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Evaluation of the mutagenicity of antimalarial products isolated from *Solanum nudum* (Solanaceae)

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Diosgenone is a major component of the hexane extract from the plant *Solanum nudum* (Solanaceae). The products from degraded and acetylated diosgenone that showed *in vitro* antimalarial activity against the FCB-2 strain of *Plasmodium falciparum* and methanol, dichloromethane and ethereal extracts of *Solanum nudum* were tested for their mutagenic activity using the Ames test with the TA-97a, TA-98, TA-100 and TA-102 strains of *Salmonella typhimurium*. These compounds were not mutagenic at the tested concentrations.

1. Introduction

The lack of therapeutic options, the increased resistance of *P. falciparum* against the available drugs [1–6] and the their mutagenicity [7–15], are important reasons to promote the search of new antimalarial drugs [16].
We have studied several plants used as antimalarials by traditional healers; one of them is *Solanum nudum* (Solanaceae), which is used in Tumaco (Colombian Pacific coast) [17–22]. In previous work, products from degraded and acetylated diosgenone obtained from stems and leaves of this plant (Scheme), showed significant *in vitro* antimalarial activity against *Plasmodium falciparum* strain FCB-2 (parasitemia of $1.15 \pm 0.87\%$ in comparison to 2.13 ± 0.34 observed in negative controls) [17]. In these experiments, IC_{50} of 21,8 μM (9 ppm) and high inverse correlation between parasitemia and concentration were seen (parasitemia $= 0.306\% - [0.151$ mg/100 ml $\times$ concentration]) [17]. Furthermore, compounds obtained from this plant have shown antimalarial activity [19, 22].
This paper reports on the testing of the mutagenic activity of products from degraded and acetylated diosgenone and methanol, dichloromethane, and ethereal extracts of *Solanum nudum* by the Ames test.

2. Investigations, results and discussion

Histidine$^+$ mutation mean produced by each concentration of the treatment with and without metabolic activation, standard deviations and controls are shown in Table 1. Results of linear regression and Kruskall-Wallis analysis are shown in Tables 2 and 3. According to these data, we concluded that neither the extracts nor the products from degraded diosgenone are direct or indirect mutagens, since no relationship was found between the dose- and the re-

Scheme

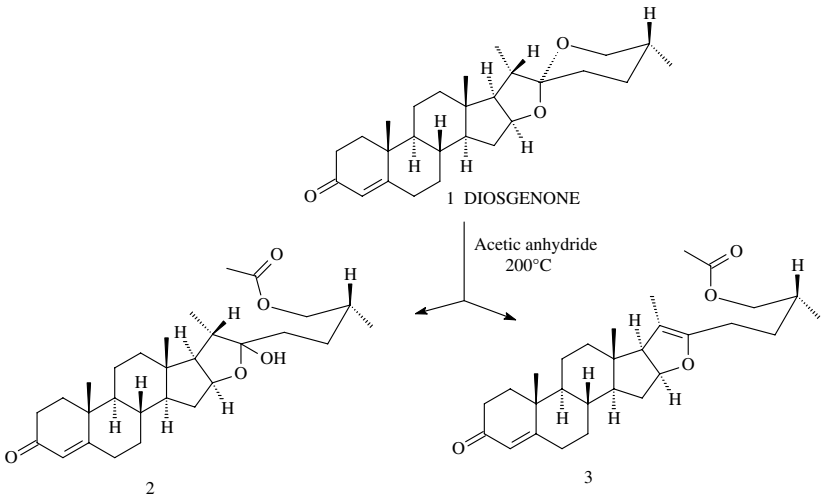

Table 1: Number of histidine$^+$ mutants/concentration of products from degraded and acetylated diosgenone and of three extracts of *Solanum nudum*. Experiments with and without metabolic activation

Substances	Dose mg/plate	Average of His$^+$ revertans/plate															
		TA-97				TA-98				TA-100				TA-102			
		+S9		−S9		+S9		−S9		+S9		−S9		+S9		−S9	
		X	±	X	±	X	±	X	±	X	±	X	±	X	±	X	±
Products from	6×10^{-4}	151	22	77	10	51	4	21	2	49	11	35	16	129	20	62	6
degraded and	1.2×10^{-3}	161	13	72	12	52	5	18	4	53	13	28	7	129	23	62	6
acetylated	2.4×10^{-3}	135	5	84	14	50	9	25	3	53	7	33	15	113	6	66	14
diosgenone	4.8×10^{-3}	153	18	80	5	49	6	29	6	48	15	29	11	116	10	67	14
	9.4×10^{-3}	122	12	78	15	43	2	22	4	56	10	25	7	116	18	59	17
Water	0.1 ml	120	14	91	10	55	7	27	9	45	16	28	7	102	6	55	6
2-Aminofluorene	1×10^{-2}	941	277	70	12	2715	21	55	12	857	89	30	6	186	36	55	13
Methanol extract	0.078	115	34	71	8	21	9	14	5	74	6	63	9	129	30	70	9
	0.156	104	20	67	15	20	9	12	5	74	9	63	9	149	38	65	8
	0.312	125	22	74	13	15	9	13	6	80	14	58	9	151	11	65	10
	0.625	134	13	83	17	19	9	16	10	79	11	61	10	146	14	72	11
	1.25	156	27	79	21	18	13	12	11	77	13	61	9	143	13	76	5
DMSO 50%	0.1 ml	120	23	76	7	18	10	18	1	77	6	63	9	129	22	80	14
2-Aminofluorene	1×10^{-2}	1062	210	74	4	1991	322	97	47	988	67	71	11	217	42	91	15
Dichloromethane	0.078	140	9	88	15	36	18	19	4	55	9	56	11	191	15	77	8
extract	0.156	131	8	78	8	36	15	20	6	61	12	52	11	102	5	71	13
	0.312	131	15	83	6	46	5	24	4	64	12	49	1	109	4	68	5
	0.625	132	7	94	6	35	12	19	3	58	7	51	12	110	7	74	6
	1.25	119	10	88	11	42	11	20	4	50	16	53	10	109	8	75	15
DMSO 75%	0.1 ml	127	18	86	6	35	15	25	5	56	11	47	9	148	35	78	8
2-Aminofluorene	1×10^{-2}	699	109	88	17	3532	838	29	1	889	110	53	7	166	31	146	90
Ethereal extract	6×10^{-4}	101	27	48	19	36	20	15	8	57	22	29	7	264	28	129	14
	1.2×10^{-3}	106	15	50	17	38	22	19	11	63	23	32	5	297	38	132	18
	2.4×10^{-3}	93	19	55	27	38	18	21	8	66	24	33	14	285	7	134	17
	4.8×10^{-3}	98	28	52	25	37	13	22	9	52	6	34	10	287	28	143	11
	9.7×10^{-3}	92	24	53	31	35	15	17	6	65	24	38	8	269	34	152	26
Water	0.1 ml	140	36	100	34	43	13	18	10	50	11	37	17	233	24	111	37
2-Aminofluorene	1×10^{-2}	1043	343	80	18	2744	431	38	14	1050	238	41	13	104	20	117	10

+S9: experiments in presence of S9 fraction; −S9, experiments without S9 fraction; X: average of mutants His$^+$/plate; ±: standard deviation; PVP: polyvinyl; 2AF: 2 aminofluorene (10 μg/plate)

Table 2: Covariance correlation of the number of revertants and the dose of products from degraded and acetylated diosgenone or the extracts of *Solanum nudum*

Substances	Strains	r and r^2 coefficients			
		With S9		Without S9	
		r	r^2	r	r^2
Products from degraded	TA-97	−0.525673	27.63%	0.072792	0.53%
and acetylated diosgenone	TA-98	−0.523639	27.42%	0.249865	6.24%
	TA-100	0.150381	2.26%	−0.251521	6.33%
	TA-102	−0.0850676	0.72%	0.070035	0.49%
Methanol extract	TA-97	0.593411	35.21%	0.258458	6.68%
	TA-98	−0.0774759	0.60%	−0.000330	0.00%
	TA-100	−0.166071	2.76%	0.713598	50.92%
	TA-102	0.0499758	0.25%	0.389059	15.14%
Dichloromethane extract	TA-97	−0.508719	25.88%	0.22451	5.04%
	TA-98	0.0985677	0.97%	0.002222	0.00%
	TA-100	−0.135655	1.84%	−0.010202	0.01%
	TA-102	−0.107336	1.15%	0.174384	3.04%
Ethereal extract	TA-97	−0.164116	2.69%	0.0510041	0.26%
	TA-98	−0.0524367	0.27%	−0.0031025	0.00%
	TA-100	0.0360017	0.13%	0.303551	9.21%
	TA-102	−0.0876159	0.77%	0.446539	19.94%

Table 3: Statistical analysis of variance (ANOVA) using the Kruskal Wallis test for the mean of revertants according to dose of products from degraded and acetylated diosgenone or the extracts of *Solanum nudum*

Substances	Strains	Anova			
		With S9		Without S9	
		K-W	P	K-W	P
Products from degraded and acetylated diosgenone	TA-97	15.142	**0.004416**	2.402	0.662340
	TA-98	9.180	0.056760	12.929	**0.011628**
	TA-100	2.221	0.695147	1.778	0.776459
	TA-102	12.705	0.176396	8.110	0.523122
Methanol extract	TA-97	11.190	**0.024511**	5.109	0.276271
	TA-98	3.175	0.528910	3.295	0.509723
	TA-100	7.959	0.538270	23.074	**0.006032**
	TA-102	3.334	0.503557	6.872	0.142822
Dichloromethane extract	TA-97	8.886	0.064024	8.331	0.080174
	TA-98	2.543	0.636972	6.197	0.184901
	TA-100	7.460	0.188632	1.953	0.855638
	TA-102	15.381	**0.008854**	4.476	0.483072
Ethereal extract	TA-97	1.543	0.818938	0.366	0.985128
	TA-98	0.379	0.984166	3.753	0.440425
	TA-100	1.624	0.804516	1.609	0.807247
	TA-102	3.482	0.480637	5.634	0.228197

sponse. The values of the coefficient of determination r^2 were very low, with a maximum of 35.21 for the TA-97a strain treated with the methanol extract. This value indicates that less than 36% of the results could be explained in function of the dose (Table 2). According to the approach of positive if a "two fold or more increment", a mutagenic positive answer never occurred, as it can be seen in Table 1. In addition, *p* values associated to *F* test were >0.05 for all the strains assayed, except in TA-97a strain with the degraded diosgenone and the methanol extract (Table 3).

This work demonstrated that the number of spontaneous revertants observed in negative controls (water and DMSO) of *Salmonella typhimurium* strains was below the established range for the Ames test [23–24]. This reduction of spontaneous revertants was not caused by loss of the R-factor plasmid since bacteria used to prepare culture broth were obtained from master plates that contained 25 μg/ml of ampicillin per plate, and 2 μg/ml ampicillin were added to vials for freezing [24]. Similarly, decrease of spontaneous mutants was not due to a lower bacterial density since 0.1 ml of bacterial culture with an optical density 0.454–0.560 at a 650 nm wavelength was always used [23]. Histidine availability in plates was not responsible for the finding since reagents were always prepared strictly following the Ames protocol. Therefore, the decrease of spontaneous mutants may have been caused due to particular variations of the conditions of our laboratory. Although it is certain that the quantity of bacteria was the recommended, bacteria viability could not be guaranteed because there was no control on this variable. On the other hand, strains with 2-aminofluorene as positive controls responded appropriately, which indicates that reagents and cofactors used to induce metabolic activation were adequate. As a consequence of these indicators, values obtained in the negative control were accepted. The quantitative changes are less important since the overall performance of the test should not be altered, on the contrary, factors affecting qualitatively the results will lead to reduced test sensitivity and predictivity [26]. Therefore, we consider that the situation affects equally experimental and negative controls groups, all groups are comparable and the differences among them cannot be explained by a reduced number of spontaneous mutants.

When the results obtained with the ethereal extract were evaluated with the S*almonella typhimurium* TA-97a strain without metabolic activation, it was observed that all the revertion mean values at the different dose assayed were half of the mean observed in the negative control. We do not believe that this result was due to a toxic effect of the extract because toxicity was eliminated when the concentrations were selected to carry out the mutagenicity assays with the various strains. There is no clear explanation for this result; it could be a random observation, since the ethereal extract did not behave in the same manner with the TA-98 strain, which is also sensitive to detection of frameshift mutagens. It is important to highlight that the extract with antimalarial activity was not mutagenic in this assay.

Qadri et al., found that steroidal-oxathiolane, -thiones and disulfone presented a marked mutagenic activity on strains TA-102 and TA-104 of *Salmonella typhimurium*, with and without metabolic activation. Mutagenic activity of these steroids is probably due to a substitute group donor of electrons in position 3 and to a tyol-type heavy group in positions 5 and 6 [27]. These two groups substitute electron-donor and tyol are responsible for interaction with some reactive species to form reactive-oxygen species. This explanation would validate the fact that an increase in the number of oxygen atoms increases mutagenic activity of steroids.

Compounds 2 and 3 from degradation and acetylation of diosgenone are steroids with an electronegative group in position 3 that lacks a substitute heavy group in positions 5 and 6. Absence of mutagenic activity of these compounds in the TA-102 strain, may be due to the fact that they are stable molecules because of their αβ-insaturated connection that binds the ketone group to the structure. Compound 2 presents an OH group in position 22. This group is electronegative and a very unstable species which might have been neutralized by the adjacent hydrogen to

form water. But the absence of mutagenic activity in all the compounds may have also been due to the fact that they need a different kind of metabolism from that produced by microsomal enzymes contained in the S9 mix.

Absence of mutagenicity of methanol, dichloromethane and ethereal extracts does not mean that these raw extracts are free from natural mutagens, since extracts are a mixture of components that show similar polarity in the extraction process. Furthermore, demonstration of mutagenic activity of natural products depends on solubility and concentration of the mutagenic compound, extraction method, type of plant material, storage conditions, and method applied to detect the mutagenic potential. Similarly, quantity and composition of substances that could be accompanying a natural mutagen can mask or modify their mutagenic activity, and other aspects such as toxicity of the extract.

According to the two criteria generally considered to define positive mutagenic activity of several compounds when using the Ames test, the products from degraded and acetylated diosgenone and the three extracts, were not mutagenic. However, the most refined statistical analysis showed a significant difference in the mean of revertants dependant upon the doses of the methanol extract assayed with the TA-97a strain. Based on this, we included statistical tests within the established criteria to define the mutagenic activity to various compounds, especially when the results were negative.

Although determination of mutagenic activity of raw extracts isolated from plants is limited, it is important to carry out these tests. In Tumaco, native healers prepare plant infusions for treatment of febrile patients or they use them to prevent illness such as malaria. These studies serve the purpose of screening and testing the mutagenic potential components of *Solanum nudum* before carrying out *in vivo* assays for antimalarial activity.

3. Experimental

3.1. Reagents

Sigma 2-aminofluorene (2-AF), aroclor 1254, glucose 6-phosphate, D-biotin, and L-histidine Oxoid nutrient broth and bacteriological agar were used.

3.2. Plant material

Stems and leaves of *Solanum nudum* were collected in the Colombian Pacific coast (78°,30′ log.W;1°43′15″ lat. N. 15 meters above sea level). Universidad de Antioquia Herbarium stores a sample under the registration number 554 which is classified it as *Solanum nudum,* Solanaceae family, common name: "zapata". The New York herbarium confirmed the identification of the species. In this Colombian region, the plant is used by native healers as treatment against malaria. The collected material was dried in an oven at 40 °C for 24 h and then ground before the extraction process.

3.3. Extract preparation

Dried and ground material was extracted by percolation with hexane during eight days at room temperature. The mixture was filtered and vacuum concentrated in a evaporator in order to extract the low polarity active principles, such as fats and terpenoids; this procedure allowed us to obtain an hexane extract and a residue 1. Fat extraction was carried out with ether, and processed as before to obtain an ether extract [21].

Fat-free plant material (residue 1) was dried at room temperature and mixed again with dichloromethane in a percolator for seven days. This dichloromethane solution was then filtered and vacuum concentrated in an evaporator to obtain an extract with medium polarity compounds (dichloromethane extract) and a residue 2. The extraction process was repeated with the residue 2 using methanol, obtaining several high polarity compounds (methanol extract) [21]. Both, the methanol and dichloromethane extracts, were completely solubilised in dimethylsulfoxide (DMSO) at 50% and 75%, respectively. The ether extract, which was non-soluble at non-toxic concentrations of DMSO, was treated with polyvinyl-pyrrolidone m.w. 10000 (PVP-10) to obtain a water soluble copolymer. This copolymer was formed by dissolving one part of the ether extract and four parts of PVP-10. This mixture of PVP-10 and the extract dissolved in ether, were homogenized and evaporated, which resulted in a aqueous ether extract.

Table 4: Concentrations of the compounds tested

Compounds	Concentrations (mg/plato)
Acetylated diosgenone	0.0094, 0.0048, 0.0024, 0.0012, 0.0006
Methanol extract	1.25, 0.625, 0.312, 0.156, 0.078
Dichlorometane extract	1.25, 0.625, 0.312, 0.156, 0.078
Copolymer PVP- hexane extract	0.00975, 0.0048, 0.0024, 0.0012, 0.0006

3.4. Diosgenone preparation

Diosgenone was isolated from the hexane extract of *Solanum nudum*. The molecular formula of this steroid is $C_{27}H_{40}O_3$ (25R)-spirost-4ene-3-one [19, 20]. The concentrate obtained underwent separation in a silica gel and hexane/ ethyl acetate (8 : 2) chromatography column. The fractions obtained were further processed by TLC, samples were revealed with 50% sulfuric acid and ultraviolet light. Similar fractions were grouped and diosgenone was obtained. This compound crystallized in hexane/ethyl acetate [18]. Since diosgenone was insoluble even after treatment with PVP-10, ethanol, DMSO and Tween-80, a degradation process with acetic anhydride was carried out. This was as follows: 200 mg of diosgenone (**1**) were dissolved in 2 ml of acetic anhydride. Then, the solution was heated at 200 °C for 6 h in a sealed tube. The solution was cooled and diluted in 50 ml acidulated water (HCl 2%) to hydrolyze the acetic anhydride excess. Next, the solution was extracted 3 times with 50 ml of dichloromethane. This organic solution of dichloromethane was rinsed with water and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure and the crude product, was purified by column chromatography (silica gel eluted with hexane/ethyl acetate 8 : 2). This allowed the isolation of most of component **2** which signaled in the ^{13}C NMR spectrum at 106.9 ppm and 68.4 ppm. This corresponds to a quaternary carbon of furostanol type, assigned to the carbon C-22 and a carbon type CH_2-O corresponding to the C-26 ($C_{29}H_{44}O_5$), which was predominant (90%). Two signals on the low intensity spectrum of 109.5 and 153 ppm were also observed. This indicated the existence of a second compound with a double bond $\Delta^{20,22}$ ($C_{29}H_{42}O_4$). Compounds **2** and **3** were obtained as a mixture [17]. A water-soluble copolymer was formed with the mixture and PVP-10, and this was evaluated for its antimalarial activity and mutagenicity.

3.5. Concentrations of extracts and degraded diosgenone

Different concentrations of the extracts and degraded diosgenone were selected to be tested for their mutagenicity considering the bacteria viability in nutritive medium (the maximum non-toxic concentration). The non-toxic concentration was selected after strain TA-100 of *Salmonella typhimurium* was cultured and prepared at $1-2 \times 10^9$ colony forming units/ml. A dilution of bacteria (1 : 262144) was prepared and placed in contact with a series of two-fold dilutions of each extract or degraded and acetylated diosgenone. Nutritive agar plates without the extract or diosgenone were used as negative control. The Ames test was always performed with 1/10 of the non-toxic concentration. Concentration as shown in Table 4 were selected to evaluate the mutagenicity activity.

3.6. Ames test

The test was made following the protocol described by Maron and Ames, [23] and Mortelmans and Zeiger [24]. TA-97a, TA-98, TA-100 and TA-102 strains were donated by Dr. Bruce S. Ames (University of California at Berkeley). Different *Salmonella typhimurium* strains were used taking into consideration that each of them can detect different types of mutagens. TA-97a and TA-98 strains detect various frameshif mutagens while TA-100 strain detect mutagens that cause base-pair substitutions and TA-102 detect mutagens that cause transitions/transversions and oxidative damage. Furthermore, all these strains have other genetic markers, which make them highly sensitive to fixed mutations. Prior to the experimental phase, presence of the *Salmonella* genetic markers (*uvrB, rfa* and pKM101, pAQ1 plasmid) was confirmed for every strain.

The experimental procedure was as follows: 0.1 ml bacteria culture (16-h incubation and optical density 0.454–0.560 at a 650 nm), 0.1 ml of each concentration of the degraded diosgenone or extracts (filtered in membrane of 0.22 μm) and 0.5 ml phosphate-buffered solution (PBS) were used. During the metabolic activation phase, 0.5 ml of the S9 mix was added instead of PBS. The positive control was 2-aminoflourene and the negative control was solvent. This mixture was incubated for 20 min at 37 °C without shaking. Then, 3 ml of top agar melted at 45 °C (supplemented with 0.6%

NaCl, 0.05 mM of L-histidine and D-biotin) were added. Later, the content of the glass tube was mixed and poured into a Petri dish that contained approximately 30 ml of glucose minimal agar medium consisting of Vogel-Bonner E medium (V/B salts) supplemented with glucose (2% w/v) and 1.5% Oxoid bacteriological agar. Plates were incubated at 37 °C for 48 h.
To induce metabolic activation of the studied compounds, we used microsomal enzymes contained in the supernatant or the S9 fraction of liver of *Rattus rattus* induced with Aroclor 1254. This supernatant was obtained through centrifugation of homogenized liver at $9000 \times g$; a few minutes before using the S9 mix, cofactors NADP, glucose 6-phosphate, buffered $MgCl_2-KCl$ (pH 7.2) were added.
Three independent trials in duplicate were performed under the same experimental conditions for all the five concentrations of the degraded diosgenone and the three extracts, and for each strain. Experiments with and without the S9 mix were carried out for a total of 6 evaluations per dose.
We considered as a positive mutation when the number of reversions his^- to his^+ showed a dose-effect relationship reproducible or when the number of revertans had a two-fold increment when compared to negative controls in every plate [23–25].

3.7. Statistical analysis

Mean number of revertants on the treatment group was compared with controls using the *t* test.
Linear regression analysis was used to evaluate the variation among the number of mutants or strains which reverted the mutation, and the dose of the treatment administered to each *Salmonella* strain, with and without metabolic activation.
Kruskal-Wallis test was performed to compare the mean of mutations of *Salmonella* strains exposed to different concentrations of treatment with the degraded diosgenone or extracts.

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