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Research Article

Evaluation of the Genotoxic and Antigenotoxic Effects of Andiroba (*Carapa guianensis* Aublet) Oil and Nanoemulsion on Swiss Mice

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The *Carapa guianensis* (andiroba) oil is commonly used by the Amazon population for medicinal purposes. The objective of this study was to determine the genotoxic and antigenotoxic potential of the andiroba oil (AO) and nanoemulsion (AN) using Swiss mice. Therefore, we used the comet assay and micronucleus test. The AO predominant compounds were oleic (39.13%), palmitic (33.22%), and linoleic (16.86%) acids. AN composition obeyed the surfactant/oil ratio of 0.69, and the Tween 80/Span 80 ratio was held at 0.9. Our results showed no cytotoxicity or genotoxicity in the mice treated with AO and AN alone. However, there was a significant reduction in the polychromatic erythrocytes (PCEs) numbers in all groups treated with doxorubicin (DOX), including those pretreated with AO and AN. Thus, the samples tested did not protect against DOX. On the other hand, our results showed a large increase in micronucleus (MN) formation when the mice were treated with DOX alone; these numbers were reduced when the animals were pretreated with AO and AN. The results indicate a protective effect of andiroba on MN formation and show no evidence of genotoxicity in mice.

1. Introduction

Vegetable oils are important sources of energy and essential fatty acids [1]. Fatty acids can be distinguished as saturated and unsaturated. The fatty acids present in vegetable oils are known to be polyunsaturated because they have more than one unsaturation in their chemical composition [2]. Fatty acids have important antioxidant activities with the potential to protect biological systems against the action of reactive oxygen species (ROS) [3].

The search for molecules with biological activities is increasingly common to minimize the effects of more toxic compounds on the environment and human health. *Carapa guianensis*, which is popularly known as andiroba, belongs to the family Meliaceae. Its seeds are very oleaginous, and the seed oil has been used for a long time by indigenous people as a repellent against insect bites [4]. The Amazon population uses the andiroba oil (AO) for several other purposes, such as an analgesic, antibacterial, anti-inflammatory, anticarcinogenic, and antiallergic uses, as well as protection against arthritis,

rheumatism, and ear infections [5]. Vendramini et al. (2012) [6] showed that the oil had a powerful effect on the reproductive system of a tick species, indicating that it might be a vital natural agent capable of reducing and preventing the reproduction of these animals. Miranda Júnior et al. (2012) [7] found an important antiplasmodial activity in AO with no toxicity in mice.

Another important attribute of vegetable oils for human health is related to their demonstrated antioxidant activity. In this context, vegetable oils have been the focus of scientific interest due to the ability of natural antioxidants to protect biological systems against the action of ROS and nitrogen, which are responsible for oxidative damage to lipids, proteins, and nucleic acids [8]. Doxorubicin (DOX) is an antineoplastic used in the fight against various types of cancer and although it is quite effective for this purpose, it has limited clinical use because it causes severe side effects [9]. The mechanism of toxicity of DOX seems to be related to the production of reactive oxygen species (ROS) in the body [10, 11]. On the order hand, antioxidants might be used to decrease ROS generated by DXR. So, here we use DOX for to better investigate the possible antigenotoxic effects from andiroba.

The use of vegetable oils in traditional medicine is very common. Generally, these substances are of great interest because they can be a source of new compound structures of interest for the development of new drugs [12]. In this context, the development of new technologies has led to great strides towards improving the effect of a particular substance. For example, nanotechnology uses nanodevices to maximize the clinical benefits of a substance [13]. One advantage of the use of nanoemulsions (i.e., the development of insect repellent skin cream) is that the spreading ability of the cream; thus, the contact of the active ingredient and the protection capacity are increased due to the increased surface area.

Therefore, it is of great importance to increase knowledge on the adverse and/or protective effects of natural products, such as AO. The objective of this study was to determine the genotoxic and antigenotoxic potential of oil extracted from the seeds of *Carapa guianensis*. Additionally, we tested a nanoemulsion produced from AO and compared the effect with only AO.

2. Material and Methods

2.1. Extraction and Characterization of Andiroba Oil

2.1.1. Seed Collection and Oil Extraction. Andiroba (*Carapa guianensis* Aublet) seeds were collected in the state of Pará, Brazil (04° 16′ 34.0″ S and 55° 59′ 01.0″ W), placed in raffia bags, and transported to the Laboratory of Systematic Research in Biotechnology and Biodiversity (LabISisBio/UFPA) for processing and chemical characterization. The collection and transport permits were granted by ICMBio/SISBIO/MMA under n. 33336-2 on 07/08/2013. The andiroba plant was identified using an exsiccate (n. 191736-VGS 770) that was deposited in the IAN Herbarium of Embrapa Eastern Amazon.

Oil was extracted from andiroba seeds by fermentation using the method described by Salgado (2015) [14].

2.1.2. Preparation of Samples for Analysis. A 20.0 mg aliquot of oil was weighed in a capped conical tube (2.5 mL), and 250 μL of an aqueous 2 M NaOH solution was added. The mixture was heated at 50°C for 5 minutes and stirred at 800 rpm in a dry bath. After the hydrolysis and saponification reactions, 300 μL of hexane was added to the sample and stirred vigorously for 30 seconds to extract the unsaponifiables. The unsaponifiables were neutralized with 300 μL of 2 M HCl. The mixture was heated at 50°C for 5 minutes and stirred at 800 rpm. The unsaponifiable substances were extracted with 1000 μL of a 1 : 1 hexane : ether mixture. The solvents were evaporated in a vacuum concentrator with heating at 45°C. The unsaponifiable and saponifiable samples were derivatized by the addition of 50 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in each sample. The reaction was performed in a dry bath at 30°C for 5 minutes at 800 rpm. Then, 700 μL of dry hexane was added to each sample and mixed vigorously for 30 seconds. Finally, 500 mL of each solution was transferred to a different septum-capped vial and sent for GC/MS analysis.

Gas Chromatography Coupled to Mass Spectrometry Analysis. The gas chromatography analysis was performed using a Thermo Scientific Trace 1300 gas chromatograph (GC) coupled with a Thermo Scientific ISQ Single Quadrupole mass spectrometer (MS) with an AI-1310 autosampler equipped with a RTX-65 TG capillary column (15 m × 0.25 mm × 0.1 μm). Helium was used as the carrier gas at a flow rate of 1 mL/min. A 1.0 μL aliquot of sample was injected in 1 : 5 split mode. The injector operated at 250°C. The initial oven temperature of 40°C was ramped up to 200°C at 6°C/minute, kept at this temperature for 1 minute, ramped up to 300°C at 15°C/minute, and kept at this temperature for 5 minutes. Then, the temperature was raised to 340°C at 15°C/min and maintained for 9 minutes. The ISQ MS operated at a 280°C interface temperature, 280°C ionization source temperature, and 40–600 Da mass range. The electron ionization was 70 eV. The substances were identified by comparing the mass spectra with the WILEI2009 commercial library. The free fatty acid (FFA) concentration was determined by calculating the normalization of the peak area with the following equation: $(\%) = (Ai/\Sigma An)x100$, where Ai is the peak area of the analyte and ΣAn is the sum of the areas of all obtained peaks.

2.2. Nanoemulsion Preparation and Characterization

2.2.1. Preparation of Nanoemulsions with Different Surfactant-to-Oil Ratios. AN were prepared by a phase inversion temperature (PIT) method with the surfactant mixture (Smix) consisting of Tween 80 : Span 80 (9 : 1, w : w). In this experiment, nanoemulsions were produced with different surfactant-to-oil weight ratios (SOR = surfactant weight/surfactant weight + oil weight) to determine the effect of this variable on the colloidal properties of the formulation. Briefly, 0.5 g of AO was added to 0.75, 0.79, 0.84, 0.86, or 0.94 g of Smix, resulting in mixtures with a SOR ranging from 0.60 to 0.75. Next, 4 mL of water was added and the mixtures were heated to 95°C. This temperature was kept for 5 minutes under magnetic stirring (300 RPM). Next, the mixture was

rapidly poured into a cold test tube (−20°C) and vortexed for 2 minutes. The volumes of the nanoemulsions were increased to 5 mL with distilled water. The nanoemulsion droplet hydrodynamic diameter and polydispersity index properties were measured as described in the "Colloidal properties" section.

2.2.2. Nanoemulsion Preparation. The AN used in the biological tests was prepared as described in the "Emulsions prepared with different surfactant-to-oil ratios" section with a SOR of 0.69. Briefly, 0.5 g of AO was mixed together with 1.12 g of Smix and 4 mL of water. This mixture was heated to 95°C and stirred (300 RPM) for 5 minutes. At this point, the liquid became transparent. The mixture was immediately poured into a cold test tube (−20°C) and vortexed for 2 minutes. The volume was increased to 5 mL with distilled water. This formulation was kept at 4°C prior to use.

2.2.3. Colloidal Properties. The hydrodynamic diameter and zeta potential of the droplets contained in the nanoemulsions were measured at 25°C by photon correlation spectroscopy and electrophoretic laser Doppler velocimetry (ZetaSizer Nano ZS®, Malvern Instruments, Malvern, UK), respectively. The polydispersity index (PdI) was calculated using the equipment software based on the DLS measurements. All measurements were performed in triplicate, and the results were presented as the mean ± standard deviation (SD).

2.3. Biological Assays

2.3.1. Animals and Experimental Design. The project was approved by the ethics committee of the Federal University of Pará (03/05/2012; opinion 194-14). The animals were divided into seven treatment groups with six animals per group: AO (2000 mg/kg), AN (2000 mg/kg), DOX (40 mg/kg), AO (2000 mg/kg) + DOX (40 mg/kg), and AN (2000 mg/kg) + DOX (40 mg/kg); for AO control was used corn oil (Salada®) and as AN control the nanoemulsion surfactant was used. The dosages used in this study were based on the maximum dose without toxic effect observed (nonobserved adverse effect level (NOAEL)), obtained previously on study published of Milhomem-Paixão et al. (2016) [15]; the highest dose used by that authors was chosen that did not show any genotoxicity and therefore could be used to safely investigate possible antigenotoxicity. The control was corn oil (Salada) and the surfactant of the nanoemulsion containing a mixture of all its components except andiroba. To administer the preset doses, the AO was diluted in corn oil (Salada). The AO and AN were administered to the mice by gavage for 14 consecutive days. On the 13th day DOX was administered intraperitoneally (ip) to assess the protective potential of andiroba. The animals were euthanized on the 15th day.

2.3.2. Necropsy and Tissue Preparation. For the performance of the tests, the animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) applied ip in a volume of 120 μL/animal and sacrificed by exsanguination via cardiac puncture. All animals were examined internally for signs of abnormal coloring and organ and tissue abnormalities.

2.3.3. Comet Assay. The comet assay was performed according to the method proposed by Singh et al. (1988) [16] with alkaline comet pH > 13, with modifications.

The material was observed under a Zeiss Axioskop 2 fluorescence microscope (510–560 nm filter, 590 nm filter barrier, and 400x magnification). Ethidium bromide (20 g/mL) was used for staining. The comets were classified into migration categories based on the degree of DNA damage according to Collins et al. (1995) [17]. One hundred cells were analyzed per animal, and the damage index (DI) was calculated using the following formula: $DI = [(1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)/n] \times 100$, where n represents the total cells analyzed and $n1$ to $n4$ indicate the numbers of cells with damage levels of 0 to 4.

2.3.4. Micronucleus Test. The micronucleus test was performed in bone marrow cells collected in fetal bovine serum (FBS), following the protocols of Schmid (1975) [18], with modifications. The slides were scored blind using an Olympus BH2 light microscope with 1000x magnification. The micronuclei frequency was evaluated by counting 2000 polychromatic erythrocytes (PCEs) and 2000 normochromatic erythrocytes (NCEs). The cytotoxicity was evaluated by the percentage of PCEs (% PCE). When the population of erythrocytes reached 2000 cells during the count, the amount of the other populations was recorded. The % PCE was calculated using the formula $\%\ PCE = [PCE/(NCE + PCE)] \times 100$.

2.4. Statistical Analysis. The results were analyzed using the Instat 3.02 software. Possible differences were investigated using analysis of variance (ANOVA) or Kruskal-Wallis test ($p < 0.05$), according to distribution of the normally.

3. Results

3.1. Characterization of Andiroba Oil. The overall analysis profile of the sample composition is shown in Table 1. The three predominant substances in the oil composition were oleic acid (39.13%), palmitic acid (33.22%), and linoleic acid (16.86%).

3.2. Characterization of the Andiroba Nanoemulsion. Figure 1 shows the hydrodynamic diameter (HD) and polydispersity index (PdI) of formulations with different surfactant/oil ratio (SOR). The chosen SOR was 0.69 as described in Material and Methods. In this experiment, the Tween 80/Span 80 ratio (TSR) was held at 0.9. The ratios 0.69 (SOR) and 0.9 (TSR) gave formulations with good PdI (below 0.25, indicative of monodisperse formulation) with the lowest concentration of surfactant. Figure 2 shows the HD and PdI of the formulations when the SOR was held at 0.69; the proportion of Tween 80 and Span 80 (TSR) ranged between 0.8 and 1.0 (i.e., between 80 and 100% Tween 80).

3.3. Biological Assays. During the 14 days of exposure, no mortality or signs of toxicity or death were observed in any of the treatments. The body weights were also not affected. No change was observed in the external morphology of the organs in the macroscopic analysis.

Table 1: General profile analysis of the composition of AO sample.

RT	Class	Substances	% area
13,98	Fatty acids	Lauric acid	0,26
16,02		Myristic acid	0,36
17,86		Palmitoleic acid	1,10
18,51		Palmitic acid	33,21
21,12		Oleic acid	39,13
21,31		Linoleic acid	16,86
21,58		Stearic acid	4,70
26,36	Monoglycerides	Monopalmitin	0,52
28,00		Monoolein	1,40
30,96	Steroids and triterpenoids	Alpha-sitosterol	0,24
33,69		Trihydroxy colane 3,7,12-ethyl-24-oate	0,36
33,98		Deoxy-cericea-lactone	0,65
34,58		Gedunine	0,43
35,03		7-Oxogedunine	0,30
35,37		Efusanin A	0,27
35,98		Deacetyl-gedunin	0,26

RT: retention time (min).

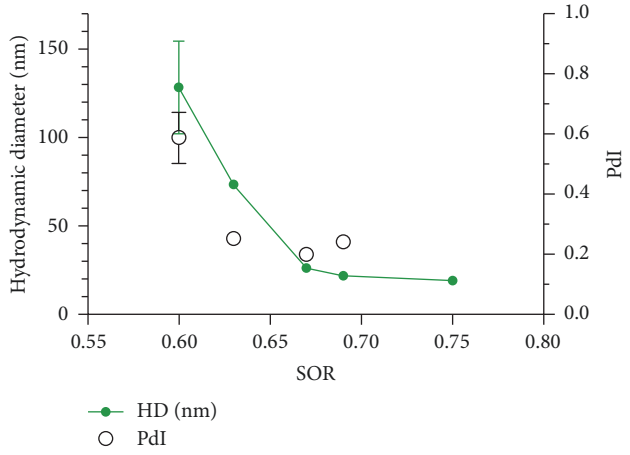

Figure 1: The hydrodynamic diameter (HD) and polydispersity index (PdI) of formulations with different surfactant/oil ratio (SOR).

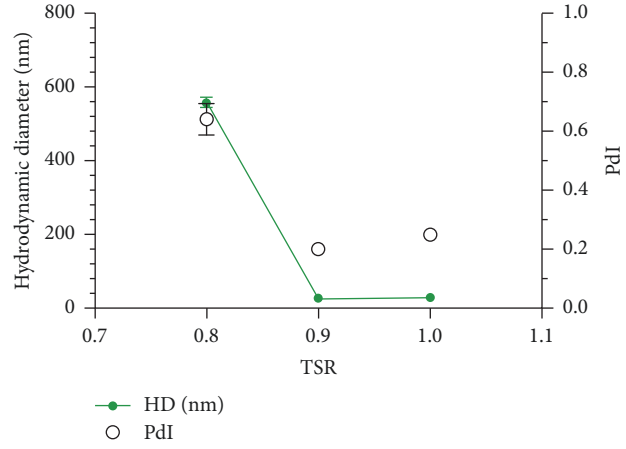

Figure 2: The hydrodynamic diameter (HD), polydispersity index (PdI), and Tween 80/Span 80 ratio (TSR) of formulations with oil/surfactant ratios (SOR) held at 0.69.

3.3.1. Comet Assay. The results of the comet assay are shown in Table 2. No significant difference was observed for any of the treatment groups.

3.3.2. Micronucleus Test. Figure 3 shows the PCE percentages after 14 days of treatment with AO, AN, and DOX and their respective controls. In the controls and groups treated with the AO and AN, the percentages of these cells remained close to 50%. However, there was a significant reduction in the numbers of these cells in all groups treated with DOX.

Table 3 shows the frequency of micronuclei (MN) in polychromatic erythrocytes (MNPCEs) and normochromatic erythrocytes (MNNCEs) in all treatment groups. There was no significant difference between the controls and groups treated with AO and AN. Treatment with DOX alone resulted in increased formation of both MNPCEs and MNNCEs. In contrast, these values were reduced in the animals pretreated with AO and AN after applying DOX: in the AO+DOX treatment group, there was a reduction of 89% and 45% in MNPCEs and MNNCEs, respectively, whereas in the group treated with AN + DOX, there was a reduction of 62% and 42% in the occurrence of MNPCEs and MNNCEs, respectively.

4. Discussion

Carapa guianensis is a plant species that provides important benefits to local populations of the Amazon primarily due to the medicinal properties of the oil extracted from its seeds

Table 2: The total damage index percentage (total DI%) in mice blood cells after 14 days of treatment with AO and AN, DOX, and their respective controls.

	AO control	AN control	AO (2000 mg/Kg)	AN (2000 mg/Kg)	AO (2000 mg/Kg) + DOX	AN (2000 mg/Kg) + DOX	DOX
	G1	G2	G3	G4	G5	G6	G7
Total DI%	20.51 (±5.52)	19.83 (±4.45)	19.10 (±2.62)	19.41 (±6.93)	20.54 (±6.95)	20.67 (±5.06)	23.21 (±8.54)

Nonparametric Kruskal-Wallis test; Dunn's multiple comparisons test.

Table 3: Frequency of micronuclei in polychromatic erythrocytes (MNEPC) and normochromatic (MNPCE) in bone marrow of Swiss mice after 14 treatments with AO, AN, and DOX and their respective controls.

	AO control	AN control	AO (2000 mg/Kg)	AN (2000 mg/Kg)	AO (2000 mg/Kg) + DOX	AN (2000 mg/Kg) + DOX	DOX
	G1	G2	G3	G4	G5	G6	G7
MNEPC	0.07 (±0,03)	0.05 (±0.04)	0.07 (±0.06)	0.10 (±0.05)	0.22 (±0.14)	0.77 (±0.26)[a,b,c]	1.37 (±0.70)[a,b,c]
Reduction (%)	-	-	-	-	89	62	-
MNENC	0.07 (±0.06)	0.07 (±0.049)	0.04 (±0.03)	0.05 (±0.04)	0.17 (±0.16)	0.22 (±0.16)	0.34 (±0.10)[c,d]
Reduction (%)	-	-	-	-	45	42	-

Note. Nonparametric Kruskal-Wallis test; Dunn's multiple comparisons test. [a]Differs statistically compared to G1. [b]Differs statistically compared to G2. [c]Differs statistically compared to G3. [d]Differs statistically compared to G4.

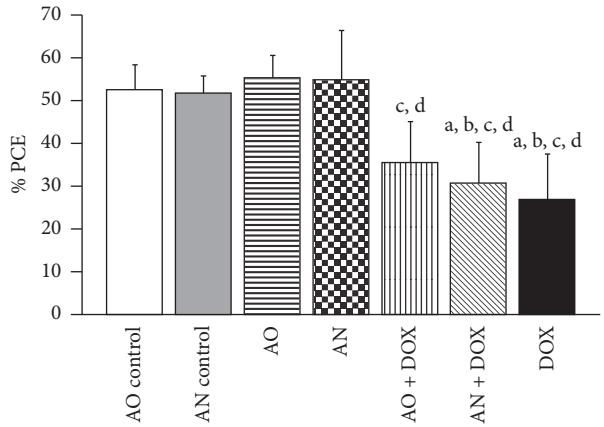

Figure 3: Percentage of polychromatic erythrocytes (% PCEs) in Swiss mice bone marrow after 14 days of treatment with AO and AN, DOX, and their respective controls. ANOVA parametric test; Tukey multiple comparisons. [a]Differs from AO control. [b]Differs AN control. [c]Differs from AO. [d]Differs from AN.

[19]. The use of plants in popular medicine is common worldwide because they represent a major source of new structural compounds that can be applied for the development of new drugs [13]. Additionally, plants can play an important role in health because they may possess antimutagenic and anticarcinogenic properties and contribute to immune system activation and protection against cardiovascular disease [20]. Research on the effects of andiroba is important due to the popular use of the oil extracted from its seeds and its potential for the production of biopharmaceuticals and the development of new technologies.

In vegetable oils, the characterization and identification of the sources of certain types of fatty acids are important to determine which are able to meet the needs for these compounds in the body given the involvement of lipid compounds in the structural composition of membranes and in metabolic functions [2]. The predominant occurrence of oleic acid (39.13%) and linoleic acid (16.86%) in the andiroba sample used in this study shows its ability to perform important biological activities. Oleic acid is known as omega-9 and linoleic acid as omega-6. These are unsaturated fatty acids and are important because they reduce plasma cholesterol levels, which can decrease the risk of developing coronary artery disease when replacing saturated fat in the diet [21, 22]. Some unsaturated fatty acids cannot be produced by the human body and must be supplied by the diet because they are essential to survival [23].

Conversely, some fatty acids have high levels of toxicity. For example, palmitic acid is a compound that can cause cell death by apoptosis [1, 24]. This cell death mechanism can be caused by excessive DNA damage in an attempt to maintain the integrity of the tissue and prevent the effects that the damage can induce [25]. However, taking into consideration the large amount of palmitic acid present in the AO sample used in this study but the absence of any evidence of genotoxicity, it is likely that in this case apoptosis is due to other pathways that do not include excessive DNA damage.

The absence of mortality and signs of toxicity and mutagenicity observed in this study show that both the AO and AN could be used safely by the population regarding genetic damage. These findings confirm the results reported by Milhomem-Paixão et al. (2016) [15] using the same exposure doses as the present study; the authors found the same major compounds from our samples: oleic acid, palmitic acid, and linoleic acid. Therefore, we consider that these compounds at the doses and administration forms tested in this study were not able to induce genotoxicity. A previous study conducted with mice to test the effect of AO extract showed no genotoxic

or mutagenic potential [26]. These authors also investigated the oil's genotoxic potential to induce abnormalities in the sperm heads of mice and did not observe any type of change after administration of the AO extract [27].

To test the protective potential of andiroba in this study, pretreatment with AO and AN was performed prior to exposure to the antineoplastic agent DOX. DOX is a commonly used compound in the treatment of various types of cancer but has limited clinical effect due to its adverse effects [28]. The decrease in the percentage of polychromatic erythrocytes (% PCE) observed in the groups treated with DOX in the MN test may have been due to both the myelosuppressive capacity of the chemotherapeutic and cellular destruction resulting from lipid peroxidation of the cell membrane [29, 30]. The decline in % PCE even in the groups pretreated with AO and AN with subsequent administration of DOX demonstrated that neither AO nor AN protected against the cytotoxicity of this chemotherapeutic agent.

Various antineoplastics are able to produce large amounts of ROS. DOX is an antineoplastic of the class of anthracyclines that has the ability to trigger ROS in the body [31]. In addition to oxidative damage to the DNA, the action mechanism of DOX to induce apoptosis in tumor cells primarily consists of the inhibition of topoisomerase II and the formation of adducts with DNA, which results in blockage of DNA and RNA synthesis and DNA fragmentation [32]. Thus, in this study we used DOX as an inducer of genotoxicity and oxidative stress to determine the protective capacity of the AO and AN.

The decrease in the formation of MN in animals pretreated with AO and AN and DOX compared with mice treated only with DOX suggests an antimutagenic effect of AO and AN. Polyunsaturated fatty acids typical of vegetable oils are known to have important antioxidant activity and therefore exert a protective effect on various types of biomolecules. They can reduce the peroxidation of molecules such as lipids, proteins, and nucleic acids [8]. Because the reduction was much more significant in the AO + DOX group (89% reduction of MNPCEs and 45% of MNNCEs) than the AN + DOX group (62% reduction of MNPCEs and 42% of MNNCEs) and because the AO was diluted with corn oil, the antimutagenic effect may have been enhanced by the joint action of the two oils. However, many studies have used corn oil as a dilution vehicle to test vegetable oils because corn oil is a good medium for this purpose [32–37]. Thus, more accurate research concerning the effect of corn oil in combination with other vegetable oils is needed.

Nevertheless, the protection evidenced in the group pretreated with AN showed the antioxidant potential of andiroba because this formulation contained no corn oil. In this context, incorporating lipid compounds into nanoparticles becomes an interesting strategy in terms of compound bioavailability because it can improve dispersion in environments with little water solubility, increase absorption, and facilitate transport inside cells due to the reduced size of the droplets produced [38]. Milhomem-Paixão et al. (2017) [39] point to the future use of nanoemulsion produced from andiroba in cosmetics and/or development of new herbal remedies and this work shows its protective potential can also be used in its favor.

5. Conclusion

In summary, the data generated in this study indicate that the AO and AN do not cause genetic damage, and they have a protective effect against micronucleus formation in mice treated with doxorubicin. The results presented here are initial, but progress in studies of the biological properties of andiroba, as well as a nanoemulsion produced from it, makes its use in the cosmetics and medicine industry promising.

Disclosure

This study is part of the doctoral thesis of Karina Motta Melo in genetic and molecular biology, under a CAPES Doctoral Scholarship.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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