained and grouped according to their Rf: S1 (fractions 1–17), S2 (fractions 18–36) and S3 (fractions 37–43).

Compounds 1, 2 and 3 were isolated from 23.4 g of the dichloromethane

extract, which was subjected to CC analysis and eluted with hexane-ethyl acetate (1 : 1 v/v) to obtain 55 fractions. Fractions 17 to 53 (5.7 g) were processed by CC and eluted with hexane-ethyl acetate (7 : 3 v/v); from this, three pure compounds were obtained: neurolenin B (1330 mg), lobatin B (270 mg), and lobatin A (50 mg).
Since the extracts were insoluble in water, four parts of polyvynilpyrrolidone (mw 10000, PVP-10) and one part of the crude extract were mixed and dried to obtain a water soluble mixture. Neurolenin B was dissolved in propylenglycol 100% and further diluted to eliminate the lytic effect on erythrocytes (0.45%).

4.3. In vitro antiplasmodial evaluation

The procedure for the *in vitro* antiplasmodial evaluation of the extracts, fractions and compounds was carried out according to Rieckmann et al. [15], with some changes introduced by Cruz-Mancipe and Fuenmayor [16]. *P. falciparum* strain FCB-2 was continuously cultured following the method described by Trager and Jensen [17, 18], using the technique of the jar and the candle in a 5% CO_2 atmosphere at 37 °C. Parasites were maintained in Petri dishes with O^+ human erythrocytes (5% haematocrit), Hepes enriched RPMI-1640, hypoxanthine, gentamicine and complemented with human serum 10% from a pool of recalcified ABO plasma, 5% sodium bicarbonate and reduced glutation, pH 7.2–7.4.
Each experiment was repeated eight times, initial parasitaemia was 0.8% and culture time was 24 h. In each experiment, three microplates (96 wells/microplate) were used to evaluate the treatment with each of the four extracts, two fractions or one compound, chloroquine (positive control) or negative control were set up. Two-fold dilutions of each treatment were made and ten doses of each treatment were evaluated. Each dose was evaluated 12 times in the case of extract/fraction and 6 times when chloroquine was added. In total, in each experiment, 120 wells with extract/fraction/compound (10 doses × 12 repetitions), 60 wells with chloroquine (10 doses × 6 times) and 88 wells with negative control were evaluated. The initial concentration (first dilution) of each treatment is shown in Table 3. In the case of the dichloromethane extract, two experiments were made under similar conditions; for this reason, results of eight experiments are included.

4.4. Controls

In each experiment, positive and negative controls were used. The positive control was chloroquine; on well 10, the chloroquine concentration was 0.01953125 ppm. The negative control consisted of parasites cultured without *E. inulaefolium.*

4.5. Interpretation of the results

The *in vitro* antiplasmodial effect was determined by the difference between the mean parasitaemia observed in wells added the plant material (extract, fraction, compound), and control wells (without plant material). The percentage parasitaemia was measured in each well as the number of parasitised red blood cells per 100 red blood cells after 24 h of treatment. This was made in order to evaluate the changes in parasitaemia at the different doses administered.
The percentage of parasitaemia inhibition by each extract/fraction was estimated by the difference between% parasitaemia of the negative control and% parasitaemia of the experimental wells.

4.6. Statistical analysis

The SGPlus 7.1 and Epi Info 6.04 programs were used for data analysis of experiments. Mean parasitaemia on each treatment (extract/fraction/compound, chloroquine, negative control) was evaluated by the Kruskal and Wallis one way variance analysis.
When the mean number of parasites in the extract/fraction/compound was lower than in the negative control, other analysis were carried out:
a) One way analysis of variance by Kruskal and Wallis to compare the parasite percentage in each dose of extract/fraction/compound.
b) Regression analysis to explore the relation between the dose of extract/faction/compound and the number of parasites (dose–response relationship).

4.7. Physical and spectroscopic data

Neurolenin B: $C_{22}H_{30}O_8$, white crystals, m.p. 157 °C, UV λ_{max} (MeOH) nm: 212, MS m/z (int. rel): 440 $[MH + NH_3]^+$ (100), 423 $[MH]^+$ (3), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.52 d, H-2), 5.93 (t, H-3), 3.01 (m, H-4), 1.84 (ddd, H-5a), 1.32 (ddd, H-5b), 4.48 (dd, H-6), 2.53 (s, H-7), 5.6 (d, H-7, H-8), 6.22 (s, H-13), 1.25 (s, H-14), 1.04 (d, H-15), 1.94 (m, H-3′), 0.79 (d, H-4′y 5′), 2.01 (s, H-2′). ^{13}C NMR (75 MHz, $CDCl_3$).
Lobatin B: $C_{20}H_{24}O_7$, UV λ_{max} (MeOH) nm: 210, 263, MS m/z (int. Rel): 394 $[MH + NH_3]^+$ (100), 377 $[MH]^+$ (15), ^{1}H-NMR (300 MHz, $CDCl_3$). δ 5.53 (s, H-2), 5.87 (m, H-5), 5.20 (m, H-6), 3.84 (m, H-7), 4.90 (dd, H-8), 3.83 (d, H-9), 6.24 (d, H-13 a), 5.71 (d, H-13 b), 1.43 (s, H-14), 2.07 (d, H-15), 1.96 (m, H-2′), 2.00 (m, H-3′), 0.81 (d, H-4′), 079 (d, H-5′). ^{13}C NMR (75 MHz, $CDCl_3$).
Lobatin A: $C_{22}H_{30}O_8$, UV λ_{max} (MeOH) nm: 219, MS m/z (int. Rel): 422 $[M]^+$ (2), 404 $[M\text{-}18]^+$ (8), ^{1}H-NMR (300 MHz, $CDCl_3$), (dd, H-2 a), 3.57 (dd, H-2b), 5.87 (t, H-3), (dd, H-5a), 2.75 (dd, H-5 b), 4.91 (ddd, H-6), 2.61 (m, H-7), 5.85 (dd, H-8), 5.65 (d, H-9), 5.68 (d, H-13b), 6.31 (d, H-13a), 1.32 (s, H-14), 1.84 (s, H-15), 1.97 (m, H-3′), 0.87 (d, H-4′), 0.85 (d, H-5′)2.13 (s, H-2″). ^{13}C NMR (75 MHz, $CDCl_3$).

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Silvia Blair
Grupo Malaria
Facultad de Medicina
Universidad de Antioquia
AA: 1226
Medellín-Colombia
sblair@catios.udea.edu.co