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Biology-genetics

Mangifera indica L.: Assessing the cytogenotoxicity of aqueous extract through micronucleus and comet assays

Mangifera indica L.: Avaliando a citogenotoxicidade do extrato aquoso através do micronúcleo e ensaio de eletroforese em gel de célula única

Ellen Borges Rocha[I], Jônatas Sennas Souza[I], Mayara Christine Silva[I], Danilo Giovanni Narciso Pastura[I], Raphael Santiago Alves[I], Hataânderson Luiz Cabral Santos[I], Helcio Resende Borba[I], Viviane Moreira Lima[I]

[I] Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brasil

ABSTRACT

Mangifera indica L., known as mango tree, is a species belonging to family Anacardiaceae, widely used in popular medicine whose leaves are used to treat several diseases. Although several studies did not detect the cytogenotoxic activity of *M. indica* leaf extract, other authors have observed these feature and highlighted the importance of further research about the toxic potential of *M. indica* leaf extract. The aim of the current research is to assess the cytogenotoxic potential of the aqueous extract of *M. indica* leaves through comet DNA assay and micronucleus test. It was possible observing significant increase in the number of micronucleated polychromatic erythrocytes (MNPCE) in mice subjected to the treatment with the extract, in addition to significant increase in DNA damage index in comparison to the negative control group. The extract also presented cytotoxic effect caused by significant reduction in the polychromatic:normochromatic erythrocyte (PCE/NCE) ratio in comparison to the negative control group. The extract of *M. indica* leaves is cytogenotoxic under the conditions used in the current research; this outcome highlighted the importance of comparative studies applied to the several used processing methods, as well as to cultivation conditions, to certify the safety in the popular use of medicinal plants.

Keywords: Medicinal plant; Genotoxicity; Cytotoxicity; Phytochemical; Infusion

RESUMO

Mangifera indica L., conhecida como mangueira, é uma espécie pertencente à família Anacardiaceae, amplamente utilizada na medicina popular, cujas folhas são utilizadas no tratamento de diversas doenças. Embora vários estudos não tenham detectado a atividade citogenotóxica do extrato da folha

de *M. indica*, outros autores observaram essa característica e destacaram a importância de novas pesquisas sobre o potencial tóxico do extrato da folha de *M. indica*. Sendo assim, o trabalho objetivou analisar o potencial citogenotóxico do extrato aquoso das folhas de *M. indica*, através do ensaio de DNA cometa e do teste do micronúcleo. Foi possível observar um aumento significativo no número de eritrócitos policromáticos micronucleados (MNPCEs) nos camundongos submetidos ao extrato, além de um aumento significativo no índice de danos ao DNA, quando comparado ao controle negativo. O extrato também apresentou efeito citotóxico, com uma redução significativa na relação de eritrócitos policromáticos/eritrócitos normocromáticos (PCE/NCE) quando comparado ao controle negativo. Conclui-se que o extrato obtido a partir das folhas de *M. indica* é citogenotóxico nas condições utilizadas neste trabalho; estes resultados reforçam a importância de estudos comparativos aplicados aos diversos métodos de processamento utilizados, bem como às condições de cultivo, para atestar a segurança no uso popular de plantas medicinais.

Palavras-chave: Planta medicinal; Genotoxicidade; Citotoxicidade; Fitoquímica; Infusão

1 INTRODUCTION

Mangifera indica L., popularly known as mango tree, is a large tree belonging to family Anacardiaceae, native to Asia, and widely grown in tropical and subtropical regions worldwide. The species' bark, roots and leaves are used in popular medicine*;* its leaves are used to treat diseases such as pain, diarrhea, dysentery, fever, cough, throat affections inflammation, diabetes and burns among others (SEVERI *et al*., 2009; YAKUBU and SALIMON, 2015; VILLAS BOAS *et al*., 2019). Studies about the chemical components of *M. indica* date back to the 20th century and the scientific interest in its biological properties has grown in recent years. The species' biologically active components, once isolated, have several therapeutic applications, including antifungal, antibacterial, antiparasitic, anticancer, antioxidant, antidiabetic activity, among others (BATOOL *et al*., 2018; SEVERI *et al.*, 2009).

The wide array of applications and use in popular medicine of *M. indica* leaf extract highlight the importance of studies regarding its cytotoxic and/or genotoxic potential. Previous studies did not detect any genotoxic activity in aqueous extract from *M. indica* leaves (LIMA *et al.*, 2016; REDDEMAN *et al.*, 2019; VILLAS BOAS *et al.*, 2019). Other studies reported anticarcinogenic activity in aqueous extract from *M. indica* leaves (BONTEMPO and ORSOLIN, 2016). However, Reddeman *et al*. (2019) reported significant increase in the number of

chromosomal aberrations in assay performed with mammalian cells, *in vitro,* during a whole set of tests. Such outcomes highlight the need for further research that contribute to the safe use of extracts in the production of medicines, as well as to planning informational actions about the correct use of medicinal plants by the general population.

The aim of the current research was to assess the cytogenotoxicity of aqueous *M. indica* leaf extract based on the micronucleus and comet DNA assays carried out with mice's peripheral blood.

2 MATERIAL AND METHODS

2.1 Sample collection and identification

M. indica leaves at different ages ranging from young to mature ones were collected at Rio de Janeiro Federal Rural University (UFRRJ) campus, Seropédica Municipality, Rio de Janeiro State/Brazil. The herbarium technician Thiago Azevedo Amorim from the Botany Department of UFRRJ recognized the botanical material; a voucher specimen was deposited in the University´s Herbarium under number RBR 36380.

2.2 Preparation of aqueous *M. indica* leaf extract

Immediately after the collection, leaves were taken to the Laboratory of Plant Genotoxic Activity (LAGEP) of UFRRJ; they were spread on a table and left to air dry at room temperature (28 °C) in the dark for 72 hours. The naturally dried leaves were then placed in amber flask until extract preparation.

The leaf extract was obtained through infusion, which is the preparation procedure often used by the population. Dried and ground leaf samples (5.0 g in weight) were placed in glass vials filled with 100 mL of distilled water at a temperature of 90 °C. The vials were immediately cover and kept under this

condition for 10 minutes to obtaining extract at concentration of 50 mg/mL, which was used in micronucleus, comet assay and phytochemical analysis. Crude extracts were filtered through cotton cloth in order to remove residues, after they returned to room temperature. Fresh extracts were prepared before their in the experiments, in a daily basis.

2.3 Extract phytochemical screening

Phytochemical analysis was carried out with 50 mg of extract, based on the techniques described by Hossain *et al*. (2013), Cai, Shi and Gao (2011), Saklani *et al*. (2012), Morsy (2014) and Kumar (2014) to identify and quantify the chemical constituents in the aqueous extract. The aqueous extract was dried through evaporation, in water bath, at 50 °C. Screening was performed to reduce sugars, saponins, tannins, cardiac glycosides, polysaccharides, anthraquinones, flavonoids and alkaloids. Benedict's reagent was adopted to detect the reducing sugars. Froth formation in test tube was generated through the vigorous shaking of diluted samples with saponins. Kedde's reagent detected the presence of cardiac glycosides. Lugol evidenced the presence of polysaccharides. Dragendorff, Mayer, Sonnenschein and Bouchardat's reagents were used to detect the alkaloids. Anthraquinones were verified through Borntränger test. Flavonoids were tested through the Shinoda test. Gelatin solution test, ferric chloride reagent and the lead acetic test were adopted to detect the tannins.

2.4 Animals and experimental design

Male and female albino Swiss mice (*Mus musculus*) in the age group 10 weeks and body mass of 25 g were provided by the Central Animal House of UFRRJ. Polyethylene cages housed ten animals, each; the cages were stored at constant temperature of 22°C ±1 °C and 12-h light/dark cycle. Standard feed pellets and water were provided *ad libitum*.

Assays were carried out with groups of 10 animals (five of each sex) through oral gavage (except for the positive control group). Group I: animals treated with a single dose (2,000 mg/kg) of aqueous *M. indica* leaf extract, following OECD guidelines (2016); Group II (positive control): animals treated with a single dose of cyclophosphamide (50 mg/kg) through intraperitoneal application; Group III (negative control): animals only treated with water.

Experimental procedures were performed in compliance with the regulations from National Council for Animal Experiment Control (CONCEA) and were approved by The Ethics Committee on Animal Use of the Rio de Janeiro Federal Rural University (UFRRJ) the current study (protocol 001/2016).

2.4.1 Comet assay

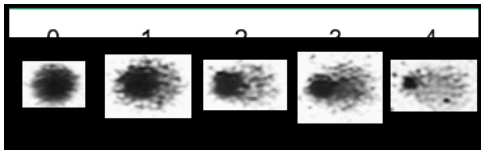
DNA comet assay followed the methods proposed by García *et al*. (2004), with modifications. Three drops of peripheral blood were collected from the retro orbital plexus of each animal 24 hours after the treatment (BERNO *et al*., 2016). Slides coated with normal melting point agarose (1.5%) were prepared with a mixture of 10μL of cell suspension from each animal and 90μL of low melting point agarose (0.75% v/v), at 37^{o}C. The mixture was spread on the slides with the aid of coverslip; both were stored in refrigerator for 10 to 20 minutes - care was taken to avoid direct exposure to light in order to prevent further damages to DNA. Next, the coverslip was carefully removed, the slides were immersed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, and pH 10.0) and protected from light for 24 hours, at 4^{o}C. After lysis, slides were incubated in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13) for 20 minutes at 4°C to allow DNA denaturation. Electrophoresis was performed in this same buffer, at 25 V, 300 mA, for 20 minutes, at 4°C. The slides were then incubated in neutralization solution (0.4 M Tris-HCl, pH 7.5) for approximately 5

minutes. These procedures were repeated twice. The slides were washed twice in distilled water and left to dry at 37°C, for 2 hours.

Afterwards, the slides were fixed in fixation solution (15% trichloroacetic acid, 5% zinc sulfate heptahydrate, and 5% glycerol) for 10 minutes, washed twice in distilled water and dried at 37°C, for two hours. Next, they were rehydrated for 5 minutes in distilled water, transferred to staining bowl and stained for 35 minutes under moderate agitation, at 37°C, in the dark. The staining solution was prepared at assay time; it was composed of 66 mL of solution A (5% sodium carbonate) and 34 mL of solution B (0.1% Ammonium nitrate, 0.1% Silver nitrate, 0.25% Tungstosilicic acid and 0.15% formaldehyde). Subsequently, the slides were washed three times in distilled water, immersed in 90 mL of stop solution (1% acetic acid), for 5 minutes, washed three times in distilled water, again, and left to dry at room temperature.

Slides were observed in Olympus BX binocular optical microscope (400x magnification). One hundred cells were assessed per animal (from two slides), 1.000 cells per treatment. Visual assessment classified damages based on tail intensity into five classes, namely: from undamaged (0) to maximum damage (4) (Fig. 1). Damage index was calculated by adding the number of cells identified in each class and multiplying it by the class value.

Figure 1 – Level of DNA damage in the comet assay. Class 0 represents undamaged cells and class 4 represents maximum damage level. 400X magnification



Source: Author (2021)

2.4.2 Micronucleus assay

Micronucleus followed the methods proposed by Andrade, Perazzo and Maistro (2008), with modifications. Peripheral blood (20 µL) was collected from the retro orbital of each animal, 48 and 72 hours after the aforementioned treatment, following OECD guidelines. Samples were deposited on slide in order to prepare the smears, which were fixed in methanol and stained with Leishman's solution.

In total, 4,000 polychromatic erythrocytes (PCE) from each Swiss mouse were scored: 2,000 cells from 48-hour blood samples and 2,000 cells from 72-hour sample. The cytotoxic potential evaluation counted on 1,000 cells per animal; it was carried out to determine the polychromatic:normochromatic erythrocyte (PCE/NCE) ratio. All slides received codes to prevent identification and were evaluated in common optical microscope (100X magnification).

2.5 Statistical analysis

Bioestat 5.0 software (AYRES *et al*., 2007) was used in the statistical analysis. The statistical treatment applied to DNA comet assay data was based on Student's t-test; all groups were compared to the negative control group. Micronucleus test data were assessed through χ^2 test and Yates' correction. $P < 0.05$ was statistically significant.

3 RESULTS

3.1 Comet assay

Based on DNA comet assay results, the aqueous *M. indica* leaf extract caused significantly increased the number of damaged cells in comparison to the

negative control group, which confirms the genotoxic potential of this extract (Table 1).

Table 1- Mean peripheral blood damage index of mice 24 hours after treatments

Treat.	Conc (mg/kg)	total number of cells	DI
NC	dH_2O	1,000	$8.9 \pm 9.08^{(b)}$
PC	100	1,000	$36.3 \pm 22.8^{(a,c)}$
AQ MI	2,000	1,000	$24.6 \pm 19.27^{(c)}$

(a), (b) and (c) – values followed by different letters recorded significant difference (P<0.05) in the T-*Student* test. Treat. – Treatment; NC – negative control; PC – positive control; AQ MI – aqueous *M. indica* leaf extract; Conc. – concentration; DI – damage index
Source: Author (2021)

3.2 Micronucleus assay

Results recorded 48 hours after the treatment (Table 2) have shown significant increase in the number of micronucleated polychromatic erythrocytes (MNPCE) in mice treated with the extract in comparison to the negative control group. There was significant reduction in the number of MNPCE 72 hours after the treatment (Table 3).

Table 2. Frequency of micronucleated polychromatic erythrocytes (MNPCE) in mice, 48 hours after treatments

Treatment	Number of assessed cells	PCE	MNPCE		PCE:NCE ratio
			Nº	%	
NC	20,000	19974	$26^{(b)}$	0.13	$0.021^{(a)}$
PC	20,000	19917	$83^{(a)}$	0.415	$0.0006^{(b)}$
AQ MI	20,000	19950	$50^{(c)}$	0.25	$0.013^{(c)}$

(a), (b) and (c) – values followed by different letters in the same column were statistically difference (P<0.05) in the χ^2 test; NC – negative control; PC – positive control; AQ MI – aqueous *M. indica* leaf extract; PCE – polychromatic erythrocytes; NCE - normochromatic erythrocytes; MNPCE - micronucleated polychromatic erythrocytes.
Source: Author (2021)

Table 3. Frequency of micronucleated polychromatic erythrocytes (MNPCE) in mice 72 hours after treatments

Treatment	Number of assessed cells	PCE	MNPCE		PCE:NCE ratio
			Nº	%	
NC	20,000	19992	8[(b)]	0,04	0,015[(a)]
PC	20,000	19977	23[(a)]	0,115	0,001[(b)]
AQ MI	20,000	19995	5[(b)]	0,025	0,001[(b)]

(a) and (b) – values followed by different letters in the same column were statistically difference ($P<0.05$) in the χ^2 test; NC – negative control; PC – positive control; AQ MI – aqueous *M. indica* leaf extract; PCE – polychromatic erythrocytes; NCE - normochromatic erythrocytes; MNPCE - micronucleated polychromatic erythrocytes.
Source: Author (2021)

It is possible observing differences in the PCE:NCE ratio between treatments, as well as in its genotoxic effect. Animals subjected to aqueous *M. indica* leaf extract reported significant PCE:NCE ratio reduction in comparison to the negative control group, which has proven the cytotoxic activity of this extract (Tables 2 and 3).

3.3 Extract phytochemical screening

The phytochemical analysis applied to the aqueous extract detected the presence of reducing sugars, saponins and tannins (Table 4).

Table 4. Phytochemical analysis applied to aqueous *M. indica* leaf extract.

Secondary compounds	Aqueous extract
Reducing sugars	+
Saponins	+
Cardiac glycosides	-
Polysaccharides	-
Alkaloids	-
Flavonoids	-
Tannins	+
Anthraquinones	-

(-) Absence, (+) Presence
Source: Author (2021)

4 DISCUSSION

Comet DNA assay is a widely used method to assess initial DNA damages since it allows detecting several lesions, including the breaking of single and double strand and alkali-labile sites (COLLINS *et al.*, 2008). Results in the current research have shown that the aqueous *M. indica* leaf extract significantly increased DNA damages in comparison to the negative control group, and this outcome indicates the genotoxic potential of this extract. Other tests, such as the micronucleus test, are required to confirm the genotoxicity of this extract since DNA injuries are subject to correction. Micronuclei from acentric chromosome fragments or from whole chromosomes were not associated with the spindle; therefore, they were not included in the main nucleus, which indicates clastogenic and aneugenic effects (ALBERTINI *et al.*, 2000). The aqueous *M. indica* leaf extract has significantly increased the number of MNPCE in comparison to the negative control group, 48 hours after the treatment, and this finding proves the extract's genotoxicity. The assessment conducted 72 hours after the treatment has shown MNPCE reduction in peripheral blood samples from all groups, which indicates that this amount of time was not ideal to assess the genotoxic potential of the herein tested extract - other research have shown similar results (BERNO *et al.*, 2016; SCHNEIDER *et al.*, 2016; SILVA *et al.*, 2020). In addition to genotoxicity,

micronucleus test allowed detecting the extract's cytotoxicity, since there was significant PCE:NCE ratio reduction in the animals treated with the aqueous extract in compared to the negative group. The research group in charge of the current study had already shown the cytotoxic potential of *M. indica* leaves, which was proven by assessing their aqueous extract - at lower concentrations (15mg/mL and 30 mg/mL) than that used in the present study - through the *Allium cepa* system (LIMA *et al.*, 2016)

Studies carried out by other authors with aqueous *M. indica* leaf extract based on methodologies similar the herein adopted one did not detect the genotoxic and cytotoxic potential of this extract (REDDEMAN *et al.*, 2019; VILLAS BOAS *et al.*, 2019). Villas Boas *et al*. (2019) conducted comet DNA assays and micronucleus test with mice treated with aqueous *M. indica* leaf extract (125, 250, 500 and 1000 mg/kg) - from leaves collected in Mato Grosso do Sul State/Brazil -, for 28 days; they noticed significant PCE:NCE ratio increase. The authors attributed such an outcome to the extracts' cytoprotective effect. It is important highlighting that the extract, in the aforementioned study, was obtained by boiling the pulverized material in water for 4 hours. The extraction process is the first step towards finding the bioactive compounds of medicinal plants; several methodologies based on different solvent types have been used for this purpose, many of them depending on heating at different temperatures and on incubation time. However, several organic compounds are sensitive to temperature, they can lose integrity and biological activity due to degradation at heating (MOREIRA *et al*. 2019). Lau *et al*. (2013) observed that different extraction methods (hot and cold aqueous extraction) resulted in *Lignosus rhinocerotis* mushroom extracts (used in popular medicine to treat different diseases, including cancer) presenting different features; cytotoxic components were only found in extract obtained through cold aqueous extraction method. Reddeman *et al*. (2019) conducted series of toxicological exams with aqueous *M. indica* leaf extract from China, which had 60% mangiferin. Leaves were subjected to extraction process with

water and ethanol; extracts were mixed and standardized to contain 60% – 65% mangiferin. Micronucleus exam was conducted with bone marrow from mice treated with doses of 500, 1,000 and 2,000 mg/Kg of extract, but it did not detect any genotoxic effect. However, the chromosomal aberration test performed *in vitro* displayed the clastogenic effect of this extract. The authors of the aforementioned study attributed such an outcome to variations in the biological effects of the assessed components, depending on their concentration. For example, low concentrations of polyphenols, such as curcumin, can induce chromosomal aberrations and act as antioxidant at higher concentrations. Differently from what was observed by Villas Boas *et al*. (2019), there was no significant change in the PCE:NCE ratio; however, there was small decrease in the PCE:NCE ratio at dose of 2000mg/kg.

It is important highlighting that secondary metabolites, which are responsible for the biological activity of plant extracts, undergo concentration and activity variations influenced by abiotic and biotic factors, such as light, water availability, temperature, soil composition, interactions with microorganisms, as well as by plant features such as phenology and ontogeny (PAVARINI *et al*. 2012). Therefore, changes in these abiotic and biotic factors can explain differences in biological effects observed in previous research.

The phytochemical analysis carried out in the current research detected the presence of reducing sugars, saponins, and tannins. Other authors have already reported the cytotoxic and genotoxic effect of saponins and tannins (ALMEIDA *et al.*, 2016; BUYUKLEYLA *et al.*, 2012; LABIENIEC and GABRYELAK, 2003; LIU *et al.*, 2011) and detected saponins and tannins in *M. indica* leaf extracts (CHIRAYATHL *et al*. 2019; VILLAS BOAS *et al*., 2019; YAKUBU and SALIMON, 2015)

Abiotic and biotic factors influence the biosynthesis and accumulation of saponins and tannins, as well as of other metabolites (PAVARINI *et al.*, 2012); changes in their concentration can alter the biological effect of a metabolite.

Labieniec and Gabryelak (2003) reported that components such as tannins can behave as antioxidants or pro-oxidants, depending on their concentration.

Therefore, a wide variety of interactions between mutagenic and protective components can modulate the toxic and genotoxic activity of plant extracts, which have different results depending on concentration of components and preparation mode. It is necessary encouraging the expansion of studies comparing different preparation methods and plants growth conditions, in order to standardize protocols used in popular medicine and certify safety in the use of medicinal plants extracts.

5 CONCLUSION

Aqueous *M. indica* leaf extract is genotoxic and cytotoxic under the herein used conditions. In addition, such an outcome highlights the importance of comparative studies applied to the different processing methods, as well as to cultivation conditions, in order to certify the safety of medicinal plant using.

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Authorship contributions

1 – Ellen Borges Rocha:
Pharmaceutical, Academic in pharmacies
https://orcid.org/0000-0002-9219-4660 • ellenagn@yahoo.com.br
Contribuition: Investigation, Formal analysis – DNA comet assay

2 – Jônatas Sennas Souza:
Pharmaceutical, Academic in pharmacies
https://orcid.org/0000-0002-3296-4432 • jonatassennas@hotmail.com
Contribuition: Investigation, Formal analysis – DNA comet assay

3 – Mayara Christine Silva:
Pharmaceutical, Graduated in pharmacy
https://orcid.org/0000-0002-9974-3044 • mayarachristine.silva@hotmail.com
Contribution: Investigation - Phytochemical

4 – Danilo Giovanni Narciso Pastura:
Biological science, Graduated in biological sciences
https://orcid.org/0000-0002-8986-2613 • danilognp22@gmail.com
Contribution: Investigation, Formal analysis – Micronucleus assay

5 – Raphael Santiago Alves:
Pharmaceutical, Academic in pharmacies
https://orcid.org/0000-0002-8286-6910 • raphaosantiago@yahoo.com.br
Contribution: Investigation, Formal analysis – Micronucleus assay

6 – Hataânderson Luiz Cabral Santos
Biological science, Graduated in Biological Sciences
https://orcid.org/0000-0003-0086-3108 • hluizcabral@gmail.com
Contribution: Investigation, Formal analysis – Micronucleus assay

7 – Helcio Resende Borba:
Veterinary science, Douctor´s in Veterinary Sciences
https://orcid.org/0000-0002-9809-2462 • borbahr4@gmail.com
Contribution: Conceptualization, Supervision, Writing – review & editing

8 – Viviane Moreira Lima:
Biological Science, Douctor´s in Biological Sciences (Genetics)
https://orcid.org/0000-0001-7930-8312 • vilima@ufrrj.br
Contribution: Conceptualization, Project administration, Supervision, Writing – original draft

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