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Genotoxicity of *Brosimum gaudichaudii* (Moraceae) and *Caesalpinia ferrea* (Fabaceae) *in Astyanax* sp. (Characidae) based on a comet assay

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Genet. Mol. Res. 17 (4): gmr18100
Received August 02, 2018
Accepted December 10, 2018
Published December 17, 2018
DOI http://dx.doi.org/10.4238/gmr18100

ABSTRACT. The plants *Brosimum gaudichaudii* (inharé) and *Caesalpinia ferrea* (jucá) are widely distributed throughout Brazil and are considered medicinal. Inharé has been popularly used as a blood purifier, for the treatment of skin and vitiligo, while jucáis considered analgesic, anti-inflammatory and anticancer. These therapeutic properties have been attributed to phytochemicals such as coumarins, flavonoids and tannins. However, the mechanisms of action of most of these phytochemicals have not yet been fully elucidated and they may compromise human health. Consequently, the evaluation of the genotoxic effect of these plant extracts is fundamental for the determination of safe doses for human consumption. We evaluated the genotoxic effect of various concentrations of extracts of *B. gaudichaudii* and *C. ferrea* using comet assay of erythrocytes from *Astyanax* sp. exposed *in vivo*. In the comet assay indicated the tail migration of DNA increased

significantly in the group of cells exposed to *C. ferrea* for various treatments and the olive tail movement exhibited a significantly higher extent of DNA damage, indicating the potential genotoxicity of the extract. On the other hand, it is premature to claima lack of genotoxic effect for *B. gaudichauddi* extracts since our experimental design was not able to rule out apotential effect of DNA damage as the concentration of 20mg/L seemed to increase the likelihood of genotoxicity. Thus, larger doses of *B. gaudichauddi* extracts should be tested in future studies of the kind. Our investigation provided valuable data for two species of plants widely used in folk medicine in different regions of Brazil. We recommend caution when using thses species for their medicinal properties.

Key words: Genotoxicological Evaluation; Phytoconstituents; Inharé; Jucá; Medicinal Plants

INTRODUCTION

Over the centuries, plants have been used for therapeutic purpose by many communities and ethnic groups. According to the World Health Organization (Robinson and Zhang, 2011), between 65 and 80% of the world population, especially from developing countries, currently use medicinal plants as remedies. Brazil is rich in biodiversity and has an enormous variety of natural resources. The use of medicinal plants in Brazil is strongly influenced by cultural miscegenation, combining the use of exotic species introduced by Africans and Europeans since the time of colonization and the use of local indigenous plants from the repertoire of Brazilian native nations. The use of natural products for treating health problems has been a common global practice since the emergence of human communities (Veiga et al., 2005).

The popular use of phytochemicals for therapeutic purposes has resulted in the discovery of medicinal or toxic properties of many plants (Veiga et al., 2005; Manso et al., 2014).However, most medicinal species need further studies before allowing them to be used for human and animal consumption, especially when it comes to their mutagenic potential (Marques et al., 2003).

Among plants considered with medicinal potential in Brazil are *Brosimum gaudichaudii* (Moraceae) and *Caesalpinia ferrea* (Fabaceae). *Brosimum gaudichaudii* is popularly known as “mama-cadela”, “algodão do campo” or “inharé”, consumed *in natura* and used for construction and paper production (Sano and Almeida, 2008). The leaves and bark of *B. gaudichaudii* are widely used as folk medicine in various regions of Brazil (Lorenzi and Matos, 2009) to treat skin blemishes and vitiligo (De Farias et al., 2015).

Caesalpinia ferrea, commonly known as “pau-ferro” or “jucá”, is considered a forage crop as the trees are common fodder for cattle in extensive rearing conditions in the Northeast region of Brazil (Nascimento et al., 2002). Jucá has been economically explored providing wood for construction, besides its use in folk medicine (Lucena et al., 2007; Sousa et al., 2014). The root of *C. ferrea* is used as an antipyretic and the decoction of stem bark is used to treat diabetes and rheumatism (Gomes, 2003). The extract of *C. ferrea* fruits has analgesic, anti-inflammatory, antihistaminic, antiallergic, anticoagulant, larvicidal

activities hypoglycemic, and anticancer activities (Nakamura et al., 2002; Cavalheiro et al., 2009; Vasconcelos et al., 2011; Monteiro et al., 2011;Pereira et al., 2012; Soares et al., 2018) and has also been used for other therapeutic purposes, such as the treatment of bronchopulmonary conditions and gastrointestinal disorders (Magalhães, 2015).

Medicinal plants are known to contain numerous biologically active compounds and, although they have proven pharmacological properties, can cause harm, including DNA damage, which lead to mutations. Therefore, it is important to screen medicinal plants using genotoxicity assays, such as the comet assay (Ribeiro et al, 2003). The comet assay (single-cell gel electrophoresis) is a simple method widely used for measurement of DNA strand breaks in eukaryotic cells. The comet assay has been useful for the detection of DNA single-strand breaks, alkali-labile sites, and incomplete excision repair events in human cells (Collins 2004; Collins 2014; Carbajal-López et al., 2016).

Herein we describe the results of the comet assay for ethanol extracts from the bark of *B gaudichaudii* and from the seeds of *C. ferrea* on erythrocytes of the *Astyanax* sp, after *in vivo* exposure to different extract concentrations.

MATERIAL AND METHODS

Sample collection

The botanical specimens were collected from Cidelândia, in southern Maranhão, Brazil. The fresh material was placed in an oven at 45ºC and submitted to drying for 20 days. Subsequently, the bark was fragmented and seeds were removed from the fruits for *B. gaudichaudii* and *C. ferrea*, respectively. The bark and seeds were pulverized in a multifunctional, industrial-grade mill. From each specimen collected, part of the botanical material was deposited in the Laboratory of Research and Studies of Natural Products of Pontifical Catholic University of Goiás (PUC-Goiás).

To obtain the ethanol extracts, 160g of each pulverized sample were homogenized in ethanol. The blends were transferred to percolators lined with paper filter and cotton; 800 mL of 70% ethanol was added. The filtrates obtained were evaporated at 50°C for 5 hours.

One hundred specimens of *Astyanax* sp. without distinction of sex were obtained from a local commercial distributor in Goiânia, Goiás, Brazil. They were acclimatized for a period of 15 days in the laboratory, being fed once a day. Following acclimatization, 42 animals were transferred to treatment tanks. In total, fish were separated into 14water tanks in groups of three animals/tank and observed for 48 hours. Subsequently, the animals were exposed to concentrations of 5, 10, and 20mg/L of extracts of *B. gaudichaudii* and *C. ferrea*, for 96 hours, except for the non-exposed control group. For both extracts, treatments were carried out in duplicates. Additionally, the non-exposed groups were also carried out in duplicates. After animal exposure, fish were euthanized with 5% xylocaine to have their gills collected. Tissue samples were placed in conical tubes containing 1mL of fetal bovine serum. Blood samples we obtained by rinsing the gills' tissue with fetal bovine serum. Blood cell suspensions were homogenized and centrifuged for 3 minutes at 1000rpm. After washing, blood cells were pooled by treatments. The two control samples were also pooled together.

Comet Assay in *Astyanax sp.*

The comet assay was performed according to Singh et al. (1988), with modifications by Figueiredo (2012), using the biological samples collected from exposed and unexposed animals. Two hundred nucleoids were analyzed from the polled replicates for each treated and control experiments. For image capture, an epifluorescence microscope (AxioImager 2® Carl Zeiss – Germany) with a 515-560nm excitation filter, and a 590nm barrier filter assisted with ZEN® software was used. The parameters considered in our study were comet tail length, percentage of DNA in the tail of the comet, and Olive tail moment. The CometScore^{TM} software (version 1.5) was used to estimate the level of DNA damage.

Statistical analysis

Kruskall-Wallis test was used for the analysis of parameters considered in the comet assay. Due to the pooling of samples from different individuals, unequal variances could not be ruled out. Thus, multiple comparisons using Bonferroni post hoc procedure was applied. In order to test the effect of the dose on the Olive tail moment, linear regression was used. The tests were performed using IBM SPSS Statistic®, version 21 (IBM Corp, USA) at a confidence interval of 95% and a significance level of 5% ($p \leq 0.05$) was considered.

Ethical considerations

The present study was approved by the Ethics Committee for the Use of Animals from the Pontifical Catholic University of Goiás under protocol number 6244160316/2016.

RESULTS

After 96h post-treatment, the observed survival rates for *Astyanax* sp. were 6/18 (0.33) and 4/18 (0.22), and 2/6 (0.33) for *B. gaudichaudii* and *C. ferrea* extracts, and untreated animals, respectively. No significant differences were found among the three groups. The genotoxic activity of the plant extracts was measured using the comet assay. The results of three parameters of the comet assay for the treatment of Astyanax sp.with the different concentrations of *B. gaudichaudii* and *C. ferrea* are summarized in Tables 1 and 2, respectively.

About 2.5 x more genomic damage was observed in erythrocytes of fish exposed to *C. ferrea* extracts. For *B. gaudichaudii*, the dose of 20mg/L was able to increase about 60% ($p = 0.017$) the tail length of the comet from *Astyanax* sp. erythrocytes when compared to the negative control (Table 1).

Table1. Sample size, means and standard deviations of the parameters analyzed by the comet assay to evaluate the genotoxicity of different concentrations of *Brosimum gaudichaudii*on blood cells of *Astyanax* sp.

Groups analyzed	n	Tail length	% of DNA in tail	Olive tail moment
Negative Control	4	3.17 ± 3.93	6.18 ± 5.66	0.33 ± 0.74
5mg/L	6	2.65 ± 4.62	5.22 ± 4.90	0.20 ± 0.62
10mg/L	3	2.01 ± 5.13	5.51 ± 4.30	0.14 ± 0.40
20mg/L	3	5.13 ± 11.10*	6.98 ± 6.15	0.57 ± 1.56

*Statistically significant ($p < 0.0001$), Kruskall Wallis test and multiple comparisons using Bonferroni post hoc test.

As is shown in Table 2, the tail length parameter demonstrated an average of 2.5x increase in the level of DNA strands breaks in erythrocytes exposed to doses of 5, 10, and 20mg/L of *C. ferrea* extract. For doses of 5 and 10 mg/L, we observed a 3x increase when compared to the negative control, while cells exposed to 20mg/L showed a 2x increase in the tail length when compared to the negative control. The Olive Tail Moment (OTM) parameter exhibited a significantly higher extent of DNA damage in cells treated with different doses compared to the negative control. Figure 1 shows the dose-response relationship between different concentrations of C. *ferrea* extract on the OTM of *Astyanax* sp erythrocytes.

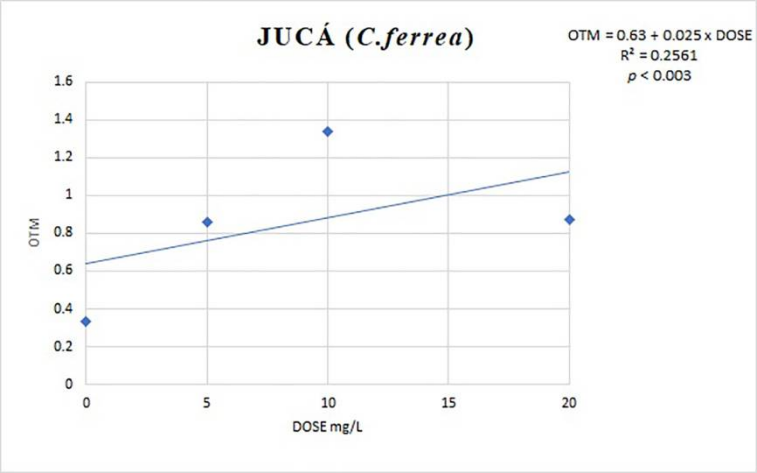

Figure 1. A scatter plot of the potential dose-response of *Caesalpinia ferrea* (Jucá) extract to predict increments in the Olive tail moment, when using erythrocytes of *Astyanax* sp. treated in vivo. Each distribution point corresponds to the arithmetic mean of 200 comets analyzed from polled replicates for each treated and control experiment.

Table 2. Sample size, means and standard deviations of the parameters analyzed by the comet assay to evaluate the genotoxicity of different concentrations of *Caesalpinia ferrea* on blood cells of *Astyanax* sp.

Groups analyzed	n	Tail length	% of DNA in tail	Olive tail moment
Negative Control	4	3.17 ± 3.93	6.18 ± 5.66	0.33 ± 0.74
5mg/L	5	8.97 ± 11.85*	6.48 ± 4.99	0.86 ± 1.57*
10mg/L	4	9.16 ± 10.40*	8.87 ± 9.30*	1.34 ± 2.62*
20mg/L	5	6.96 ± 6.36*	8.69 ± 8.07*	0.87 ± 1.38*

*Statistically significant ($p < 0.0001$), Kruskall Wallis test and multiple comparisons using Bonferroni post hoc test.

DISCUSSION

Several tests using living organisms, especially aquatic species, has been used for the analysis of the potential toxicological effect of various chemicals on the genomes. Various species of fish are used in genotoxic and mutagenic assays as they are considered good indicators for the detection of aquatic pollutants and toxic substances (Buschini et al. 2004). According to Sponchiado et al. (2016), in order to extrapolate genotoxic effects from animal models to humans, vertebrates are the recommended animals as they are both phylogenetically and physiologically closer to humans.

The comet assay was used to analyze the genotoxic potential of plants extracts in erythrocytes of *Astyanax* sp. exposed to different concentrations of *B. gaudichaudii* and *C. ferrea*. We used three recommended parameters to evaluate the potential genotoxicity of these medicinal plants. Tail length indicates the amount of DNA migrating out of the nucleus. Thus, the size of the DNA fragments, which is proportional to the amounts of strand breaks and alkali-labile sites in the genome, will contribute to the length of the comet tails. The second parameter reported is the % DNA in the tail; which is directly proportional to the amount of damaged DNA (Kumaravel and Jha, 2006; de Diana et al., 2018). The OTM is the product of the aforementioned parameters.

The extract of *B. gaudichaudii* at a concentration 20mg/L was able to increaseby1.6x the tail length of comets of fish erythrocytes in relation to the control of untreated fish. This observed effect may indicate genotoxicity of *B. gaudichaudii* a thigher doses. However, the results obtained here are not sufficient for such a claim. Thus, additional tests are required in order to understand to potential risk associated with the consumption of Inharé's extract by humans or animals. At the moment, we can not rule out genotoxicity for this extract.

Genotoxic effects of the furocoumarins (psoralen and bergapten) compounds present in *B. gaudichaudii* have been reported by Jacomassi (2006). Psoralens intercalate in the DNA molecules resulting in DNA adducts. Moreover, psoralens also induce the production of oxygen and superoxide radicalsand these reactive oxygens species are highly genotoxic and may contribute to DNA damage in exposed cells (Varanda et al., 2002). Varanda et al. (2002) also observed mutagenic activity of methanolic extracts of the root bark of *B. gaudichaudii* in a *Salmonella typhimurium* assay and on Chinese Hamster Ovary cells, the authors supposed psoralen and bergapten might contribute to the extract mutagenicity. The possibility of toxicity of a complex mixture of *B. gaudichaudii* extracts must be investigated. Currently, the plant is used to produce a natural medicine widely used for the treatment of vitiligo in Brazil (Leão et al., 2005).

On the other hand, according to the comet assay parameters, we report a genotoxic effect of *C. ferrea* extracts after exposing *Astyanax* sp. *in vivo* to all concentrations of a complex mixture obtained from the seeds when compared to the control group. In addition to the genotoxic effect of *C. ferrea*, we also observed a clastogenic response to exposure at a dose of 20mg/L, represented by a significant reduction in tail length. This difference in tail length was also significantly different from that of the two other doses used in the treatment regimen. Higher doses of C. *ferrea* reduced the tail length of the comets. A possible explanation is that when cells are exposed to a complex mixture of Jucáseed, they produce larger DNA fragments, most likely due to a clastogenic effect. Further studies to understand the biological effect of Jucá extract on vertebrates must be carried before the product is released for human and animal consumption. The current results suggest that C. *ferrea* is able to cause DNA strand breaks and a higher density in the % of DNA in the tail. This effect was noticed in a dose-response curve (Figure 1) where about 25% of the variability in the data could be significantly explained by the increase in the dose of C. *ferrea* extract.

The genotoxic and clastogenic effects observed in the comet assay with *Astyanax* sp. erythrocytes, following exposure to different concentrations of *C. ferrea* extracts, could be explained by the presence of bioactive compounds, such as flavonoids, saponins, tannins, coumarins, steroids, phenolic compounds, gallic acid, catechin, epicatechin, and ellagic acid

or by the activation of caspasesto regulate apoptosis (Wyrepkowski et al., 2014). Activation of caspases leads to DNA fragmentation, which is demonstrated by an increase in average tail length of comets and to its extreme to cell death, as we hypothesize this as the potential biological effect of *B. gaudichaudii* on fish erythrocytes. According to Castro et al. (2004), flavonoids, tannins and terpenoids are chemical compounds of plants that have been identified as carcinogenic and mutagenic. Santos (2006) found phenolic compounds (flavonoids and tannins), in *B. gaudichaudii* and *C. ferrea*, induced mutations in an Ames test and a micronucleus test using mouse blood cells. On the other hand, according to Silva et al. (2015), aqueous extract of *C. ferrea* pods are cytotoxic forroot cells of *Allium cepa*, causing inhibition in cell division. Wyrepkowski et al. (2014) worked with ethanolic extract from the stem bark of *C. ferrea* using a *Salmonella* microsome assay and identified absence of a mutagenic effect.

The application of genotoxicity tests, such as the comet assay, using model species with phylogenetic proximity to humans is important because close species could respond to genomic damage in similar ways and may be indicative of potential genotoxicity allowing the understanding of how the damage can manifest as a mutation, numerical changes or chromosomal recombination using other markers, such as micronuclei and chromosome aberrations (Sponchiado et al., 2016).It is premature to affirm a genotoxic effect of *B. gaudichaudii* extract in fish erythrocytes. However, the comet assay demonstrated genotoxic potential for *C. ferrea* extract. Additional studies are needed to evaluate genotoxicity and mutagenic potential of *B. gaudichaudii* and *C. ferrea* extracts since both species are widely used in folk medicine throughout Brazil. Thus, we recommend caution when using these species for their medicinal properties.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors thank FAPEG and CAPES for funding this research.

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