Cytogenetic evaluation of α, β-amirina obtained in resin of *Protium heptaphylum* (Aubl.) Marchand in polychromatics erythrocytes of mice swiss.

Lucas Rodrigues do Rego*[a,c], Everton Pantoja Vale[a,d,e], Danilo Dheyvison Nascimento Pureza[a,b] , Fernando Antônio de Medeiros[a,b,c] and Moacir de Azevedo Bentes Monteiro Neto[a,b,c].

[a]Genetic and Biomolecular Research Group (GPCBIO), Department of Biological Sciences and Health, Federal University of Amapá, Rod. Juscelino Kubitscheck, km 02, S / N, Macapá-AP, 68903-419, Brazil.

[b]Post-graduate Program in Pharmaceutical Sciences, Department of Biological Sciences and Health, Federal University of Amapá, Rod. Juscelino Kubitscheck, KM 02, S / N, Macapá-AP, 68903-419, Brazil.

[c]Post-graduate Program in Health Sciences, Department of Biological Sciences and Health, Federal University of Amapá, Rod. Juscelino Kubitscheck, KM 02, S / N, Macapá-AP, 68903-419, Brazil.

[d]Graduate Program on Pharmaceutical Innovation, Federal University of Amapa, Rodovia Juscelino Kubitschek, Km 2, Jd. Marco Zero, Macapa, AP, Brazil.

[e]Laboratory of Quality Control, Bromatology and Microbiology, Federal University of Amapá, Rod. Juscelino Kubitscheck, KM 02, S / N, Macapá-AP, 68903-419, Brazil.

E-mail address: lucasrego.bio@outlook.com, everton.pantoja.vale@gmail.com, danilodv_nascimento@hotmail.com, fernandomedeiros1973@gmail.com and moacir.monteironeto@yahoo.com.br (Respectively)

*Author corresponding: Telephone: +55 96 98102-2625 / email: lucasrego.bio@outlook.com

Abstract

The Amazon Rainforest has a great variety of medicinal plants, among them we can highlight the "Almecegueira" or "Breu Branco" (Protium heptaphylum) in Portuguese, the producer of a greenish-white resin that hardens when it touches the air, known by its gastroprotective and anti-inflammatory effects. These effects are attributed to a triterpene mixture of α and β amirine, predominant in the resin. The purpose of the study is to obtain a cytogenetic profile to the α, β-amirine mixture obtained in the resin of P. heptaphylum. For this, the micronucleus test was used in peripheral blood and bone marrow; administering solution in Swiss mice with the dosages of 1mg/Kg, 3mg/Kg, and 10mg/Kg, diluted in 5% DMSO, the effects were observed in 24h and 48h after the treatment. For the test in peripheral blood the mice's caudal vein was punctured, while for the bone marrow test, the femurs of the animals were obtained from which bone marrow samples were taken. It was found that in peripheral blood, the administration of the compounds did not cause genotoxicity in 24h and 48h, in contrast, antigenotoxicity was, for concentrations 1; 3 and 10mg/kg, respectively 10%; 12%; 67% in 24h and 9%; 15%; 73% in 48h. In the bone marrow, no genotoxicity was observed, as for antigenotoxicity was observed that for

concentrations 1; 3 e 10mg/kg the percentage of reduction was respectively: 11%, 15%, and 30% in 24h and 13% 16% 33% in 48h. It is concluded that the studied compound can be an alternative for treatments in the future since it presents low toxicity and high antigenotoxic potential.

Keywords: Almecegueira; α, β-amirine; Amazon Rainforest.

1. Introduction

Since the beginning of man's history, nature has been a source of a search for health problems. The use of plants to treat and prevent diseases has already been used for thousands of years. Studies carried out, along with the remains of the first hominids, could prove the use of medicinal plants for the most diverse purposes for about 60 thousand years ago (Karam, 2013). Plants are capable of producing a large quantity amount of bioactive molecules resulting from their metabolism and most plants naturally produce a great variety of bioactive molecules, called secondary metabolites, which can be obtained from different parts of the plants (Punjab et al., 2012). A wide range of biological activities is described for these of secondary metabolites, such as, for example, antitumor, anti-inflammatory, antioxidant, antibacterial, antiparasitic, antiviral, among others (Oliveira et al., 2011). The Amazon Rainforest owns a wide variety of medicinal plants, among which we can highlight the "Almacegueira" or "Breu Branco" (*Protium heptaphylum)* in Portuguese, producer of a greenish-white resin that hardens in contact with air, popularly used for its gastroprotective and anti-inflammatory effects (Araujo et al., 2011; Zappi,, 2015; Brasil, 2018). In the literature, there are some secondary metabolites obtained through *Protium. Heptaphylum* resin, among them a considerable part is a triterpene mixture of α e β amirine from which a series of derivatives such as formate, acetate, benzoate, and cinnamate can be obtained (Bandeira et al., 2001; Bandeira et al., 2007).

The arimines are pentacyclic triterpenes that can be divided into ursane, olean, and lupeol, and present basic differences in chemical structure, only in the aromatic ring "E", so then, when the ring is positioned ate C20 and contains two methyls it's called β – amirine, in contrast when the methyl radical is linked to C19 it's called α – amirine (Bandeira et. al.,2007; Florentino, 2018). The triterpenes eeing present as major constituents of resin produced by a large number of plant species, among which are the species of the genus Protium (Dias, Hamerski and Pinto, 2011). They perform important functions such as defense (phytoalexins), insect repellent, agents of pollen attraction, defense agents against herbivores, pheromones, allelochemicals, plant hormones, and because they function as signaling molecules (Bruneton, 1993; Mccaskill and Croteau, 1997; Verpoorte, 2000). Its use is widespread, mainly in folk medicine as healing, expectorant, anticâncer, antiviral, antibacterial, antifungal, antidiuretic, giardiacide, and acetylcholinesterase enzyme inhibitor (Bandeira, 2007), anti inflammatory activity, antiarthritic and analgesic (Dias, Hamerski and Pinto, 2011). Aragão et al., (2002) showed that the mixture of α.- and β.-Amirine has an antinociceptive effect and anti-inflammatory activity using 50mg/kg b.w.

Toxicological genetics, according to Ribeiro (2003), evaluates potential genotoxic effects, which are considered important to cancer development. Nucleic acids are often exposed to various genotoxic substances both from endogenous and exogenous sources, such as ultraviolet radiation, and substances that the individual makes use of or come in contact with (Neto, 2011; Ermolaeva and Schumacher, 2014). For

this type of experiment, there are techniques such as the micronucleus test. Through this test, it is possible to evaluate the measure of chromosomal damage and segregation errors. The micronucleus result from the production of acentric fragments or chromosomes that are delayed in their migration to the poles of the cell in anaphase. Any cell can be used for testing as long as it has undergone at least one division but bone marrow cells and blood cells are often used although effective tests using cells from other organs such as liver and embryos, embryos pre-implanted, and ovarian tumor cells (Countryman, 1976).

The micronucleus test in peripheral blood is a good option among the micronucleus testing objects, as a single drop of blood produces a large number of available cells for testing, without the need for any special processing or a trained cytogeneticist for analysis. Studies prove greater effectiveness of the Giemsa staining method for environmental studies, besides being a more reliable and practical method to be done. The bone marrow micronucleus test has more complex procedures, but it may provide even greater reliability, since the number of polychromatic erythrocytes is much higher in bone marrow samples than in peripheral blood, making it possible to analyze a considerable number of young cells in a shorter period compared to the blood smearing analysis methodology, therefore it presents itself as a more reliable methodology then the test on peripheral blood erythrocytes (Majone et al., 1988; Silva and Nepomuceno, 2010).

In this context this study aims to elucidate the knowledge towards α, β-amirine, and, since it has wide use and taking into account that the dosages considered safe for consumption are not established in the literature, in addition to their activity is not fully explained, thus constituting a potential risk for indiscriminate use. Accordingly, this study evaluated the genotoxic, antigenotoxic and cytotoxicity effects of the extract of the mixture α, β-amirine obtained from *P. heptaphylum* extract in vivo.

2. Material and methods

2.1. Materials

Doxorubicin (CAS. No: 23214-92-8) (DXR) from Rubidox® (Life Comercio de Medicamentos e Manipulação LTDA EPP, Santo Andre, SP – Lote: 1084504). All the other reagents were pure grade and used according to the manufactures´ instructions.

1.2. Vegetable material collection

The collection of plant material (resin) happened at a preservation área in octuber 2016 at Sítio São Benedito, located at Manoel Francisco de Souza street, number 414, in the São Lázaro neighborhood, coord: Lat. 00º4’ 626052” W, located in Macapá, Amapá, Brazil. A crude hexanic extract was produced from this resin. The identification of mentioned plant to obtain a resin sample and making of desiccate, registered at Herbarium Amapaense (HAMAB), of Institute of Studies and Research of Amapa State (IEPA) under the registration number 019059.

1.3. Purification of the hexanic resin extract

After obtaining the resin, a collum chromatography of 50g of 0,063-0,2mm silica gel was performed, assembled in hexane, using 1,5 g of hexanic extract from Resina do Breu. The solvent system

used started with 100% hexane, followed by: 1000ml of hexane + Dichloromethane 7:3; 500 ml of hexane + dichloromethane 4:6; 250 ml dichloromethane 1:1; 200 ml of dichloromethane + methanol 9:1; 200 ml of dichloromethane + methanol 8:2; 200 ml of dichloromethane + methanol 6:4; 200 ml dichloromethane + methanol 5:5. Altogether 108 fractions of this system were collected, which were gathered, observing their chemical similarities and evidenced in a chromatographic plate revealed in iodine particles. After gathering the fractions, a DMSO 5% solution containing α, β amirine was produced.

1.4. Chromatographic analysis

To confirm the sample used, gas chromatography analysis coupled with mass spectrometry was performed. It was made on Agilent equipment, coupled to an Agilent mass spectrometry analyzer. A 5% phenylmethyl polysiloxane column, 40 m x 0,30 mm x 0,10 μm, with flow division 15:1 and transfer at 290 °C, was used with Helium gas. Initially, the temperature of the chromatographic chamber was 110°C for 5 min, increasing the temperature by 15 °C/min up to 290 °C and then, increasing by 2 °C/min up to 300 °C, stabilizing this condition for 15 min.

1.5. Positive control (DNA damage induction)

The chemotherapeutic DXR (Rubidox® Smartfarma Ltda., São Paulo, SP) was used as DNA damage inducer in peripheral blood cells (positive control) (De Azevedo Bentes Monteiro Neto et al., 2011; Alves et al., 2013). The drug was dissolved in distilled water and administered intraperitoneally (i.p.) (0.3 mL / animal). The concentration of DXR (15 mg/kg body weight - b.w.) was set according to previous studies (Franke et al., 2005; Venkatesh et al., 2007; Vale et al., 2020).

1.6. Animals

Fifty male healthy Swiss mice (6 - 7 weeks and approximately 25 ± 5 g b.w.) were obtained from the Multidisciplinary Biotherm Center for Biological Research at Animal Laboratory Science Area (CEMIB) of the State University of Campinas (UNICAMP), Campinas - SP, Brazil. Each experimental group consisted of five animals. The experimental protocols used in this study were in accordance to the legislation for animal experimentation established by the Brazilian National Council of Animal Experimentation (CONCEA). All experimental protocols were reviewed and approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Amapa (UNIFAP) under the protocol number 010/2019

2.7. Micronucleus test peripheral blood

The genotoxic, antigenotoxic and cytotoxic effects of α, β-amirine were evaluated using the micronucleus test in polychromatic erythrocytes of Swiss mice. The used concentrations of α, β-amirine, were 10, 3, e 1 mg / kg b.w., according to the literature, was administered orally by gavage (0.5 mL), to observe the processes in 24h and 48h, according to the method described by Macgregor et al. (1980). For the antigenotoxic evaluation, immediately after the extract administration, the animals were treated with an intraperitoneal injection (i.p.) of DXR (0,3 ml/15 g b.w.) and subsequently performed the analysis in 24h and 48h. The Positive Control group was DXR via intraperitoneal, the Negative Control group was

treated by gavage only with water, the Dimethylsulfoxide (DMSO) group was treated by gavage (0,5 ml/25g b.w.), and the DMSO + DXR group was treated with DMSO by gavage followed by intraperitoneal injection of DXR as previously explained (Matos et al. 2013).

Each animal´s tail tip was cut and a drop of blood was dripped and then dragged over microscopy clean blades and dried at room temperature. Subsequently, they were fixed in absolut methyl alcohol for 5 minutes and again dried at room temperature. After 24 h, the blades were stained with diluted Giemsa dye (Ministry of Health, 2009) for 20 minutes. The blades were then washed to remove the excess of dye, dried at room temperature and examined in an optical microscope (Opton TIM-2008) on the immersion objective (100x).

2.8. Micronucleus test bone marrow

Bone marrow MN preparations were made using Sehmid (1976) modified method. Where 5% bovine serum albumin (BSA) was prepared in phosphate buffered saline (PBS; pH-7.2), it was used as a suspension medium. The bone marrow suspension was centrifuged at 1000 rpm and the pellet was resuspended. A drop of suspension was spread on clean, air-dried slides. The dried slides were fixed in methanol and stained with buffered May-Grunwald-Giemsa (pH 6.8).

Micronucleated polychromatic erythrocytes (PCEMNs) in bone marrow and peripheral blood samples were calculated from 2000 cells and the nuclear division index (NDI) was determined as the ratio of polycyclic erythrocytes (PCE) to normochromatic erythrocytes (NCE) (PCE/NCE) based on 400 erythrocytes per slide (Mersch-Sundermann et al., 2004). The reduction in the frequency of PCEMNs was calculated according to the following formula (Waters et al., 1990): % Reduction = (A-B) / (A-C) x 100.

Where A corresponds to the damages from the treatment with DXR (positive control), B is the group treated with PGFP plus DXR, and C is the negative control group.

2.8. Statistical analysis

The data were analyzed statistically, by another researcher who did not participate in the other stages of this study, using the variance test (ANOVA) for entirely random experiments, with the calculation of the F statistic and its respective “p-value”. In cases where $p< 0.05$, treatment averages were compared using the Tukey method, with the calculation of the minimum significant difference for $\alpha = 0.05$. The program used will be GraphPad Prism 8.

3. Results and discussion

3.1 Chromatographic analysis

The chromatographic gas layer analysis was performed according to the protocols of Rodrigues (2010). The results found for the samples obtained in the chromatography column previously performed were similar to the results observed in the literature, confirming the presence of the compound analyzed as α, β - amirine.

3.2 Nuclear Division Index - Cytotoxicity

The cytotoxicity of treatments was measured using the IDN. The data showed the absence of cytotoxicity of the treatments in all concentrations evaluated, however, it is noted that there is a significant difference between the treatment groups and the positive control, the latter where a greater occurrence of PCE was noted than in the others; leading to the belief that the administration of DXR produced cytotoxic effects to the cells, possibly sensitizing the mass production of new cells that are still launched into peripheral blood, which would explain the significant increase in the occurrence of PCE in the CP group. Also, the behavior of the control groups with DMSO solvent was observed, in these there was no significant difference concerning the groups treated with α, β - amirine, which shows that the use of the solvent did not cause an increase in the IDN in the animals, a fact which occurred similarly in the treatment groups where there was no correlation between the treatment and the IDN, as shown in Table 1 and 2.

3.3 Genotoxicity

Initially, it was observed that the treatments performed, in all dosages of α, β - amirine used (10 mg / kg; 3 mg / kg; 1 mg / kg) did not produce genotoxic effects in both the 24h and 48h analysis in Bone Marrow and Pheripheral blood, when comparing the Control groups control and treatment it's evident that there was no correlation between treatments and the increase in the frequency of micronuclei ($p > 0.05$), as shown in Table 1. The found results corroborate with the literature, studies have shown that the use of α, β - amirine does not confer genotoxicity. In the literature, treatments with higher concentrations are found, for example, 50 mg per kg p.c. of α, β - amirine that also did not produce genotoxicity in subacute assays. (Venkatesh et al., 2007; Aparecida et al., 2006).

Regarding the analysis of the frequency of micronuclei in bone marrow, there was no significant increase ($p > 0.05$) in the frequency of micronuclei correlated to the administration of any administered concentration, as shown in Table 1. The same can be said of the IDN, both in peripheral blood and in bone marrow, the treatment groups were statistically similar to the control groups where there were no changes, except for the Positive Control group which, due to the administration of DXR, had an increased frequency of micronuclei and IDN. The data obtained are following the observations in the literature where there is no evidence of the toxicity of the compound administered (Aparecida et al, 2006).

Table 1 – Nuclear division index and absolute and relative frequency of micronuclei found in peripheral blood and bone marrow, 24h and 48h after Genotoxic treatment with α, β - amirine, by each group. 10,000 cells were analyzed for each group.

Treatment	Pheripheral Blood			Bone Marrow		
	IDN	MNEPCs		IDN	MNEPCs	
	Mean and deviation	Nº	%	Mean and deviation	Nº	%
CN 24h	0.060 ± 0.0071	14	0.14	0.060 ± 0.0071	13	0.13
DXR 24h	0.1 ± 0.0045	78	0,78	0.11 ± 0.0045	150	1.5
DMSO 24h	0.061 ± 0.0071	16	0.16	0.061 ± 0.0071	15	0.15
1mg/kg b.w. 24h	0.056 ± 0.0055	12	0.12	0.056 ± 0.0055	15	0.15
3mg/kg b.w. 24h	0.056 ± 0.0089	13	0.13	0.056 ± 0.0089	14	0.14

10mg/kg b.w. 24h	0.058 ± 0.0084	15	0.15	0.058 ± 0.0084	17	0.17
CN 48h	0.056 ± 0.0054	14	0.14	0.056 ± 0.0054	12	0.12
DXR 48h	0.10 ± 0.0044	78	0,78	0.10 ± 0.0044	163	1.63
DMSO 48h	0.062 ± 0.0045	14	0.14	0.062 ± 0.0045	15	0.15
1mg/kg b.w. 48h	0.064 ± 0.0055	12	0.12	0.064 ± 0.0055	12	0.12
3mg/kg b.w. 48h	0.068 ± 0.0045	13	0.13	0.068 ± 0.0045	13	0.13
10mg/kg b.w. 48h	0.062 ± 0.0045	15	0.15	0.062 ± 0.0045	13	0.13

3.4 Antigenotoxicity

In the second step, analyzes of the antigenotoxicity of the treatment for the 24 hours since the administration of DXR were performed, where it was shown that there is a correlation between the frequency of micronuclei and the treatments ($p < 0.05$) as shown in the Table 2. Comparing the results of the highest concentration (10 mg / kg) and the results of the micronucleus frequency of the Positive Control (DXR) and DMSO + DXR groups, there was a significant difference between them, where the group treated before the application of DXR with 10 mg / kg provided a reduction in genotoxicity in 67% in 24h, followed by the groups 3 mg / kg and 1 mg / kg, which obtained 12% and 10% reduction, respectively. In 48h, a reduction was observed for concentrations of 10, 3, and 1 mg / kg of α, β - amirine of 73%, 15%, and 10% respectively (Waters et al, 1990).

This effect is believed to be due to the antioxidant potential of the studied triterpenoid compound, already described in other studies, as evidenced in the literature. In vitro studies have shown that triterpenes have anti-cancer effects on about 26 hematological markers, which would explain the reason why there was a significant decrease in the frequency of micronuclei observed at 10 mg/kg concentration (Fabiyi et al. 2012).

The results obtained are similar to those found in the literature, Carneiro et al. (2017) describe, in his study that he used Astrocaryum aculeatum oil, an antigenotoxic activity that varied from 35 to 38% in 24h and 63 to 66% in 48h, observing a slight increase of the percentage of protection of genetic material against the DXR administered in the analysis made 48h after administration of the concentrations, which is due to the protective effect of the administered compounds, which were able to further reduce the effects of DXR even during the peak of genotoxicity in 48h (Okoye et al., 2014).

Besides the antigenotoxic effect, other characteristics are attributed to α, β - amirine. Mainly anti-inflammatory effects are described, but there is also an anti-nociceptive effect both when administered orally and intraperitoneally. Some studies have pointed out an anti-hyperglycemic and hypolipidemic effect, although the physiological mechanism that corroborates this effect is not known (Aragao et al., 2008).

The results obtained in the analysis of the bone marrow, which are described in Table 2, were also similar to those described in the literature. A high level of significance was observed in the treatment performed at a concentration of 10 mg / kg p.c, thus showing, as well as data from the analysis of peripheral blood, that treatments with lower concentrations were not able to produce significant effects to decrease the frequency of micronuclei found, becoming the percentage of reduction statistically similar to the Controls that used DMSO. Nevertheless, it is important to note that despite the significant decrease in the

percentage of reduction observed in the analysis of peripheral blood and bone marrow (67% in 24h and 73% in 48h in peripheral blood; 30% and 33% in 24h and 48h respectively, bone marrow), it is still considered a high percentage of protection, especially when observing the dosage administered, which in this study was substantially lower than other previous studies, a fact that makes this study so relevant (Okoye et al., 2014; Aparecida et al., 2006).

Table 2 – Nuclear division index and absolute and relative frequency of micronuclei found in peripheral blood and bone marrow in the period of 24h and 48h after Antigenotoxic treatment with α, β - amirine + DXR, by each group. 10,000 cells were analyzed for each group.

Treatment	IDN	MNEPCs		Reduction
	Peripheral Blood			
	Mean and deviation	N	%	%
CN 24h	0.060 ± 0.0071	14	0.14	-
DXR 24h	0.1 ± 0.0045	78	0.78	-
DXR + DMSO	0.066 ± 0.0054	59	0.59	29%
1mg/kg b.w. + DXR 24h	0.063 ± 0.0044	73	0.73	11%
3mg/kg b.w. + DXR 24h	0.062 ± 0.0044	70	0.7	12%
10mg/kg b.w. + DXR 24h	0.066 ± 0.0054	35	0.35	67%
CN 48h	0.056 ± 0.0054	14	0.14	-
DXR 48h	0.10 ± 0.0044	78	0.78	-
DXR + DMSO 48h	0.062 ± 0.0044	59	0.59	29%
1mg/kg b.w. + DXR 48h	0.064 ± 0.0054	72	0.72	9%
3mg/kg b.w. + DXR 48h	0.062 ± 0.0046	68	0.68	15%
10mg/kg b.w. + DXR 48h	0.061 ± 0.0042	31	0.31	73%
	Bone Marrow			
CN 24h	0.060 ± 0.0071	13	0.13	-
DXR 24h	0.11 ± 0.0045	150	1.5	-
DXR + DMSO	0.066 ± 0.0054	128	1.28	16%
1mg/kg b.w. + DXR 24h	0.063 ± 0.0044	134	1.34	11%
3mg/kg b.w. + DXR 24h	0.062 ± 0.0044	129	1.29	15%
10mg/kg b.w. + DXR 24h	0.066 ± 0.0054	109	1.09	30%
CN 48h	0.056 ± 0.0054	12	0.12	-
DXR 48h	0.10 ± 0.0044	163	1.63	-
DXR + DMSO 48h	0.062 ± 0.0044	133	1.33	19%
1mg/kg b.w. + DXR 48h	0.064 ± 0.0054	138	1.98	13%
3mg/kg b.w. + DXR 48h	0.062 ± 0.0046	126	1.26	16%
10mg/kg b.w. + DXR 48h	0.061 ± 0.0042	100	1	33%

The values are presented as mean ± S.D. and analyzed by ANOVA and Tukey's test.

Conclusion

Cytogenetic studies are not the only studies necessary for the implementation of new drugs on the market, but they are an important guiding parameter that indicates a path to be followed. The results clearly show the mainly genotoxic potential of the compound studied, evidenced in the highest concentration administered, obtaining up to 73% protection in 48h compared to the administered DXR, when the effect is evaluated on peripheral blood and 33% in 48 hours when the bone marrow evaluations were observed. The effects observed in this study and the literature show the great pharmacological potential of the compound α, β - amirine, which has many activities not even explained in the literature. It's also important to mention that the dosages were minimal compared to previous results already mentioned and the similar results show that the treatment, although in a reduced dosage, could provide similar results, which represents a considerable advance in research involving the studied compound. Being able to provide in the future a less aggressive treatment based on an Amazonian product that can be extracted with a certain ease, in addition to being a plant not exactly Amazonian, but that occurs in a large part of the national territory. Therefore, it is worth emphasizing the importance of continuing studies like this, which aim to add knowledge to Brazilian society, especially Amazonian, which has so many plants of medical interest available for the production of drugs for treatment not only in the area of oncology but also in the several pathologies.

Conflict of interest

The authors declare no conflicts of interest.

Author Contributions

Lucas Rodrigues do Rego designed the study, performed the toxicity, Cytotoxicity, Genotoxicity and Antigenotoxicity assays, and drafted the manuscript. Everton Pantoja Vale assisted in Micronucleus test. Danilo Dheyvison Nascimento Pureza assisted in Micronucleus test. Moacir de Azevedo Bentes Monteiro Neto designed the study, drafted the manuscript, and interpreted the data.

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