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Evaluation of mutagenic activity of several antimalarial extracts from *Eupatorium inulaefolium*

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Eupatorium inulaefolium is used as an antimalarial agent by traditional healers of the Tumaco region (Nariño-Colombia). Several extracts of this plant have been tested by our laboratory and *in vitro* antimalarial activity against the FCB-2 strain of *Plasmodium falciparum* has been confirmed. For this reason, the mutagenic effect of the methanol, dichloromethane, and hexane extracts of *Eupatorium inulaefolium* (number 83377 university of Antioquia herbarium) were evaluated using the Ames test. None of the extracts evaluated had mutagenic effects on TA-98 or TA-100 strains of *Salmonella typhimurium*.

1. Introduction

As early as 480 (B.C), Hipocrates recognized the clinical characteristics of malaria [1]. From that period mankind has tried to control the disease. Nevertheless, malaria remains today as the most frequent parasite infection worldwide, causing the death of approximately 2.5 million people per year [2]. In Colombia the situation is serious and massive migrations as a results of the violence as well as the inadequate living conditions in rural areas, have contributed to the increasing prevalence of malaria during the last decades. At present, 72% of the Colombian population lives in malaria endemic areas; the number of cases is increasing every year, fluctuating from 2.3/1000 inhabitants in 1967 to 7.1/1000 in 1997 [3].

Due to the limited number of effective antimalarials, the emering resistance of *Plasmodium falciparum* to the commonly used antimalarial drugs [4–8] and their mutagenic activity [9–12], the Malaria Group of the university of Antioquia has been working for more than 10 years in the search for new therapeutic and prophylactic alternatives from plants used by the traditional medicine in Colombia. With this purpose an ethno-medical and ethno-botanical study was carried out in Tumaco (Southwest region) to evaluate the variety of plants that the population used as antimalarials. Among these plants were *Austroeupatorium inulaefolium* or *Eupatorium inulaefolium* which possess antimalarial activity *in vitro* against the FCB-2 strain of *Plasmodium falciparum*. The antimalarial activity of the methanol, dichloromethane, hexane, and ethanol extracts, the fraction 2 (obtained from the hexane extract) and Neurolenina B, a sesquiterpene lactone derived from the dichloromethane extract, was performed according to the Rieckmann techique. *P. falciparum* growth rate inhibition was 95% with 1513 ppm of methanol extract, 98% with 1000 ppm of dichloromethane extract, 86% with 80 ppm of hexane extract, 99% with 3520 ppm of ethanol extract, 99% with 80 ppm the fraction 2 and 94% with 29.54 ppm of Neurolenina B. IC_{50} were 127 ppm (methanol extract), 25.05 ppm (dichloromethane extract), 23.99 ppm (hexane extract) 201.6 ppm (ethanol extract), 6.07 ppm (fraction 2) and 2.14 (Neurolenina B).

One of the necessary steps in the search for new therapeutic compounds is to study the toxicity and mutagenicity of the candidate substance. This article reports on an evaluation of the mutagenic activity of the methanol, dichloromethane, and hexane extracts obtained from *Eupatorium inulaefolium* using the Ames test.

Bacterial tests for mutagenicity (and especially the Salmonella/plate incorporation test), are now the most widely used. Numerous validation studies over the past 18 years have shown that such assays have high sensitivity (in the order of 70–80%) for the detection of genotoxic carcinogens in rodents [13].

The Ames test uses several strains of *Salmonella typhimurium*, with a reversible mutation in the histidine operon, as a consequence of this mutation they are unable to grow in a minimal medium which lacks histidine. They also possess the *rfa* and *uvrB* mutations that increase their membrane permeability to larger molecules and they have the pKM101 plasmid which contains various copies of the histidine gene and therefore increases the detection mutagenic compounds in *Salmonella* strains. The TA-98 strain is used to detect various frameshift mutagens and the TA-100 strain detect mutagens that cause base-pair substitutions in the DNA [14, 15].

2. Investigations and results

Table 1 shows the mean number of his^+ mutants induced by each concentration of the extracts when the test was carried out with and without metabolic activation, the

Table 1: Mutagenic evaluation of three extracts from *Eupatorium inulaefolium*

Compounds	Concentration (mg/plate)	Average of mutants His^+ per plate							
		TA-98				TA-100			
		+S9[a]		−S9[b]		+S9		−S9	
		$\overline{X}$[c]	DS[d]	$\overline{X}$	DS	$\overline{X}$	DS	$\overline{X}$	DS
Methanol extract	3.0	34	12	37	13	128	11	107	21
	1.5	39	12	32	7	108	12	99	15
	0.75	41	11	29	5	108	14	96	7
	0.375	43	14	24	9	110	12	86	13
	0.187	34	16	28	12	98	6	90	11
Dichloromethane extract	1.0	43	8	23	7	109	12	82	25
	0.5	44	10	27	4	119	19	85	30
	0.25	43	7	24	6	117	19	85	8
	0.125	39	3	21	4	101	17	93	22
	0.0625	41	4	25	3	102	28	101	33
Hexane extract	0.08	43	11	27	11	106	24	78	7
	0.04	37	14	26	6	114	13	74	17
	0.02	42	10	28	8	101	13	78	16
	0.01	43	15	27	8	111	14	78	8
	0.005	37	11	29	12	122	18	74	12
Water		39	11	26	8	110	13	92	14
PVP-10[e]	444	42	10	26	6	110	14	91	14
2-AF[f]	10 μg	379	136	27	9	569	131	91	14

[a] +S9, experiments in presence of S9 fraction; [b] −S9, experiments without S9 fraction; [c] $\overline{X}$, average of mutants His^+/plate; [d] DS, standard deviation; [e] PVP, polyvinyl pyrrolidone with molecular weight 10000 (444 mg/plate); [f] 2AF, 2 aminofluorene (10 μg/plate)

Table 2: Comparison of the average numbers of mutant colonies of each bacteria strain in relation to the tested extracts concentrations

Extract	Strain	[a] +S9		[b] −S9	
		K-W[c]	Value P	K-W	Value P
Methanol	TA-98	2.024	0.731434	5.212	0.266222
	TA-100	13.095	0.010823*	5.588	0.232113
Dichloro-	TA-98	2.314	0.678155	4.990	0.288277
methane	TA-100	5.681	0.338507	2.572	0.765570
Hexane	TA-98	2.724	0.605050	0.306	0.989391
	TA-100	6.885	0.142105	0.450	0.978147

[a] +S9, experiments in the presence of S9 fraction; [b] −S9, experiments without S9 fraction; [c] K-W, value of the analysis of variance comparing more than two average numbers; [d] At least one of the concentrations differs from the others

standard deviations, the mean number of revertants corresponding to the negative controls, water and polyvinylpyrrolidone (PVP-10), and the mean number of mutants in the positive controls (2-aminofluorene).

Statistically significant differences between the mean number of his^+ revertants in the positive control (2-AF) and the negative controls (H_2O and PVP-10) were found. Significant differences were also seen between the mean number of revertants induced by the evaluated extracts and the positive control. On the other hand, the comparison between the mean number of the revertants from the different extracts and the negative controls did not show statistically significant differences.

A Kruskal-Wallis analysis was made to compare the variance of the mean number of revertants for each *Salmonella* strain according to the concentration of the different extracts. In general, no significant differences were observed between the results of each compound when this was used at different concentrations. However, in the case of the methanol extract with the TA-100 strain (Table 2), a statistically significant difference was found between the highest concentration (3.0 mg/plate) and the other tested concentrations (1.5, 0.75, 0.375, 0.187 mg/plate).

The regression analysis showed an r coefficient of 0.61, this means that there was a positive dose-response relationship between the number of revertants and the concentrations of the extract, the variable "concentration" explains only 36% of the change of the revertants number.

We conclude that none of the evaluated extracts from *Eupatorium inulaefolium* are direct or indirect mutagens for the *Salmonella* strains used in this study, since the number of reversions his^- to his^+ did not show a directly proportional increment with the extract concentrations used. Therefore, a positive dose-response relationship can be excluded.

3. Discussion

The mean number of spontaneous revertants in our the negative controls (water and PVP-10) for the TA-100 strain was 110 colonies, a value that is below previously published data (120–200 colonies/plate) [14], but the standard number observed in laboratories in Medellín (Colombia) is about 85 colonies. Therefore, we concluded that the tests carried out in this study were successful and our results are reliable. The quantitative changes are less important since the overall performance of the test should not be altered, while facts affecting qualitatively the results might give a reduction in the test sensitivity and predictability [14].

The mean number of revertants in the positive controls with 2-AF was low, especially with the TA-98 strain, compared to previous test (2745 colonies/plate). However, the results of the extracts are reliable since the differences between the number of revertants in the positive and negative control were significant. The result of the positive control with 2-AF may have been affected by the quality of the S9 fraction prepared for the assays; this quality depends on various factors such as the individual susceptibility of each animal to the effects of the mixture of Aroclor 1254 and phenobarbiton. This might have induced a different pattern of enzymes from those required for a better metabolism of 2-AF. In previous tests the positive controls were carried out after induction of the enzymes with Aroclor 1254 only. The preparation of the S9 fraction for the detection of various classes of genotoxic carcinogens is extremely complex, therefore the effects are variable. In general, the use of phenobarbitone in combination with β-naphthoflavone as a safer substitute for Aroclor 1254, has been acceptable for routine screening [13].

The absence of mutagenic activity of the methanol, dichloromethane, and hexane extracts from *Eupatorium inulaefolium* is an encouraging result for the possible use of these extracts as antimalarials. The Ames test is a very reliable tool for screening the mutagenic activity of diverse substances since it has high sensibility in detecting point mutations, and high predictive values when compared with other genotoxic tests carried out *in vitro* in eukaryotic cells or *in vivo,* in more complex organisms [14, 16]. However, it is important to purify the compounds from *Eupatorium inulaefolium* which have demonstrated antimalarial activity, in order to further assess the mutagenic activity with other tests such as the exchange of sisters chromatids and the induction of clastogenesis in mice.

4. Experimental

4.1. Plant processing and extract preparation

The vegetable material (stem and leaves) was collected in the Colombian towns of El Bagre (northwest region) and Tumaco (Southwest region). It was air dried and then ground in a blade mill.

To obtain the active principles of low polarity such as fats and terpenoids (hexane extract) the plant was subjected to hexane in a percolator for seven days. The defatted vegetable material was air dried and subjected to another extraction process with dichloromethane for seven days; the obtained solution was filtered and vacuum concentrated in a rotavaporator to obtain an medium polarity extract (dichloromethane extract). The extraction process was repeated with methanol to obtain a series of high polarity compounds (methanol extract).

The three tested extracts were insoluble in water therefore it was necessary to use polyvinyl-pyrrolidone, a substance with a molecular weight of 10000 (PVP-10), to obtain a water-soluble copolymer (four parts of PVP-10 to one part of extract) [17].

4.2. Concentration of extracts

The extract concentrations for the Ames test were the same as those used for the evaluation of the *in vitro* antimalarial activity of the plant *Eupatorium inulaefolium*, keeping in mind the toxicity for the strains used in the test. This toxicity was evaluated in a 24-hour culture of TA-100 strain of *Salmonella typhimurium* (growth density of $1-2 \times 10^9$ bacteria/ml and work dilution 1 : 262.144) with a series of two-fold dilutions of each extract. The maximum not toxic concentrations were selected.

Bacteria cultured in nutrient medium without extract were used as a negative control and the number of colonies was compared with that of the cultures exposed to the extracts. The following concentrations were selected to evaluate the mutagenic activity:

Methanol extract: 3.0, 1.5, 0.75. 0.375, 0.187 mg/plate

Dichloromethane extract: 1.0, 0.5, 0.25, 0.125, 0.0625 mg/plate

Hexane extract: 0.08, 0.04, 0.02, 0.01, 0.005 mg/plate.

4.3. Preparation of the "S9 mix" fraction

The "S9 mix" fraction was prepared from the liver of a *Rattus rattus* male treated with Aroclor 1254 (500 mg/kg in one dose injected intraperitoneally) and phenobarbitone (1 mg/ml for five days, diluted in water and administered ad libidum), in order to induce the liver production of microsomal oxygenases such as the P-450 cytochrome. The extracted liver was homogenized, mixed with 0.15 M KCl and centrifuged at 9.000 g for 10 min at 4 °C to obtain a supernatant that constituted the "S9 mix" fraction; this supernatant was distributed in 1 ml vials and kept at −70 °C. A few minutes before being used, the cofactors NADP, Glucose 6-phosphate and $MgCl_2$−KCl buffer at pH 7.2, were added [14].

4.4. Ames test

This test was made following the protocol described by Maron et. al. [13, 14]. *Salmonella typhimurium* TA-98 and TA-100 strains were donated by doctor Ames, of the University of California, Berkeley (USA). These strains were conserved in plastic vials of 1 ml at −70 °C at the Laboratorio de hemoparasitos of Universidad de Antioquia.
The experiments were carried out with and without metabolic activation (S9 fraction) and the samples were preincubated for 20 min at 37 °C before being plated in the minimal agar dishes [21]. When the procedure was completed, the dishes were incubated for 48 h at 37 °C. Then, the number of colonies were counted in every dish; this number indicated the number of reversions of the mutation his^- to his^+ of the *Salmonella typhimurium* strains in minimal glucose agar medium without histidine.
Three independent duplicate assays were carried out under the same laboratory conditions for each one of the five concentrations of every extracts and every strain; in other words, every concentration was tested a total of six times.

4.5. Controls

Controls included in every mutagenicity test were: 2-aminofluorene (2-AF) as positive control (10 μg/plate), an indirect mutagen that was also an indicator for the activity of the microsomal enzymes; water and PVP-10 as negative controls.

4.6. Interpretation of the results

We considered a positive mutagenic effect when the number of reversions his^- to his^+ showed an increase directly proportional to the extract concentration [14, 15].

4.7. Statistical analysis

The Student t-test was used to compare the average number of $histidine^+$ revertants induced by the concentrations of every extract with the number of revertants in the positive and negative controls. Newmann and Keuls multiple range analysis and Kruskal-Wallis analysis were carried out to compare the variation in the number of mutants for every strain of *Salmonella* exposed to various concentrations of the extracts. Regression analysis was carried out to evaluate the covariance between the number of mutants or revertants and the extracts concentrations in each strain of *Salmonella*. This procedure was applied in the experiments with and without metabolic activation.

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