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The mutagenic potential of *Clusia alata* (Clusiaceae) extract based on two short-term *in vivo* assays

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ABSTRACT. We examined the genotoxic and mutagenic effects of a crude extract of *Clusia alata* (a potential medicinal plant) on peripheral leukocyte and bone marrow cells of mice, using the comet and chromosome aberration assays. Extracts at doses of 1000, 1500 and 2000 mg/kg were administered by gavage, and a positive control, N-nitroso-N-ethylurea (50 mg/kg) was injected intraperitoneally. Peripheral blood leukocytes were collected 4 and 24 h after the treatments for the comet assay, and bone marrow cells were collected 24 h after the treatments, for the chromosome aberration assay. The comet assay showed that *C. alata* extract causes an increase in damage to DNA in the peripheral blood leukocytes, but it was significant only with the 2000 mg/kg dose after 24 h; the extract also induced a small but significant increase in the mean number of chromosome aberrations in the bone marrow cells at doses of 1500 and 2000 mg/kg. No evidence of a significant decrease

in the mitotic index was observed. Acute consumption of high concentrations of *C. alata* extract produced some mutagenic effects in bone marrow cells.

Key words: *Clusia alata*; Comet assay; Chromosome aberrations; Single-cell gel electrophoresis

INTRODUCTION

Drugs derived from unmodified natural products or semi-synthetic drugs obtained from natural sources account for about 80% of all new drugs approved by the FDA between 1983 and 1994 (Cragg et al., 1997). This evidence supports and quantifies the importance of screening natural products.

The genus *Clusia* comprises about 250 species, which occur in tropical and subtropical regions of South and Central America. Its species occur in the form of shrubs, vines and small- to medium-sized trees up to 20 m tall, with evergreen foliage. Some species start life as epiphytes, then develop long roots that descend to the ground and eventually strangle and kill the host tree, in a manner similar to banyans. The species of this genus produce a large amount of latex, which is rich in polyprenylated benzophenones (Cerrini et al., 1993). These substances exhibit a wide range of significant biological and pharmacological activities, e.g., anti-inflammatory, antimicrobial (Iinuma et al., 1996), antifungal and anti-HIV activity (Gustafson et al., 1992). Bees that pollinate these flowers collect and use the resin for nest building. Resin secretion in flowers may have originated as a defense mechanism against herbivores; and this resin can contain some toxic substances (Armbruster, 1984).

In view of the potential therapeutic use of *Clusia* extracts and the absence of any data on its genetic toxicity in eukaryotes, we examined the *in vivo* genotoxic and mutagenic effects of *Clusia alata* crude extract, in terms of DNA damage in peripheral blood leukocytes and the induction of chromosome aberrations in bone marrow cells of mice.

MATERIAL AND METHODS

Plant material

Dried leaves of *C. alata* (300 g) were submitted to dynamic maceration with ethanol (2000 mL, 99.0%) for 4 h. The macerate was filtered and the procedure repeated. Concentration of the extract under reduced pressure gave 27.84 g (yield 9.28%) of crude ethanol extract.

Chemicals

N-nitroso-N-ethylurea (ENU, CAS No. 759-73-9) was used as the DNA damaging agent in comet and chromosome aberration assays and was dissolved in phosphate buffer, pH 6. The other main chemicals were obtained from the following suppliers: normal melting point agarose (Cat. No. 15510-019; Invitrogen); low melting point agarose (Cat. No. 15517-014; Invitrogen); sodium salt *N*-lauroyl sarcosine (L-5125; Sigma) and ethylenediaminetetraacetic acid (EDTA; Merck).

Animals and assay procedures

The experiments were carried out in 12-week-old male Swiss albino mice (*Mus musculus*), weighing 25-30 g. The animals were acquired from the animal house of the Universidade Estadual Paulista (UNESP), Botucatu, State of São Paulo, Brazil, and kept in polyethylene boxes (N = 6), in a climate-controlled environment (25 ± 4°C, 55 ± 5% humidity) with a 12-h light/dark cycle (7:00 am to 7:00 pm). Food (Nuvital CR1 - Nuvital) and water were available *ad libitum*. Animals were divided into two experimental groups of six animals each. *Clusia alata* extract was administered in a single dose of 0.5 mL by gavage, at concentrations of 1000, 1500, and 2000 mg/kg body weight, chosen on the basis of our acute toxicity studies in mice, which was above 2000 mg/kg. The negative control group received distilled water. The positive control group received an intraperitoneal injection of ENU at 0.05 g/kg.

The chromosome aberration (CA) assay was performed on bone marrow cells, according to recommendations of Preston et al. (1987a). Swiss mice were injected intraperitoneally with 0.4 mL 0.16% colchicine, 120 min before euthanasia, which occurred 24 h after the experimental treatment. Bone marrow cells were collected from one femur by flushing in KCl (0.075 M, at 37°C), and incubated at 37°C for 25 min. The material was centrifuged and the cell pellet was fixed in aceto-methanol (acetic-acid:methanol, 1:3, v/v). Centrifugation and fixation (in the cold) were repeated three times, and the final suspension was prepared in 0.5 mL fixative, dropped onto slides, flame dried, and stained the following day in 5% buffered Giemsa, pH 6.8. Cytogenetic analysis of the slides was performed with a light microscope using a 100X oil immersion lens. One hundred well-spread metaphases containing 40 ± 2 chromosomes were analyzed per animal in a blind test, making up a total of 600 metaphases per control and treatment group. CAs were classified according to Savage (1976) as gaps, breaks, deletions, fragments, rings, and dicentric chromosomes. Since the cytogenetic significance of the gaps is still not well established, the statistical analysis was carried out excluding gaps. The mitotic index (MI) was calculated for 1000 cells per animal, totaling 6000 cells per group. MI values are reported as percent in this study.

The comet assay (single-cell gel electrophoresis, SCGE) was carried out by the method described by Speit and Hartmann (1999), which is based on the original study of Singh et al. (1988) and includes modifications introduced by Klaude et al. (1996), with additional modifications. Four and 24 h after the treatment, peripheral blood leukocytes from Swiss mice were collected. A 10-µL aliquot of cells from each animal was mixed with 120 µL 0.5% low melting point agarose at 37°C, and rapidly spread onto microscope slides pre-coated with 1.5% normal melting point agarose. Coverslips were added and the slides were allowed to gel at 4°C for 20 min. The coverslips were gently removed and the slides were then immersed in cold, freshly prepared lysing solution consisting of 89 mL of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL distilled water and 1% sodium lauryl sarcosine), plus 1 mL Triton X-100 (Merck) and 10 mL dimethylsulfoxide (Merck). The slides were left to stand at 4°C, protected from light, for 1 h and then placed in the gel box, positioned at the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH per 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) at 4°C for 20 min prior to electrophoresis, to allow the DNA to unwind. The electrophoresis run was carried out in an ice bath (4°C) for 20 min at 300 mA and 25 V (0.722 V/cm). The slides were then submerged in a neutralization buffer (0.4 M Tris-HCl, pH 7.5)

for 15 min, dried at room temperature and fixed in 100% ethyl alcohol for 10 min. The slides were dried and stored overnight or longer, before staining. For the staining process, the slides were briefly rinsed in distilled water, covered with 30 µL 1X ethidium bromide staining solution prepared from a 10X stock (200 µg/mL) and covered with a coverslip. The material was evaluated immediately at 400X magnification, using a fluorescence microscope (Olympus BX 50) with a 515-560-nm excitation filter and a 590-nm barrier filter. The animals used in this study were sacrificed by cervical dislocation. The Animal Bioethics Committee of the UNESP, Botucatu, Brazil, approved the present study on September 28, 2006 (protocol number 47/06), in accordance with the Federal Government legislation on animal care.

Scoring procedures and data evaluation

The extent and distribution of DNA damage indicated by the SCGE assay were evaluated by examining at least 100 randomly selected and non-overlapping cells on the slides, per animal. These cells were scored visually, according to tail size, into four classes, as follows: 1) class 0: no tail; 2) class 1: tail shorter than the diameter of the head (nucleus); 3) class 2: tail length 1 to 2 times the diameter of the head, and 4) class 3: tail longer than 2 times the diameter of the head. Comets with no heads and images with nearly all DNA in the tail, or with a very wide tail, were excluded from the evaluation because they probably represent dead cells (Hartmann and Speit, 1997). The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 300 (all maximally damaged).

Statistical analysis

After verifying whether the data were normally distributed, the data obtained on chromosome aberration and SCGE assays were submitted to one-way analysis of variance (ANOVA) and the Tukey multiple comparison test (Sokal and Rohlf, 1995), using the GraphPad Instat® software (version 3.01). The results were considered to be statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

When conducting a thorough hazard characterization for a chemical, it is important to determine if that chemical can induce any of the various types of possible mutations. The different mutation hazard identification assays have different abilities to detect these changes (Moore and Harrington-Brock, 2000).

The ability of *C. alata* extract to induce genotoxicity and mutagenicity was assessed in our study using comet and CA assays, respectively. The *in vivo* comet assay (SCGE) is increasingly being used in genotoxicity testing. It can be used to detect DNA damage such as strand breaks, alkali-labile sites, DNA-DNA, and DNA-protein crosslinks (Brendler-Schwaab et al., 2005). Its advantages include applicability to various tissues and/or special cell types, its sensitivity for detecting low levels of DNA damage, and the requirement of small numbers of cells per sample, among others (Singh et al., 1988; Tice et al., 2000). This approach can detect primary DNA damage that does not necessarily reflect the adverse impact a chemical might cause to the cell, since this damage can also be repaired. The results of the *C. alata* extract by the comet assay, namely data on the total number of cells with damage and scores of mice treated with 1000, 1500, and 2000 mg/kg,

as well as the negative and positive controls (ENU, 50 mg/kg) are presented in Tables 1 and 2. As expected, ENU, the positive control, induced a significant increase in DNA migration in leukocytes ($P < 0.001$). No statistically significant difference between treated versus untreated animals was observed for *C. alata* extract at the most of the doses tested, or at both times of leukocyte collection (4 and 24 h) ($P > 0.05$). A statistically significant increase of total cells with DNA damage was observed only at the dose of 2000 mg/kg (collected after 24 h; $P < 0.05$). When exposed to three concentrations of the extract, most of the cells examined on the slides were undamaged, a few cells showed minor damage (class 1) and very few showed a large amount of damage (classes 2 and 3). Furthermore, there were no significant differences in DNA migration between the three extract concentrations tested and between the two times of leukocyte collection (4 h, before the DNA cell repair process, and 24 h, after the DNA repair process).

Table 1. DNA migration in the comet assay for the assessment of genotoxicity of *Clusia alata* extract in peripheral blood cells (collected 4 h after the treatment) from male (M) Swiss mice *in vivo*.

Treatments	Animals	Total[1]	Comet class				Scores
			0	1	2	3	
Control	M_1	15	85	14	1	0	16
	M_2	13	87	12	1	0	14
	M_3	9	91	8	1	0	10
	M_4	11	89	11	0	0	11
	M_5	11	89	9	2	0	13
	M_6	10	90	7	3	0	13
	Mean ± SD	11.5 ± 2.1					12.83 ± 2.13
Clusia alata extract	M_7	9	91	9	0	0	9
(1000 mg/kg)	M_8	7	93	7	0	0	7
	M_9	11	89	11	0	0	11
	M_{10}	10	90	10	0	0	10
	M_{11}	7	93	7	0	0	7
	M_{12}	9	91	9	0	0	9
	Mean ± SD	8.8 ± 1.6					8.83 ± 1.60
Clusia alata extract	M_{13}	15	85	14	1	0	16
(1500 mg/kg)	M_{14}	10	90	10	0	0	10
	M_{15}	11	89	9	2	0	13
	M_{16}	10	90	10	0	0	10
	M_{17}	12	88	11	1	0	13
	M_{18}	12	88	10	2	0	14
	Mean ± SD	11.6 ± 1.8					12.66 ± 2.33
Clusia alata extract	M_{19}	12	88	12	0	0	12
(2000 mg/kg)	M_{20}	14	86	14	0	0	14
	M_{21}	12	88	11	1	0	13
	M_{22}	16	84	16	0	0	16
	M_{23}	12	88	11	1	0	13
	M_{24}	9	91	9	0	0	9
	Mean ± SD SD	12.5 ± 2.3					12.83 ± 2.31
N-nitroso-N-ethylurea	M_{25}	66	34	64	2	0	68
(50 mg/kg)	M_{26}	58	42	57	1	0	59
	M_{27}	64	36	62	2	0	66
	M_{28}	70	30	68	2	0	72
	M_{29}	70	30	66	4	0	74
	M_{30}	56	44	54	2	0	58
	Mean ± SD	64.0 ± 5.9*					66.1 ± 6.58*

*Significantly different from the negative control ($P < 0.001$). [1]Total number of damaged cells (classes 1 + 2 + 3).

Table 2. DNA migration in the comet assay for the assessment of genotoxicity of *Clusia alata* extract in peripheral blood cells (collected 24 h after the treatment) from male (M) Swiss mice *in vivo*.

Treatments	Animals	Total[1]	Comet class				Scores
			0	1	2	3	
Control	M_1	5	95	5	0	0	5
	M_2	4	96	4	0	0	4
	M_3	5	95	5	0	0	5
	M_4	6	94	6	0	0	6
	M_5	10	90	10	0	0	10
	M_6	6	94	5	1	0	7
	Mean ± SD	6.0 ± 2.0					6.16 ± 2.13
Clusia alata extract	M_7	14	86	14	2	0	14
(1000 mg/kg)	M_8	9	91	9	0	0	9
	M_9	10	90	10	1	0	10
	M_{10}	10	90	9	1	0	11
	M_{11}	8	92	8	0	0	8
	M_{12}	11	89	11	1	0	11
	Mean ± SD	10.3 ± 2.0					10.5 ± 2.07
Clusia alata extract	M_{13}	6	94	5	1	0	7
(1500 mg/kg)	M_{14}	13	87	12	1	0	14
	M_{15}	8	92	6	1	1	8
	M_{16}	11	89	10	1	0	12
	M_{17}	9	91	9	0	0	9
	M_{18}	12	88	9	2	1	16
	Mean ± SD	9.8 ± 2.6					11.0 ± 3.57
Clusia alata extract	M_{19}	10	90	10	0	0	10
(2000 mg/kg)	M_{20}	7	93	7	0	0	7
	M_{21}	15	85	14	1	0	16
	M_{22}	13	87	11	2	0	15
	M_{23}	13	87	13	0	0	13
	M_{24}	15	85	15	0	0	15
	Mean ± SD	12.1 ± 3.1*					12.66 ± 3.50
N-nitroso-N-ethylurea	M_{25}	56	44	53	3	0	59
(50 mg/kg)	M_{26}	52	48	51	1	0	53
	M_{27}	63	37	62	1	0	64
	M_{28}	64	36	62	2	0	66
	M_{29}	62	38	57	4	1	68
	M_{30}	67	33	64	3	0	70
	Mean ± SD	60.6 ± 5.5**					63.33 ± 6.31**

*Significantly different from the negative control ($P < 0.05$). **Significantly different from the negative control ($P < 0.001$). [1]Total number of damaged cells (classes 1 + 2 + 3).

To obtain a parameter for comparison with the SCGE assay, we also applied a CA test. The mouse *in vivo* CA assay has been recognized as one of the sensitive endpoints to test the mutagenicity of chemicals and/or industrial pollutants (Preston et al., 1987a). Mutagenic effects occur when the test substance induces heritable mutations (damage that can pass to daughter somatic cells). Table 3 shows data on the induction of CAs in bone marrow cells of mice following *in vivo* exposure to different doses of *C. alata* extract. A single exposure in mice, of three high doses of the extract, enhanced the average number of CAs at metaphase at the three doses tested, being statistically significant at doses of 1500 and 2000 mg/kg. As expected, the animals treated with ENU had a significantly higher number of CAs. The CAs observed were chromatid and chromosome

breaks, gaps, deletions, acentric fragments, and ring chromosomes. Chromatid breaks were present at higher frequencies than in the other types, and were induced by all doses tested. Although the number of gaps at the three concentrations tested of the extract was increased when compared with the control group, the gaps were excluded from the statistical analysis since they are considered by some investigators to be of debatable genetic significance, since their presence does not always lead to CAs in the subsequent cell divisions (Preston et al., 1987a; Brusik, 1987).

Table 3. Mitotic index (MI) and distribution of the different types of chromosomal aberrations (CA) observed in male (M) Swiss mice leukocytes treated with a *Clusia alata* extract, and respective controls.

Treatments	Animal	MI (%)	Chromosomal aberrations				OA	Total CA without gaps	Cells with aberrations
			Gaps		Breaks				
			C	IC	C	IC			
Control	M_1	2.6	0	0	0	0	1f, 1r	2	2
	M_2	3.0	0	1	1	0	0	1	1
	M_3	2.9	0	1	0	0	1del	1	1
	M_4	2.6	0	0	0	0	1f	1	1
	M_5	3.6	0	0	1	0	0	1	1
	M_6	3.1	0	0	1	0	1r	2	2
	Mean ± SD	2.9 ± 0.3						1.33 ± 0.5	
Clusia alata extract	M_7	3.2	0	0	3	1	1f	5	5
(1000 mg/kg)	M_8	3.7	0	2	3	0	0	3	3
	M_9	3.2	0	2	1	0	1f	2	2
	M_{10}	4.1	1	0	2	0	1f	3	3
	M_{11}	3.2	1	0	2	0	1r, 1del	4	4
	M_{12}	3.6	1	0	2	0	1f	3	3
	Mean ± SD	3.5 ± 0.3						3.33 ± 1.0	
Clusia alata extract	M_{13}	3.6	0	0	2	0	1f, 1del	4	4
(1500 mg/kg)	M_{14}	3.1	1	0	2	0	1f, 1r	4	4
	M_{15}	4.2	2	0	2	0	1r, 1del	4	4
	M_{16}	3.9	0	1	2	1	1del	4	4
	M_{17}	3.6	2	0	2	0	1f, 1del	4	4
	M_{18}	3.2	1	1	2	0	1f	3	3
	Mean ± SD	3.6 ± 0.4						3.83 ± 0.4*	
Clusia alata extract	M_{19}	4.6	1	1	2	0	1f, 2del	5	5
(2000 mg/kg)	M_{20}	4.5	2	0	3	0	1f, 3del	7	7
	M_{21}	3.1	1	1	3	0	2del	5	5
	M_{22}	3.2	0	1	3	0	1r, 1del	5	5
	M_{23}	3.1	1	0	4	0	1f, 1del	6	6
	M_{24}	3.6	0	1	3	0	1f, 1del	5	5
	Mean ± SD	3.6 ± 0.6						5.5 ± 0.8**	
N-nitroso-N-ethylurea	M_{25}	3.2	3	1	12	0	1f, 3r, 6del	22	20
(50 mg/kg)	M_{26}	5.2	3	2	15	1	3f, 1r, 7del	27	27
	M_{27}	6.1	2	1	12	1	2f, 3r, 8del	26	26
	M_{28}	6.3	5	2	15	3	2f, 1r, 6del	27	27
	M_{29}	5.6	5	2	11	1	2f, 7del	21	21
	M_{30}	5.2	3	1	14	1	2f, 2r, 8del	27	26
	Mean ± SD	5.2 ± 1.1**						25.0 ± 2.7**	

*Significantly different from negative control ($P < 0.05$). **Significantly different from negative control ($P < 0.001$). One hundred cells were analyzed per animal, for a total of 600 cells per treatment. C = chromatid-type; IC = isochromatid-type; OA = other aberrations; del = deletion; f = acentric fragment; r = ring; SD = standard deviation.

The MI (used as a measure to evaluate cell cycle kinetics) values obtained from the analysis of 1000 cells/animal for a sample of 30 animals (N = 6/group) ranged from 3.1 to 4.6% (means) and have shown that the MI of bone marrow cells exposed to *Clusia* extract did not decrease, compared to those in the negative control indicating that the extract does not affect cell division.

There have been no previous studies regarding phytochemical composition specifically for the *C. alata* extract; however, these data do exist for several other *Clusia* species. de Oliveira et al. (1996) reported that the main components of the floral resins from five *Clusia* species are polyisoprenylated benzophenones, and found also as minor components, clusianone and three hitherto-unknown compounds, grandone, nemorosone, and hydroxylnemorosone. Lokvam et al. (2000) and Porto et al. (2000) analyzing phytochemically floral resins and staminal oils from about 16 *Clusia* species, observed that female and male floral resin chemistry does not diverge in the chemical structure of major components, such as fatty acids and polyisoprenylated benzophenones. Differences arise in the minor components and in the ratios between the major components, i.e., the chemical identity is maintained while the ratios may change between male and female resins of the same species. Some of these *Clusia* species present antibacterial activity (Lokvam and Braddock, 1999; Porto et al., 2000). Suffredini et al. (2006) observed that *Clusia columnaris* and other Clusiaceae have antibacterial and cytotoxic activity. A survey by Porto et al. (2000) on the bioactivity of all methylated and non-methylated resins and bee nest extract using bioautography (Betina, 1973) revealed that non-methylated polyisoprenylated benzophenones are largely responsible for the antimicrobial activity of pure floral resins and of the nest extract. The data obtained in the present study regarding the MI showed that the major components of the *C. alata* extract did not produce cytotoxic effects on the eukaryotic cells of the mice analyzed.

Our data obtained in this study enable us to conclude that *C. alata* crude extract has some mutagenic activity in mice bone marrow cells under the assay conditions. Since mutations are involved in the etiology of cancer due to an increased frequency of spontaneous mutation, we recommend, based on our results, that the consumption of high doses or the chronic use of *Clusia alata* crude extract be moderated until the development of other genotoxic and mutagenic tests can objectively determine their health risk for humans.

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