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The antigenotoxic activity of latex from *Himatanthus articulatus*

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Abstract: *Himatanthus articulatus* (Vahl) Woodson (Apocynaceae) is a native plant to the Amazon popularly used to treat ulcers, tumors, inflammations, cancer, syphilis and malaria. The aim of the present study was to investigate the *in vivo* genotoxic/antigenotoxic and mutagenic potential of this plant, using the comet and the micronucleus assays in mice. Female and male adult mice were treated with different doses of *H. articulatus* latex by gavage for two consecutive days. For the experiments, the latex was serially diluted with water to 1:2 (D1); 1:4 ($D^{1/2}$) and 1:8 ($D^{1/4}$) and administered to the animals. The blood slides were exposed to hydrogen peroxide (*ex vivo*) to evaluate antigenotoxic effect. Under the experimental conditions used in this study, the latex of *H. articulatus* did not increase the frequency of DNA damage as measured by the comet assay and micronucleus test in treated mice, indicating a non-genotoxic and non-mutagenic activity. In relation to the antigenotoxicity, latex exerted protective effect against DNA damage induced by hydrogen peroxide. Therefore, our results add new information about the antigenotoxic potential of *H. articulatus* latex, which is popularly used in the Amazon to treat different pathologies.

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Introduction

The biological properties of latex are always a mystery. Even in opium, the best known example of medicinal latex and the most powerful analgesic medicine, dual effects such as dependence and addiction have been observed concomitantly (Nicolaou & Montagnon, 2008). Several studies have been published about latex properties, while some papers have reported toxic effects such as keratoconjunctivitis (Shlamovitz et al., 2009), mutagenic action (Lopes et al., 2004), irritant activity to skin (Hohmann & Molnár, 2004), cytotoxicity (Wang et al., 2008) other papers present pharmacological, anti-ulcer (Bharti et al., 2010), antinociceptive (Soares et al., 2005), anti-inflammatory (Fernandez-Arche et al., 2010), and analgesic (Dewan et al., 2000) activities. The latex consumption is one of the innumerable ways that medicinal plants are used by the popular medicine in Brazil.

In South America, research has described the use of the latex from distinct species of the Apocynaceae genus, such as *Plumeria, Allamanda* and *Himatanthus*. The latex from *Himatanthus sucuuba* showed important biological properties such as anti-inflammatory and analgesic activities (De Miranda et al., 2000), apart from the role it plays in the treatment of tumors, ulcers, asthma and tuberculosis (Suffredini & Daly, 2004), as well as the antileishmanial activity (Soares et al., 2010). This genus is widely used in phytotherapeutic treatments in distinct South American countries (Milliken, 1997; Suffredini & Daly, 2004). The pure latex of *H. articulatus* or diluted in water is frequently consumed to treat antifungal and antibacterial infections (Sequeira et al., 2009), ulcers, tumors, inflammations and cancer (Coelho-Ferreira, 2009), syphilis (Barreto et al., 1998) and malaria (Milliken, 1997).

Several parts of this plant, commonly known as *sucuba*, are usually sold in stalls and herbal healers in the north, northeast and west-central Brazil (Pinto & Maduro, 2003). However, in spite of this popularity, only one phytochemical study has been published about extracts from the bark, leaves and latex of *H. articulatus*, which reported the isolation and identification of terpenes and iridoids (Barreto et al., 1998). Chemical compositions of latex from distinct *Himatanthus* species described by different studies are presented in Chart 1.

Considering the absence of studies on the toxic effects of this plant, the aim of the present study was to evaluate the genotoxic/antigenotoxic and mutagenic activities of latex from *H. articulatus,* using the comet assay in blood and the micronucleus assay in the bone marrow and blood of mice.

Chart 1: Chemical constituents from latex of *Himatanthus* species.

Species	Chemical isolate	Class	Author
H. articulatus	α-amyrin-3β-*O*-cinnamoyl, β-amyrin-3β-*O*-cinnamoyl, acetate of lupeol, cinnamate of lupeol, cycloartenol	Terpenes	Barreto et al., 1998
H. fallax	isoplumericin, plumericin, plumieride, pinoresinol matairesinol, 7(*R*)-methoxy-8-*epi*-matairesinol	Iridoids	Abdel-Kader, 1997
H. sucuuba	15-demethylisoplumieride acid, 15-demethylplumieride acid, plumieride, isoplumieride	Iridoids	Barreto et al., 2007
	15-demethylplumieride acid, plumieride, isoplumieride	Iridoids	Silva et al., 2007
	lupeol acetate, α-amyrin and lupeol cinnamates	Terpenes	De Miranda et al., 2000
	cis-polyisoprene, arabinose, glucose, xylose, rhamnose and galactose, Na, Mg, Al, K, Ca, Mn, Fe, Sr	Miscellaneous	Silva et al., 2003
	α-amyrin cinnamates, β- amyrin cinnamates, lupeol cinnamates, lupeol acetate	Terpenes	Silva, 2000
	plumericin, isoplumericin, plumieride, isoplumieride, 15-demethylisoplumieride, 15-demethylplumieride	Iridoids	Silva, 2000
	catechol, gallic acid, myricetrin, quercitrin	Phenolic compounds	Silva, 2000
H. bracteatus;	plumieride, isoplumieride	Iridoids	Ferreira et al., 2009
H. stenophyllus; H. sucuuba			

Materials and Methods

Plant material

Himatanthus articulatus (Vahl) Woodson, Apocynaceae, latex was collected from Campus Cauame (Monte Cristo), Federal University of Roraima (UFRR) in August, 2009 (GPS coordinates 2° 51'48"N, 60° 43'01" W). A voucher specimen was deposited at the Herbarium of the National Institute of Amazonian Research, INPA Manaus, Amazonas, Brazil, recorded under number 214743.

Preparation of samples

Due to the impossibility to administer the pure latex by gavage, it was mixed with water (1:1; v/v) and stored in a refrigerator at 4 °C. For the experiments, the latex was serially diluted with water to 1:2 (D1); 1:4 ($D^{1/2}$) and 1:8 ($D^{1/4}$) and administered to the animals.

Materials/chemicals and reagents

Cyclophosphamide (CP, Genuxal, Asta Medica, Lot. n.807034) was used as a positive control.

Animals and treatments

A total of fifty male and female mice (CF-1, 5 weeks old, 29.23±1.99 g) from our breeding colony, free of specific pathogens were used. Animals were housed in cages, received commercial standard mouse cube diet (Nuvilab, CR1, Moinho Nuvipal Ltda, Curitiba, PR, Brazil) and water ad libitum, under a 12-h light/dark cycle, temperature of 23±3 °C and relative humidity of about 60%. All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). This study was approved by an internal ethics committee animal experimentation of the Lutheran University of Brazil (CEP-ULBRA 2008-018A).

The animals were divided to form groups with ten individuals per group: (five male and five female), and treated by gavage with water (negative control) or latex (D1; $D^{1/2}$; $D^{1/4}$) and the positive control group (treated with cyclophosphamide by gavage 25 mg/kg). All animals received treatment for two consecutive days (OECD, 1997). For the comet assay, peripheral blood samples were obtained 3, 6 and 48 h after the beginning of the experiment from mouse tail tips (about 15 µL) by means of small incision and immediately mixed with heparin sodium (7 µL). The animals were sacrificed by cervical dislocation after 48 h, when blood and bone marrow were collected for comet assay and micronucleus test.

DNA damage was induced *ex vivo* by exposing the blood cells to H_2O_2 0.25 mM (0-250 µmol/L diluted in PBS) on slides. In order to establish the protective ability of the latex against the oxidative stress induced by H_2O_2, the comet assay was performed with all the animals pre-exposed to latex. The slides were exposed to H_2O_2 for 5 min (*ex vivo*) (Lovell & Omori, 2008). Antigenotoxic potential was expressed as described in Kapiszewska et al. (2005), as percentage inhibition of damage index (DI) according to the expression: (I%): percentage of inhibition of DI=[H_2O_2 DI mean-DI mean of the extract with H_2O_2]/

[H_2O_2 DI mean-DI mean negative control] x 100.

Comet assay

The alkaline comet assay was performed as described by Singh et al. (1988) and as modified in Da Silva et al. (2000). Cells were obtained according to the method described by Tice (1995), where whole heparinized blood was utilized, and bone marrow perfuse of femur was homogenized with fetal bovine serum. Cells isolated from tissues (10 µL) were embedded in 95 µL of 0.75% (w/v) low melting point agarose (Gibco BRL) and the mixture added to a microscope slide pre-coated with 1.5% (w/v) of normal melting point agarose and covered with a coverslip. All slides were prepared in quadruplicate. The slide was briefly placed on ice for agarose to solidify, and the coverslip carefully removed. Then, slides were exposed to either PBS (two slides) or 0.25 mM freshly prepared H_2O_2 solution (*ex vivo* treatment) for 5 min, at 4 °C (2 slides) (Lovell & Omori, 2008). Next, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0-10.5) containing freshly added 1% triton X-100 and 10% dimethyl sulfoxide (DMSO) for at least 1 h at 4 ºC.

Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH >13) for 20 min for DNA unwinding, and electrophoresed in the same buffer. The electrophoresis conditions were 15 min at 300 mA and 25 V (0.7 V/cm). All these steps were carried out under dim indirect light.

Following electrophoresis, the slides were neutralized in 400 mM Tris (pH 7.5) and fixed (15% w/v trichloroacetic acid, 5% w/v zinc sulfate, 5% glycerol), washed in distilled water and dried overnight. The gels were re-hydrated for 5 min in distilled water, and then stained for 15 min (37 °C) with a solution containing the following sequence: 34 mL of Solution B (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, 5% w/v sodium carbonate) and 66 mL of Solution A (5% sodium carbonate). The staining was stopped with 1% acetic acid and the gels were air-dried (Nadin et al., 2001) and analyzed using a microscope.

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal; all sides were coded for blind analysis (Lovell & Omori, 2008). Cells were also visually scored according to tail size into five classes ranging from undamaged (0) to maximally damaged (4), resulting in a single DNA damage score for each animal, and consequently to each studied group. Therefore, the damage index can range from 0 (completely undamaged, 100 cells x 0) to 400 (with maximum damage, 100 x 4). The frequency (DF in %) was calculated for each sample based on the number of cells with tail versus those without.

Micronucleus assay

The micronucleus assay was performed according to the US Environmental Protection Agency Gene-Tox Program (Mavournin et al., 1990) and as modified in Da Silva et al. (2000). Whole blood smears were prepared on slides after 48 h, when animals were killed by cervical dislocation.

The bone marrow was extracted from the two femurs. Smears were prepared directly on slides with bone marrow and blood, two per animal and per tissue. Bone marrow smear was prepared with a drop of fetal calf serum. The slides were stained with 5% Giemsa, air-dried and coded for blind analysis. To avoid false negative results and as a measure of toxicity in bone marrow, the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) was scored in 1,000 cells. The incidence of micronuclei (MN) was observed in 2,000 PCE and 2,000 peripheral blood reticulocytes (RET) for each animal (*i.e.* 1,000 from each of the two slides prepared from the duplicate), using bright-field optical microscopy under 200-1000× magnification. All sides were coded for blind analysis. The test groups were compared to the respective negative controls for gender, separately and in combination.

Data analysis

Results are expressed as means±SD and statistical significance between the five groups and gender was determined by One-Way Analysis of Variance (ANOVA, Kruskal-Wallis test). In all comparisons, $p<0.05$ was considered statistical significance.

Results

The comet assay data for genotoxicity at different exposure times (3, 6 and 48 h) per gender and per group are summarized in Table 1. No differences were observed for the different exposure times in comparison to negative control. Only the positive control presented differences in mean DI, with more damaged cells than the negative control ($p<0.05$, Kruskal-Wallis test). No significant differences were found between sex for different exposure groups and exposure times.

Results of the micronucleus test in peripheral blood and bone marrow cells collected after 48 h are shown in Table 2. The Kruskal-Wallis Test revealed that mean micronuclei frequency in peripheral blood and bone marrow cells was significantly higher only in the positive control group, when compared with the negative control. No differences in micronucleus frequency were observed

between groups in the blood and bone marrow tissues, neither in PCE/NCE ratio. No difference was observed between genders.

We also checked the antigenotoxicity activity after H_2O_2 challenge (Table 3). The data in Table 4 indicate that the intervention with latex significantly increased the ability to remove the DNA damage generated by H_2O_2 in blood cells isolated from animals treated with latex (D1: 100%) after 48 h. When antigenotoxic potential was expressed as described in Kapiszewska et al. (2005), the concentration of latex used for the 48-h treatment caused a significant reduction in DNA damage. In comparison to the 3-h treatment, a DNA damage modulation of 12.85 % was observed in the 3-h treatment, while for the 48-h treatment modulation was 79.02%.

Table 1. Cytogenetic parameter (mean±SD) for blood, samples of exposed animals to latex, cyclophosphamide (positive control) and water (negative control). In each group: n=10 (five male and five female), 100 cells/animal.

Groups	Gender	Comet assay parameters per exposition period					
		Damage Index (DI)			Damage Frequency (DF)		
		3 h	6 h	48 h	3 h	6 h	48 h
Negative Control	Male	6.67±1.53	10.00±2.65	7.20±3.27	6.67±1.53	9.00±2.00	6.40±2.97
	Female	5.00± 2.00	9.00± 6.08	10.60±1.14	4.67±1.53	6.67±3.79	9.20±0.84
	Group	5.83±1.83	9.50±4.23	8.90± 2.92	5.17±1.47	7.83±2.99	7.80±2.53
Latex D¼	Male	10.33±1.53	8.00±2.65	5.00±2.35	8.00±1.00	7.00±1.00	4.40±1.95
	Female	13.00±1.53	8.67±3.51	7.40±2.10	10.00±2.65	5.67±2.08	6.60±1.30
	Group	11.70±2.42	8.33±2.80	6.20± 2.45	9.00±2.10	6.33±1.63	5.50±2.38
Latex D½	Male	12.67±1.53	9.67±0.58	5.60±1.82	10.33±1.53	7.33±1.20	4.80±1.48
	Female	14.33±0.58	7.00±1.73	6.40± 0.55	12.67±0.58	5.00±1.00	5.60±0.89
	Group	13.50±1.38	8.33±1.86	6.00±1.33	11.50±1.64	6.20±2.00	5.20±1.23
Latex D1	Male	5.67±1.15	9.33±4.73	6.40±1.70	5.33±0.58	7.00±4.00	5.60±1.42
	Female	6.67±2.52	11.67±2.08	8.00±2.35	4.33±1.15	8.33±1.53	7.20±0.37
	Group	6.17±1.83	10.50±3.51	7.20±2.15	4.83±0.98	7.67±2.80	6.40±1.51
Positive Control	Male	53.67±5.51	49.00±14.73	40.60±8.08	29.33±1.53	34.00±6.24	32.80±6.30
	Female	58.33±2.08	60.00 ±16.46	32.20 ±9.44	35.00±9.64	40.33±5.86	25.40±2.70
	Group	56.00 ±4.52***	54.50±15.22*	36.40 ±9.40*	32.17 ±6.90***	37.17±6.43*	29.10±6.01*

*Significant in comparison to negative control in the same exposure time at $p<0.05$; ***$p<0.001$; by the Kruskal-Wallis test.

Table 2. Micronuclei mean (±SD) in peripheral blood reticulocytes (MNRET) and bone marrow polychromatic erythrocytes (MNPCE) of mice exposed to latex, cyclophosphamide (positive control) and water (negative control). In each group: n=10 (five male and five female), 2,000 cells/animal.

Group	Gender	MNRET		MNPCE		Ratio (PCE:NCE)[a]
		Per gender	Per group	Per gender	Per group	Per group
Negative Control[c]	Male	1.2±0.45	1.4±0.52	1.6±0.55	1.6±0.52	1.33±0.21
	Female	1.6±0.55		1.6±0.55		
Latex D¼	Male	1.4±0.89	1.4±1.07	2.0±0.71	2.1±0.74	1.45±0.34
	Female	1.4±1.34		2.2±0.84		
Latex D½	Male	1.4±0.89	1.7±1.10	1.6±0.55	1.7±0.48	1.71±0.52
	Female	2.0±1.22		1.8±0.45		
Latex D1	Male	2.8±0.45	2.6±0.52	1.2±0.84	1.4±0.70	1.42±0.71
	Female	2.4±0.55		1.6±0.55		
Positive Control[b]	Male	12.2±2.77	11.9±1.91***	17.8±4.66	16.2±4.21***	0.82±0.34
	Female	11.6±0.55		14.6±2.44		

***Significant in comparison to negative control in same tissue at $p<0.0001$; by the Kruskal-Wallis test.

Table 3. Comet assay in peripheral blood leukocytes of mice treated with latex (D1) with and without hydrogen peroxide (0.25 mM) (*ex vivo*). Values are expressed as mean±SD.

Groups	Time	Gender	Comet assay parameters							
			Damage Index (DI)				Damage Frequency (DF)			
			Without H_2O_2		With H_2O_2		Without H_2O_2		With H_2O_2	
			Per Gender	Per Group	Per Gender	Per Group	Per Gender	Per Group	Per Gender	Per Group
Control	3h	Male	3.60±2.79	3.0±2.67	147.40±18.09	142.20±17.70	3.20±2.28	2.80±2.39	63.80±11.39	64.10±9.55
		Female	2.40±2.70		137.00±17.61		2.40±2.70		64.40±8.68	
	48h	Male	5.80±3.27	4.10±3.21	136.00±33.53	127.90±24.60	3.80±1.92	3.00±2.16	62.00±15.13	59.00±17.69
		Female	2.40±2.30		119.80±8.56		2.20±2.28		56.00±21.27	
Latex D1	3h	Male	3.20±1.48	3.70±1.60	115.20±17.17	124.30±17.00	3.00±1.40	3.40±1.50	50.40±10.67	56.80±11.24
		Female	4.20±1.79		133.40±12.20		3.80±1.60		63.20±8.26	
	48h	Male	7.00±3.32	7.00±3.06	15.40±4.98	17.90±4.93 [a,b]	5.60±2.30	5.70±2.45	14.40±5.05	16.20±4.64[a,b]
		Female	7.00±3.16		20.40±3.78		5.80±2.86		18.40±3.29	

[a]Significant in comparison to control in the same exposure time at $p<0.001$; [b]Significant in comparison to latex (3 h) at $p<0.001$; by the Kruskal-Wallis test.

Discussion

In this study, genotoxic and antigenotoxic activity of *H. articulatus* latex was tested in blood cells, and its mutagenic effect was assessed in bone marrow and blood cells in mice. The comet assay was used to detect recent DNA damage, such as single and double strand breaks, alkali-labile sites, DNA–DNA and DNA–protein crosslinks (Singh et al., 1988; Tice, 1995), while the micronucleus test was used to detect clastogenic/aneugenic activities, which leads to increasing micronucleus frequency, suggesting the occurrence of mutagenic effects at chromosomal level (Mavournin et al., 1990). The results obtained in this study showed no mutagenic nor genotoxic effects of the latex of *H. articulatus* (Table 2 and 3). Similarly, a study on latex of "Sangre de Drago", a red viscous latex extracted from *Croton lechleri*, Euphorbiaceae, cortex, no mutagenic activity was observed by means of the Ames/Salmonella test, on the *Salmonella typhimurium* strains T98 and T100, either with or without S9 activation (Rossi et al., 2003). Paiva et al. (2011) using several experimental methods to evaluate the mutagenic and genotoxic effects of *Euphorbia tirucalli* latex preparations, such as inductest, the Ames/Salmonella test and the chromotest, showed that no mutagenic or genotoxic damages were induced by all samples studied. Another aspect that is in accordance with the low toxicity found is the high occurrence of iridoids such as plumericin in the latex of distinct *Himatanthus* species. This substance was shown to be weakly cytotoxic against Sc-7 strain of *Saccharomyces cerevisiae* (Abdel-Kader et al., 1997), an observation also reported by Aponte et al. (2009) against several mammalian cancer cell lines. No data on assessments of the genotoxicity or damage effects of different species of *Himatanthus* was found in the literature.

The putative antigenotoxic effect of latex was investigated in mice blood cells, *ex vivo*, before and after a hydrogen peroxide (H_2O_2) treatment by the comet assay. Hydrogen peroxide causes DNA strand breakage by generating hydroxyl radicals close to the DNA molecule. Thus, antioxidant activity is assessed as the decreased induction of DNA breaks. Our results (Table 4) showed that animals treated with latex demonstrated an increase in cell resistance to DNA damage caused by H_2O_2-induced DNA strand breaks in blood cells, irrespective of the dose ingested (48 h > 3 h).

The phytochemical composition of this latex can help understand this antigenotoxic effect in greater depth. The presence of terpenes and iridoids has been reported to occur in the latex of distinct *Himatanthus* species (Table 1). Terpenes such as lupeol derivatives are reported to afford a hepatoprotective effect against the toxic damages induced by cadmium. Metals like cadmium are known to induce lipid peroxidation (Sunitha et al., 2001). In addition, lupeol derivatives are believed to function by modulating processes associated with xenobiotic biotransformation, with the protection of cellular elements from oxidative damage, or with the promotion of a more differentiated phenotype in target cells (Nigam et al., 2007). It has been reported that lupeol and its derivatives can be exploited as prospective targets for development of novel chemotherapeutic agents for the prevention and/or treatment of cancer (Chaturvedi et al., 2008). The amyrin derivatives are other terpenic constituents of the latex of *H. articulatus* whose protective effects against damage induced by different compounds, like tetrachloromethane (Aimin et al., 1998), acetaminophen (Oliveira et al., 2005), and 12-*O*-tetradecanoylphorbol-acetate (Medeiros et al., 2007).

In a previous study, latex components such as iridoids (catalpol) were described to present

hepatoprotective and anti-inflammatory properties (Dinda et al., 2009). This product was able to protect cell viability and reduce intracellular reactive oxygen species (ROS) formation in astrocytes from oxidant stress induced by H_2O_2, and may be an attractive candidate for the treatment of various neurodegenerative disorders associated with oxidative stress (Bi et al., 2008). Aucubin is another iridoid that was shown to have protective effects. Jin et al. (2008) demonstrated that this iridoid was able to control hyperglycemia and exert antioxidant protection of pancreatic β cell, against damage induced by streptozootocin, being appointed by the authors as a preventive agent against diabetes mellitus. The pre-treatment with aucubin in human skin fibroblast cell lines showed a significant inhibition of ROS formation and malondialdehyde levels and increased cell viability and glutathione after exposure to UVB radiation (Ho et al., 2005).

Finally, it is well described that the presence of phenolic compounds in plant extracts contributes significantly to their antioxidant potential (Dudonné et al., 2009). For this reason, we also cannot rule out the possibility that the observed protection may be related to the presence of phenolic compounds (gallic acid, catechol, myricetrin and quercitrin) described by Silva (2000).

Conclusions

Under the experimental conditions used in this study, the latex of *H. articulatus* did not increase the frequency of DNA damage as measured by the comet assay and micronucleus test in treated mice, indicating a non-genotoxic and non-mutagenic activity. In relation to the antigenotoxicity, latex exerted protective effect against DNA damage induced by hydrogen peroxide. Therefore, our results add new information about the antigenotoxic potential of *H. articulatus* latex, which is popularly used in the Amazon to treat different pathologies.

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