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Full Length Research Paper

Antioxidants from three *Swietenia* Species (Meliaceae)

Lina M. Preciado[1], Tatiana Lobo-Echeverri[1]*, Jaime A. Pereañez[2], Karol Zapata[1] and Benjamín A. Rojano[1]

[1]Group of Chemistry of Natural Products and Foodstuffs. School of Chemistry, College of Science, Universidad Nacional de Colombia-Sede Medellín, Calle 59A No 63-20 Medellín, Colombia.
[2]Program of Ophidism/Scorpionism, College of Pharmaceutical Chemistry, Universidad de Antioquia, Calle 70 No 52-21, Medellín, Colombia.

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The genus *Swietenia* (Meliaceae) has a wide variety of secondary metabolites with reported antioxidant activity, such as flavonoids and limonoids. In the present study, the antioxidant capacity, along with the phenol and flavonoid contents of the leaf extracts of three species of this genus: *Swietenia mahagoni*, *Swietenia macrophylla*, and *Swietenia humilis* were evaluated. The antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ferric reducing/antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) methods. The results showed that the three species had significant antioxidant activity and substantial contents of phenolic compounds and flavonoids. The species *S. macrophylla* was the most effective, and compounds with recognized antioxidant capability were detected by gas chromatography coupled with mass spectrometry (GC-MS). Catechin was the most abundant constituent in the active fractions, and was confirmed and quantified by high performance liquid chromatography (HPLC).

Key words: *Swietenia macrophylla*, antioxidant, (+)-catechin, flavonoids, phenols.

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are produced constantly in metabolic reactions of aerobic organisms. When there is an excess of oxygen in cells, or its reduction is not enough, ROS such as the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2), are generated (Cotgreave et al., 1988). Aerobic organisms have a natural defensive system of enzymatic and non-enzymatic mechanisms of detoxification of these radicals. However, when this endogenous system fails, the cell goes into a stage of oxidative stress responsible for cellular degeneration. In this way, free radicals and other ROS can react with proteins, lipids, and DNA, causing irreversible damages (Donaldson et al., 1996). Furthermore, oxidative stress in cells cause a wide range of diseases such as cancer, atherosclerosis, cataracts, neurodegenerative disorders, and inflammation (Aruoma, 1998). Antioxidant molecules that are able to donate electrons to stabilize free radicals

*Corresponding author. E-mail: tloboech@unal.edu.co. Tel: (+574) 430 9393.

and neutralize their effects are relevant to prevent oxidative stress. Thus, there is an increasing interest in antioxidant and free radical scavenging properties of compounds derived from plants, with numerous reports of plant products with a range of antioxidant properties. Some groups of metabolites such as terpenes, flavonoids (flavones, isoflavones, anthocyanins, flavanones), and other polyphenols (ellagic acid, gallic acid, and tannins) have shown promising antioxidant properties (Prat, 1999).

The genus *Swietenia* (Meliaceae) includes about 145 species distributed in the Neotropics. The species *Swietenia mahagoni* Jacq, *Swietenia macrophylla* King and *Swietenia humilis* Zucc are timber species widely used in traditional medicine. The three species have previous reports validating some of the traditional uses or of promising bioactivities in the laboratory. In the case of *S. mahagoni* the antagonist activity of the platelet-activating factor was determined (Kadota et al., 1989). In addition, methyl esters of the cholinergic acid with a high antioxidant activity were reported for this species (Matsuse et al., 1997). Similarly, the *in vitro* antioxidant potential of the methanol extract of seeds of *S. mahagoni* was determined using different techniques, with a positive effect to scavenge free radicals and inhibit the enzyme xanthine oxidase, responsible for generating ROS (Sahgal et al., 2009). Antioxidant properties of different organic extracts of flowers and bark were also reported for this species (Rahman et al., 2014). For *S. macrophylla*, the antioxidant potential of several limonoids was demonstrated through inhibition of superoxide anion generation in human neutrophils as a response to formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (Chen et al., 2010). In addition, from the bark of *S. macrophylla* a new compound, swietemacrophyllanin-catechin-8,7,7,2-epoxy-(methyl-4,5-dihidroxifenil propanoate) was isolated (Falah et al., 2008). This metabolite, in scavenging the DPPH radical, showed an IC_{50} of 56 µg/ml using Trolox as a standard. This was a remarkable result when compared to catechin (IC_{50} 70 µg/ml) and epicatechin (IC_{50} 59 µg/ml) (Falah et al., 2008). Previous studies on *S. humilis* were mainly on its antibacterial (López et al., 2007), antifungal (Angulo et al., 2009), and insecticidal activities (Jiménez et al., 1997).

Based on the antioxidant properties reported for *S. mahagoni* and *S. macrophylla*, the purpose of this study was to focus on the comparative evaluation of the three local species within the genus *Swietenia*. The aim of this research was to evaluate the antioxidant potential of the ethanol extract of the leaves of *S. mahagoni*, *S. macrophylla*, and *S. humilis* using different assessment methods, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), oxygen radical absorbance capacity (ORAC), and ferric reducing/antioxidant power (FRAP) assays.

MATERIALS AND METHODS

All of the reagents used for the antioxidant assays and organic solvents for extraction and fractionation, along with HPLC grade water were obtained from Sigma Aldrich® (St. Louis, MO, USA). For thin layer chromatography aluminum, silica gel (G-60) plates of 0.25 mm (F_{254}) were used (Merck, Darmstadt, Germany). The derivatizing agent used (BSTFA+TMCS) was purchased from Supelco (Bellefonte, PA, USA).

Plant

For the extraction and biological evaluation, dried leaves (2 kg) of *S. humilis, S. macrophylla* and *S. mahagoni* were collected in the tropical and pre-mountain forests of Medellin, Colombia, at 1600 m.s.l (6° 15'41" N, 75° 34'35.5" W). A voucher sample of each species remains at the Herbarium "Gabriel Gutiérrez Villegas" (MEDEL) (Universidad Nacional de Colombia, Medellin branch) under accession numbers TL-102, TL-103 y TL-104, respectively. Samples were identified by Leon Morales (Universidad Nacional de Colombia).

Extraction of plant material

The leaf plant material of the three species (500 g each) was air-dried, milled, and extracted by percolation at room temperature overnight with 90% ethanol (1 L × 100 g). The crude ethanol extract was filtered and concentrated under reduced pressure, using a rotary evaporator (Büchi R-144) at a temperature below 40°C. The resultant ethanol extract was mixed with distilled water to 10%, and then defatted with hexane. Subsequently, the ethanol-aqueous extract was evaporated and lyophilized.

Fractionation of the most active species

The most active species in the antioxidant panel of bioassays was selected for further fractionation. Thus, the ethanolic extract of *S. macrophylla* leaf material was fractionated using a silica gel 60 F_{254} open column, eluting with a gradient of dichloromethane-acetone-methanol, beginning with the less polar solvent and ending with methanol. According to the chromatographic profile obtained by thin layer chromatography (TLC), twenty-five initial fractions were reduced to eight final fractions that were evaluated in the bioassays. After testing, the active fractions F4 and F5 were submitted to GC-MS and HPLC analysis.

Characterization of compounds using gas chromatography-mass spectrometry (GC-MS)

The chemical profiles of the ethanolic extracts of the three species of *Swietenia* and the most promising fractions of *S. macrophylla* were analyzed using a gas chromatograph (Agilent 6890), coupled with a mass spectrometer (Agilent 5973), employing a capillary column of fused silica (Agilent HP-5, 0.25 mm × 30 m × 0.25 µm) covered with 5% phenyl methyl siloxane. All of the samples (extract and fractions) were derivatized before injection according to the method described by Silici and Kutluca (2005). One mg of each sample was diluted in 50 µl of pyridine and a mixture of 100 µl of BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide) with 1% of

trimethylchlorosilane (TMCS) was added. The mixture was heated for 30 min at 100°C. For each sample, 5.0 µl was injected, using helium gas grade 5 (AGA Fano S.A., UAP 99.999%) at a flux of 1.0 ml/min (lineal velocity 37 cm/s). The injection used the split-less mode with an initial temperature of 200°C for 3 min, which was raised to 250°C and maintained for 1 min. Finally, the temperature was raised by 2°C per minute to a maximum of 350°C for 60 min. To obtain the mass data, the detector was fixed at 350°C. The run was made using the SCAN mode between *m/z* 30 to 800. The chromatograms were analyzed with Automated Mass Spectral Deconvolution and Identification System (AMDIS) software, and the spectral database NIST 98 (2001). The identification of compounds was done though comparison of the mass spectral fragmentation patterns of each compound with the databases mentioned.

Determination of (+)-catechin and (-)-epicatechin in the most active species

The confirmation and quantification of (+)-catechin and (-)-epicatechin in the ethanolic extract and active fractions of *S. macrophylla* was performed through HPLC analysis. The separation of both compounds was done using an ultra-aqueous C_{18} column with a particle size of 5 µm (250 mm × 4.6 mm, Merck). As a mobile phase, methanol (A), and formic acid (0.1%) were used in a gradient system of elution of 0.01 min 60% of A; 5 to 12 min 80% of A; 13 to 14 min 60% of A. The mobile phase flux was 1.0 ml/min. The identification of the compounds in the active fractions of *S. macrophylla* was done by comparison with standard samples of (+)-catechin and (-)-epicatechin (Sigma).

Antioxidant determination

The leaf ethanolic extracts of *S. humillis* (Sh), *S. macrophylla* (Smc), and *S. mahagony* (Smh), and the fractions of *S. macrophylla* were ran in an antioxidant panel of assays. All tests were done in triplicate and expressed in internationally accepted units, such as Trolox equivalent antioxidant capacity values (TEAC) through the construction of a standard curve using Trolox®. For the FRAP assay, the antioxidant potential was expressed in vitamin C equivalents (VCEAC). All of the spectrophotometric experiments were carried out in a Multiskan Spectrum UV-Vis plate reader (Thermo Scientific, Finland).

DPPH assay

To quantify the free radical scavenging capacity of the leaf ethanolic extracts of the three species of *Swietenia* and fractions of *S. macrophylla*, the DPPH assay was done according to the method of Brand-Williams et al. (1995) with some modifications (Peyrat-Maillard et al., 2000). The stock solution was 20 mg/L of DPPH dissolved in methanol. The working solution was 990 µl of the stock solution with 10 µl of the extract or fractions at different concentrations. A standard sample was 990 µl of methanol with 10 µl of the sample, and a simple standard of 990 µl DPPH with 10 µl of solvent. Samples reacted for 30 min at room temperature, in the dark. The absorbance was measured at 517 nm. Results were expressed in µM of Trolox equivalent per g of extract.

ABTS$^{\bullet+}$ assay

This technique was performed following the procedure by Re et al. (1999). For the biological evaluation, 10 µl of the extracts or fractions, and 990 µl of the ABTS solution were mixed. Samples reacted for 30 min at room temperature, in the dark. The reference sample was an ABTS solution and the solvent used to dissolve the sample. The absorbance of all samples was taken at 734 nm. Results were expressed in µM of Trolox equivalent per g of extract.

Ferric reducing/antioxidant power assay (FRAP)

This method evaluated the antioxidant potential of the samples according to its capability to reduce Fe^{+3} to Fe^{+2} in a complex with the ferrous tripyridyl triazine complex reagent (TPTZ) (Benzie and Strain, 1996). The stock solution included acetic acid-sodium acetate (pH 3.4), with TPTZ and $FeCl_3$. The working solution was 900 µl of the stock solution, 50 µl of the extracts or fractions, and 50 µl of distilled water. After 60 min of reaction, the absorbance was determined at a wavelength of 593 nm. The standard curve was done using ascorbic acid as a standard reference and results expressed as mg of ascorbic acid per g of extract.

Oxygen radical absorbance capacity assay (ORAC)

This method evaluated the ability of the extracts and fractions to trap peroxil radicals ($ROO^{\bullet}$) responsible for the discoloration of the fluorescent probe fluorescein. The procedure was performed following the literature (Ou et al., 2001; Atala et al., 2009), using Trolox as standard and at controlled conditions of temperature at 37°C and pH at 7.4. Solutions of fluorescein 1×10^{-2} M in PBS (75 mM) and AAPH 0.6 M in OPBS (75 mM), were used. The working solution consisted of 21 µl of fluorescein, 3 µl of PBS, 30 µl of the extracts or fractions, and 50 µl of AAPH. Readings were done at a ʎ of excitation spectrum of 493 nm and slit of excitation 5, ʎ of emission of 515 nm and slit of emission 13, with attenuator of 1% and without attenuator plate. The protective effect was calculated using the differences of the areas under the curve (AUC) of the decrease of fluorescein, between the standard and the sample (extracts or fractions). This was compared with the Trolox curve and results were expressed in micromoles equivalents of Trolox per gram of extract. The area under the fluorescence decay curve (AUC) was calculated as:

$$\text{ORAC} = \frac{(AUC - AUC°)}{(AUC_{Trolox} - AUC°)} \; f \; [Trolox]$$

Where *AUC* is the area under the curve of the extracts or fractions, *AUC°* is the area under the curve for the standard sample, AUC_{Trolox} is the area under the curve for Trolox, and *f* is the factor of dilution of the extracts.

Total phenols assay

The determination of total phenols in the extracts was performed by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). An amount of 50 µl of the extracts was mixed with 125 µl of the Folin reagent, and 400 µl of sodium carbonate 7.1% (w/v), adding distilled water up to 1000 µl. The reading was done at 760 nm and a comparison was established with the standard curve using gallic acid as the phenolic standard. Results were expressed as mg of equivalent of gallic acid per grams of extract.

Table 1. Antioxidant activity and contents of total phenols and flavonoids for the ethanolic extracts of *Swietenia humilis, S. macrophylla* and *S. mahagoni.*

Species	DPPH* µmol Tx/g extract	ABTS* µmol Tx/g extract	FRAP* mg ascorbic acid/g extract	ORAC* µmol Tx/g extract	PHENOLS* mg gallic acid/g extract	FLAVONOIDS* mg catechin/ g extract
Swietenia humilis	89.0 ± 0.5	2479.0 ± 162.6	152.2 ± 3.4	11519.8 ± 211.6	250.9 ± 21.6	165.5 ± 14.9
Swietenia macrophylla	161.9 ± 2.5	4944.6 ± 465.9	276.7 ± 0.7	4576.8 ± 854.1	290.5 ± 19.1	225.1 ± 15.0
Swietenia mahagoni	107.1 ± 0	4076.1 ± 114.1	192.5 ± 2.2	1255.2 ± 23.1	146.4 ± 3.0	286.8 ± 21.7

*The result of each experiment is presented as the mean ± standard deviation (n=3).

Table 2. Major compounds detected through GC-MS in leaf ethanolic extracts of *Swietenia humilis. S macrophylla* and *S. mahagoni*.

Compound	Abundance (%) in *S. humilis*	Abundance (%) in *S. macrophylla*	Abundance (%) in *S. mahagoni*	Fragments of the derivatized compound (*m/z*)
Protocatechuic acid	7.96	12.2	6.10	193 (99.9), 73 (47.1), 370 (42.0), 355 (25.9), 311 (15.1), 194 (14.7), 371 (13.1), 281 (9.3), 356 (9.0), 223 (7.7)
Gallic acid	0	12.7	0	281 (99.9), 458 (66.4), 73 (52.4), 443 (32.4), 459 (27.2), 282 (22.4), 460 (13.2), 444 (12.4), 283 (92), 355 (6.4)
(+)-Catechin	9.50	17.7	13.3	368 (99.9), 73 (72.0), 355 (35.0), 369 (34.0), 370 (16.0), 650 (13.0), 267 (12.0), 356 (12.0), 383 (10.0), 179 (8.0)

Flavonoids total assay

The determination of flavonoids was done following the colorimetric method described my Marinova et al. (2005) with some modifications. An amount of 100 µl of extracts was mixed with 30 µl of $NaNO_2$ 5% (w/v), 30 µl of $AlCl_3$ 10% (w/v), and 200 µl of NaOH 1 M, adding distilled water up to 1000 µl. The reading was done at 510 nm and a comparison was established with the standard curve using (+)-catechin as the standard flavonoid. Results were expressed as mg of equivalent of (+)-catechin per grams of extract.

Statistical analysis

To identify the variation in the biological activity, a one-way analysis of variance (ANOVA) was carried out. When significant differences were detected ($\alpha \leq 0.05$), a Turkey's range test, with a confidence level of 95%, was done to establish the differences in each level of activity. Additionally, correlations among data obtained for the antioxidant activity of the fractions of *S. macrophylla* were calculated using Pearson's correlation coefficient. All tests were done in triplicate and expressed as the median and the standard deviation using R, version 2.15 (R Development Core Team, 2012).

RESULTS

The three species of *Swietenia* showed antioxidant properties in the different assays, and considerable phenol and flavonoid contents in the different assays (Table 1). These results were supported by the ANOVA of the antioxidant potential of the leaf extracts (Figure 1). In the mass spectra of the ethanolic extract of *S. humillis*, *S macrophylla*, and *S. mahagony*, fragmentation patterns of phenolic compounds were detected. The most abundant metabolites in each species are shown in Table 2.

Due to the highest antioxidant potential and the abundance of phenolic compounds, *S. macrophylla* was selected as the most promising lead from the three species evaluated. In order to elucidate the capability of this particular species to

Table 3. Antioxidant activity and contents of total phenols and flavonoids for the fractions of *Swietenia macrophylla.*

Fraction	DPPH* μmol Tx/g extract	ABTS* μmol Tx/g extract	FRAP* mg ascorbic acid/g extract	ORAC* μmol Tx/g extract	PHENOLS* mg gallic acid/g extract	FLAVONOIDS* mg catechin/g extract
F1	16.7 ± 1.9	57.8 ± 2.4	2.7 ± 0.3	36.5 ± 1.7	4.3 ± 0.8	21.2 ± 1.1
F2	36.5 ± 3.3	55.4 ± 3.9	0.7 ± 0.0	75.9 ± 9.5	5.2 ± 0.2	6.7 ± 0.3
F3	121.4 ± 2.8	167.2 ± 6.2	8.3 ± 0.8	152.1 ± 11.7	8.0 ± 0.9	14.7 ± 0.6
F4	4514.7 ± 302.6	3959.4 ± 188.0	360.2 ± 13.1	5079.9 ± 509.7	128.9 ± 7.8	240.2 ± 7.8
F5	3163.2 ± 353.5	3039.0 ± 113.2	226.2 ± 13.2	4481.3 ± 526.3	130.3 ± 11.4	153.4 ± 8.9
F6	571.04 ± 17.9	530.2 ± 8.1	103.5 ± 3.1	1960.8 ± 126.62	55.7 ± 3.2	63.9 ± 2.1
F7	569.8 ± 7.3	282.7 ± 20.7	11.1 ± 0.1	516.9 ± 64.9	132.8 ± 3.3	161.5 ± 13.3
F8	371.47 ± 40.4	579.13 ± 32.9	73.1 ± 1.5	697.2 ± 77.4	49.6 ± 1.4	65.5 ± 6.7

*The result of each experiment is presented as the mean ± standard deviation (n=3).

trap different free radicals in diverse systems, statistical correlations between each of the antioxidant results were performed (Figure 2). To support the aforementioned results, fractionation of the ethanolic extract of *S. macrophylla* and further bioassays were performed for each fraction. There was differential in the antioxidant activity between the eight fractions derived from the leaf extract (Table 3), with the most significant activity found in fractions F4 and F5 (Figure 3).

In an attempt to verify if the compounds detected initially by GC-MS in the leaf extracts were present in fractions F4 and F5, the same method was used for each fraction. As a result, gallic acid was not found in either fraction, while protocatechuic acid was detected only for F5 in a 14.3% level of abundance. Catechin was present in both fractions, in a 7.8% level of abundance for F4 and 17.9% for F5. To support these findings, the determination of catechin and epicatechin in the ethanolic extract of *S. macrophylla* and in fractions F4 and F5 was carried out by HPLC. The ethanolic extract, (+)-catechin was quantified as 2.096 mg/g and (-)-epicatechin as 0.869 mg/g. In fraction F4, (+)-catechin was 1.009 mg/g and (-)-epicatechin as 1.396 mg/g, while in fraction F5 (+)-catechin, 0.184 mg/g and (-)-epicatechin 0.097mg/g. These metabolites were not detected in the other fractions by HPLC.

DISCUSSION

Even though in the three species of *Swietenia* there was an evident antioxidant property and substantial phenol and flavonoid contents, the best results were observed for *S. macrophylla* (Table 1). The results obtained by the different techniques were significantly variable, offering diverse potential according to the types of metabolites present in each plant species (Figure 1). For the DPPH, ABTS and FRAP assays, the species *S. macrophylla* had the highest activity followed by *S. mahagoni*, which was in accordance with previous reports (Falah et al., 2008; Matsuse et al., 1997; Rahman et al., 2014; Sahgal et al., 2009). In particular, for the DPPH and ABTS assays for both species, the latest assay showed higher values, which could be due to the low selectivity of the radical ABTS, which reacted with hydroxylated compounds independently of its antioxidant potential (Roginsky and Lissi, 2005). In contrast, the radical DPPH, has a steric inaccessibility that causes a slow reaction of some compounds that could even make them seem inert to this radical (Prior et al., 2005). In addition, if the antioxidant potential of the extracts is due to the presence of flavonoids or other phenols (Es-Safi et al., 2007), it has to be considered that the radical DPPH does not react with flavonoids that do not have hydroxyl groups in the B ring, or phenols that have only one hydroxyl group (Roginsky and Lissi, 2005). This could be another reason for the differences in the values between the DPPH and the ABTS assays, apart from the low selectivity of the ABTS radical or the steric inaccessibility of the DPPH radical.

In the particular case of the FRAP assay, the presence of metabolites with the ability to stabilize a free radical molecule through a single-electron transfer mechanism (SET) was also evidenced for *S. macrophylla*. This could point to the presence of metabolites with a high reducing potential that is related to molecules with a high degree of hydroxylation or highly conjugated polyphenols (Pulido et al., 2000). This is supported by the significant total phenolics content for this species (Table 1). In contrast, in the ORAC evaluation *S. humilis* presented the highest number of TEAC equivalents, with interesting results, since no reports of antioxidant activity were found for this species. In this case, we suggest that *S. humilis* leaves could have a higher content of antioxidant hydrophilic metabolites with a capability to trap peroxide radicals $ROO^{\cdot}$ through a hydrogen atom transfer (HAT) mechanism.

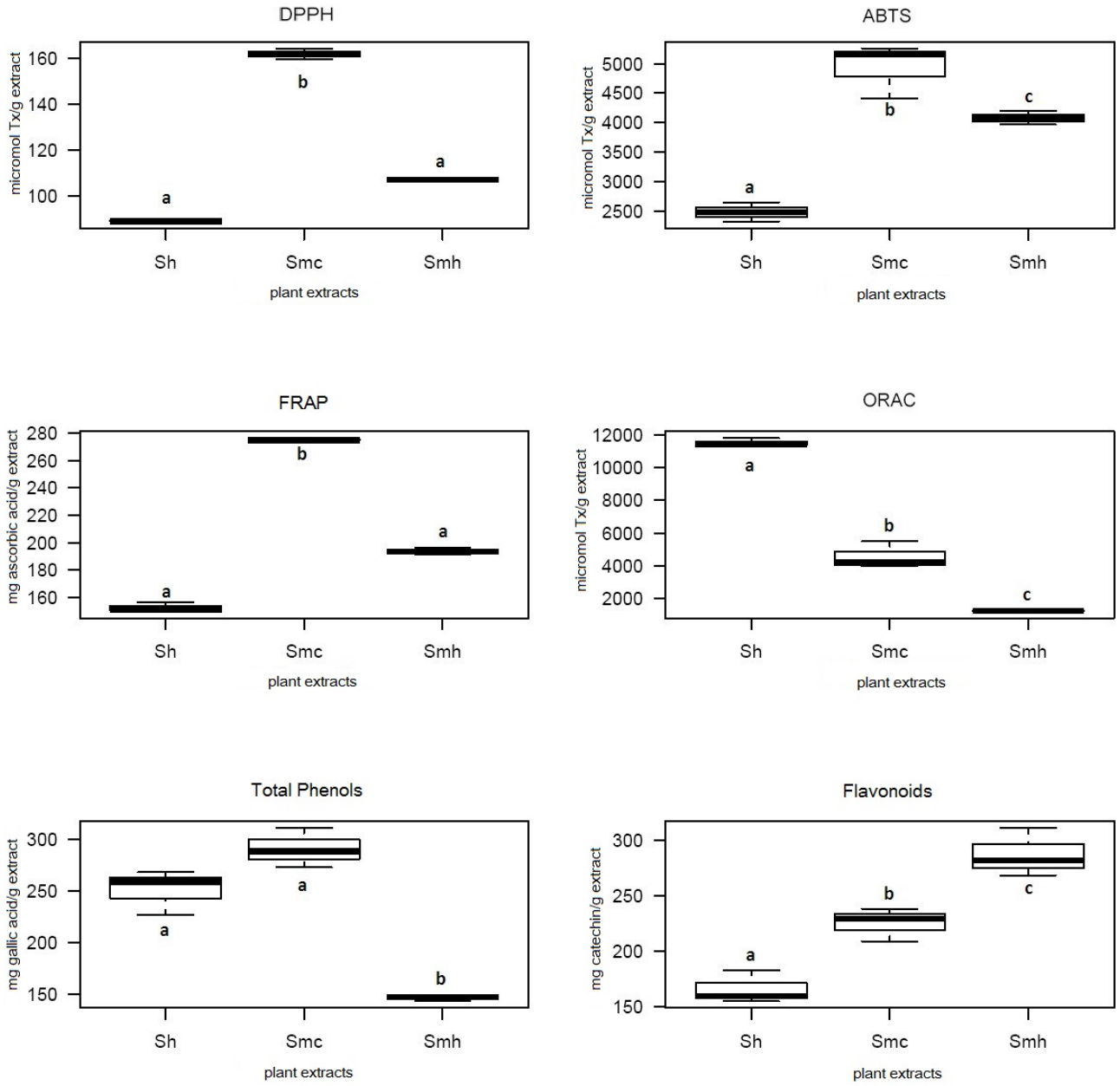

Figure 1. ANOVA of each of the antioxidant assays between the species *Swietenia humilis* (Sh), *S. macrophylla* (Smc) and *S. mahagoni* (Smh). Samples with different letters represent the significant differences according Tukey test ($\alpha \leq 0.05$).

The phenolic metabolites protocatechuic acid and catechin were detected differentially in each sample, as mayor compounds while gallic acid was detected only in *S. macrophylla* (Table 2). The fragmentation pattern for the derivatized samples acquired through MS was in accordance with the literature (Proestos and Komaitis, 2013). The identification of catechin was based on the spectral data of the derivatized sample, which presented a molecular ion of *m/z* 650 and a characteristic base peak (*m/z* 368) originated from the excision of the heterocyclic ring through a retro diels alder fragmentation pathway (Zeeb et al., 2001). The TMS derivative of gallic acid presented a molecular ion of *m/z* 458 and a characteristic base peak of *m/z* 28, while the TMS derivative of protocacheuic acid evidenced a molecular ion of *m/z* 370 and a base peak of *m/z* 193. These metabolites are recognized in the literature for its high free radical scavenging capability that is determined by

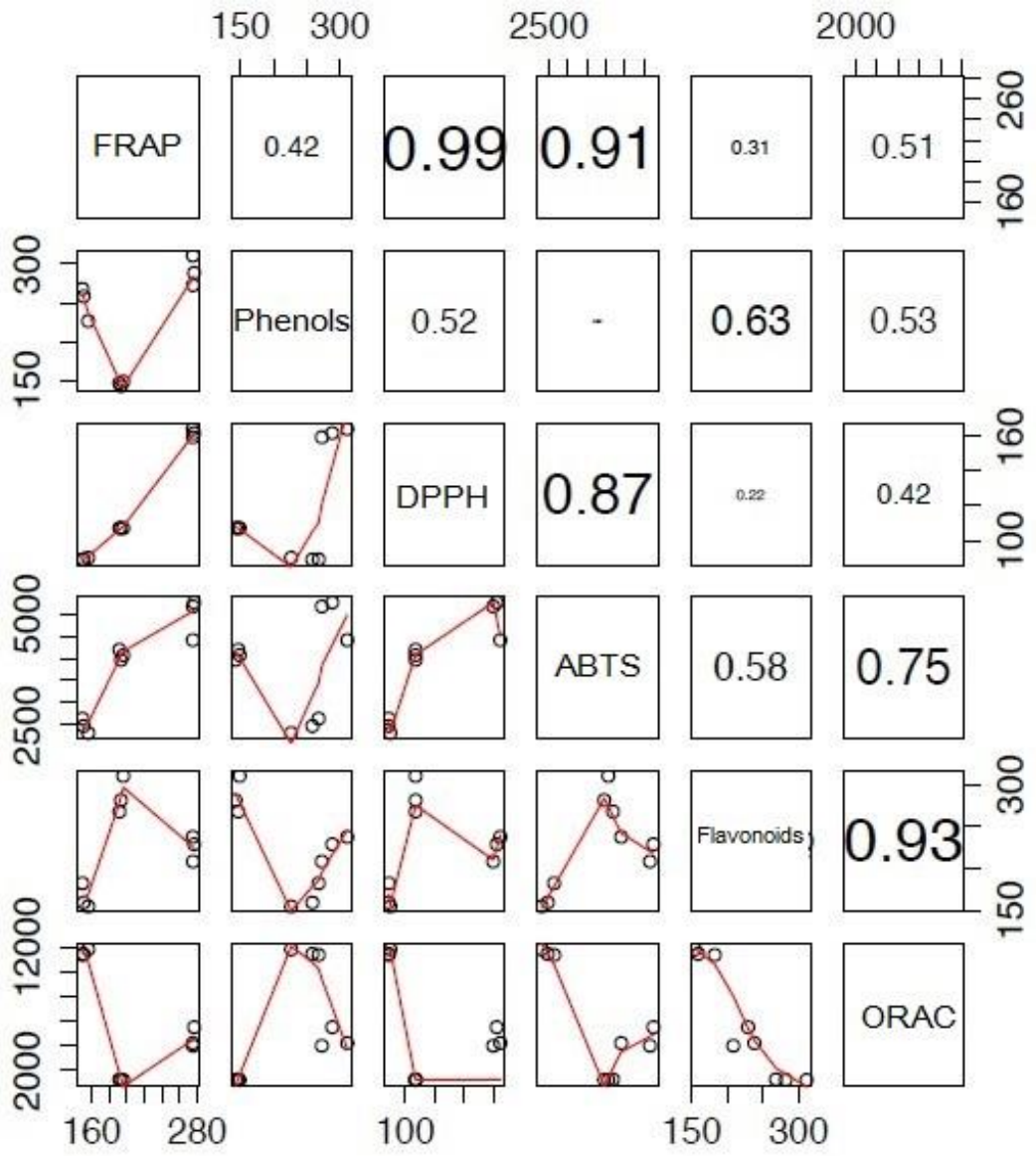

Figure 2. Correlation between the antioxidant assays for *Swietenia macrophylla.*

their ability to act as donors of electrons or hydrogen atoms, the stability of the antioxidant molecule in its radical form their reactivity with other antioxidants, and the capacity to chelate metals (Rice-Evans et al., 1997). Particularly, the detection of catechin in the three plant extracts was relevant, since this flavonoid is known to be an excellent free radical scavenger (Pedrielli et al., 2001). It has been demonstrated that catechin isomers have a lower reduction potential than vitamin E, suggesting that their electron donor capability is higher (Jovanovic et al., 1996).

Based on the overall results, *S. macrophylla* was the most promising lead, which was in accordance with previous studies (Tan et al., 2009). In the statistical correlations between each of the antioxidant results of this particular species, the high correlation between FRAP and DPPH (R^2= 0.99) and FRAP with ABTS (R^2= 0.91) (Figure 2) suggests that the reducing agents present in *S. macrophylla* react through a single electron transfer mechanism (SET). The correlation between ABTS and DPPH was also observed in sorghum and its products, evidencing a similar mode of action (Awika et al., 2003). In contrast, the high correlation among the flavonoid contents and the ORAC assay (R^2=0.93) could evidence that the flavonoids in this extract have a high capability to trap peroxil radicals, which is the case for

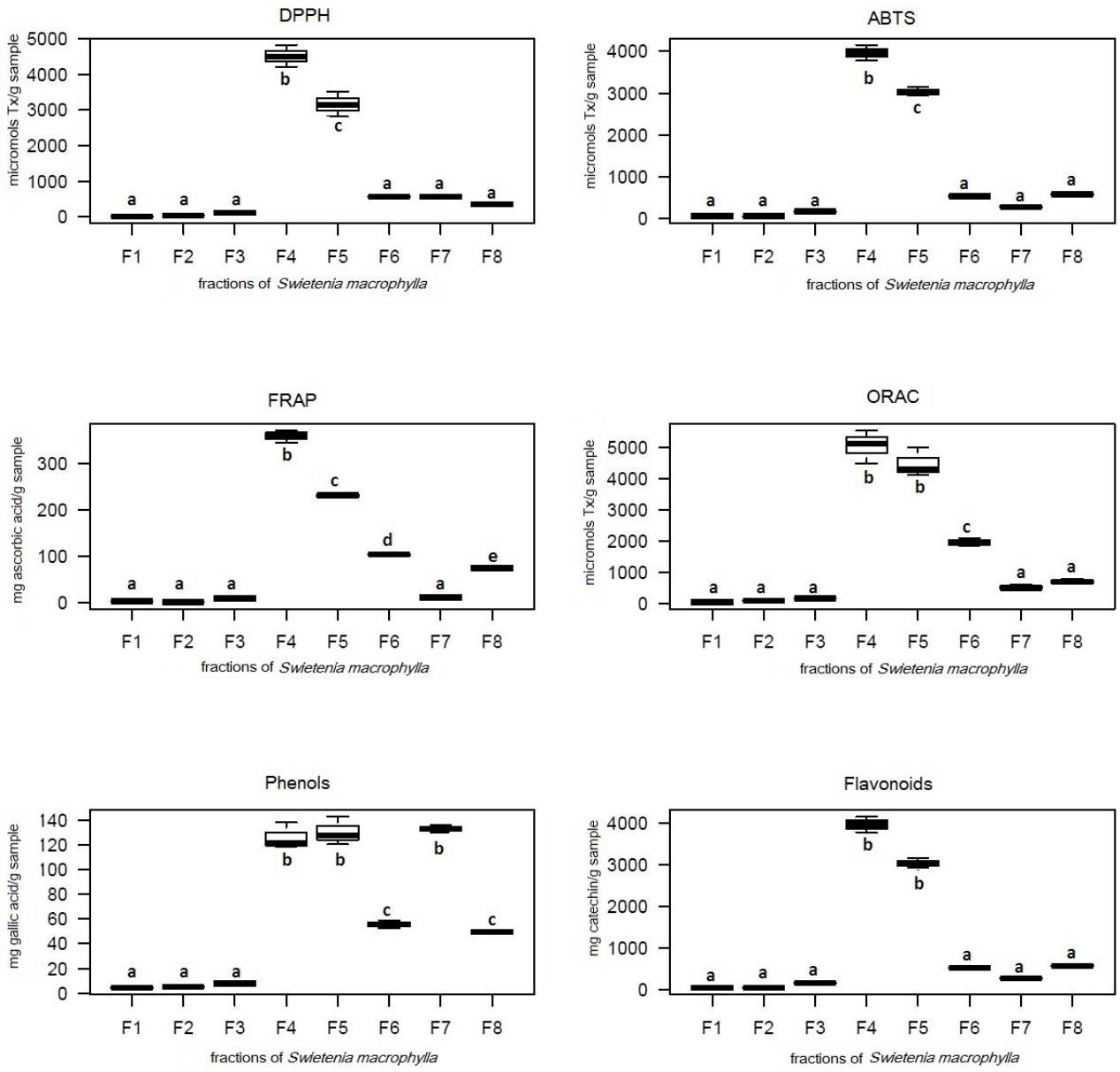

Figure 3. ANOVA of each of the antioxidant assays between the fractions of *Swietenia macrophylla.* Samples with different letters represent the significant differences according Tukey test ($\alpha \leq 0.05$).

catechin (Tan et al., 2009). As a group, flavonoids are the most diverse phenols, with a high potential as free radical scavengers, and other biological activities (Middleton et al., 2000).

As seen in Table 3, the fractions of *S. macrophylla* exhibited a differential antioxidant activity. The most promising fractions were F4 and F5, with significant differences when compared statistically with the other fractions (Figure 3). In this way, in the GC-MS analysis of these fractions (F4 and F5), protocacheuic acid was present only in fraction F5, while catechin was present in both fractions. In accordance with previous results, catechin as a major compound detected in both fractions could be one of the compounds implicated in the bioactivity, as evidenced in the significantly high activity in the biological tests (Figure 3). It is relevant to point out that both fractions (F4 and F5) had no statistical difference in the bioactivity in the ORAC assay, supporting the presence of hydrophilic antioxidants specific to trap peroxil radicals, such as catechin (Tan et al., 2009). In the determination of catechin and epicatechin in the ethanolic extract of *S. macrophylla* and

in fractions F4 and F5 by HPLC it supports the aforementioned results and strongly suggests the involvement of these metabolites in the observed bioactivity.

Finally, it is important to point out that many assays have been frequently used to estimate antioxidant potentials in natural products, in conducting other studies regarding the isolation or standardization of biological extracts. These techniques have shown different results among crop species and across laboratories. Some authors pointed out that the ORAC assay was the most relevant because it utilized a biologically relevant radical source (Prior et al., 2003). Nevertheless, all the different methods to determine the antioxidant potential have been validated in different studies, and the potential of the three species evaluated in this research was demonstrated as sources of potential antioxidant molecules, with *S. macrophylla* as the most promising lead.

Conclusion

The *Swietenia* species evaluated in this study exhibited a high free radical scavenging activity, in which correlations among the different assays that contributed to the inference of the type of metabolites and conditions of the mode of action. The additional results obtained with *S. macrophylla* support the correlations of the bioactivity and the implication of flavonoids, such as catechin in the results. It would be relevant to conduct further studies of isolation and identification of other compounds in fractions F4 and F5 to explore their antioxidant potential.

Conflict of Interests

The authors have not declared any conflict of interests.

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Toxicogenetic profile of rats treated with aqueous extract from *Morinda citrifolia* fruits

Germano Pinho de Moraes[1], Marcus Vinícius Oliveira Barros de Alencar[2,3]*, Md. Torequl Islam[2,4], Lidiane da Silva Araújo[4], Débora Cássia Vieira Gomes[2,3], Rodrigo Mendes de Carvalho[3], Dione Corrêa[1], Márcia Fernanda Correia Jardim Paz[1,3], Paulo Michel Pinheiro Ferreira[2,5,6], Ana Amélia de Carvalho Melo-Cavalcante[2,3,5], Alexandre Ferraz[1] Ivana Grivicich[1] and Jaqueline Nascimento Picada[1]

[1]Post-Graduation Program in Cellular Biology Applied to Health (PPGBioSaúde), Lutheran University of Brazil, Canoas, Brazil.
[2]Postgraduate Program in Biotechnology, Federal University of Piauí, Teresina, Brazil.
[3]Laboratory of Genetic Toxicology, Federal University of Piauí, Teresina, Brazil.
[4]Laboratory of Research in Experimental Neurochemistry, Federal University of Piauí, Teresina, Brazil.
[5]Postgraduate Program in Pharmaceutical Sciences, Federal University of Piauí, Teresina, Brazil.
[6]Laboratory of Experimental Cancerology, Department of Biophysics and Physiology, Federal University of Piauí, Teresina, Brazil.

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Morinda citrifolia **((Family: Rubiaceae)) is extensively used in traditional medicine due to its anti-inflammatory, antimicrobial, antitumoral, and anti-hypertensive activities. However, there is no substantial data about hepatotoxic and toxicogenetic effects. This study evaluated biochemical changes and hepatotoxic, genotoxic, and mutagenic effects of aqueous extract of the fruit of *M. citrifolia* (AEMC) in liver, bone marrow, and peripheral blood cells. Animals (*Rattus novergicus*, 5 males and 5 females) were divided into negative control, positive control (Cyclophosphamide 25 mg/kg), and AEMC (2.5, 5, and 10 mg/kg, by gavage). AEMC induced increase of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), especially at 10 mg/kg in female (174.8 ± 50.7, 221.4 ± 24.6, and 174.7 ± 14.3 U/L) and male (156.5 ± 21.6, 183.7 ± 21.5, and 147.3 ± 17.8 U/L) (p<0.05). Histological analysis of livers showed inflammatory cell infiltration, nuclear fragmentation, microvacuolization, cellular swelling, points of inflammatoy necrosis, and discrete microvesicular steatosis. DNA damage in hepatocytes was found in both genders, mainly at 10 mg/kg (Frequency of Damage: 78.1 ± 4.5 and 70.4 ± 7.3%; Index of Damage: 107.6 ± 14.2 and 136.0 ± 26.9 for male and female, respectively). Similar results were observed in bone marrow cells. The AEMC 5 and 10 mg/kg induced micronucleus formation (4.4 ± 0.8 and 7.8 ± 1.1; 7.4 ± 1.1 and 9.6 ± 1.4 for peripheral blood and bone marrow cells, respectively) (p<0.05). These findings suggest clastogenic and/or aneugenic effects and genetic instability activated by AEMC, indicating precaution regarding the consumption of formulations or folk preparations based on this plant.**

Key words: Hepatotoxicity, genotoxicity, mutagenicity, *Morinda citrifolia*, noni.

INTRODUCTION

Popular knowledges associated with geographic and economic barriers to obtain health care contribute to the use of medicinal plants such as teas and extracts, especially in developing countries to supply primary basic

requirements (Firenzuoli and Gori, 2007; WHO, 2011; Akram et al., 2014; Tuttolomondo et al., 2014; Araujo et al., 2015). However, continuous exposure to synthetic or natural chemicals present in these preparations may lead to structural and functional damage to macromolecules, due to possible toxic effects, which can be measured by several genotoxic and mutagenic testing methodologies (Speit and Rothfuss, 2012; Hussin et al., 2014).

Morinda citrifolia Linn (Fam.: Rubiaceae) is habituated to the Southeast Asia and is distributed through several settlers of the Pacific Islands (Wang et al., 2002; Samoylenko et al., 2006; Ebeling et al., 2014). Aerial parts of this plant are commonly used in folk medicine as antibacterial, antiviral, antifungal, antitumoral, anthelmintic, contra-ceptive, hypotensive, anti-inflammatory, antioxidant, immunomodulating (Nayak and Shettigar, 2010), as well as antidopaminergic and antiadrenergic (Pandy et al., 2014). Moreover, its fruit has medicinal effects on allergy, arthritis, asthma, bacterial infections, cancer, diabetes, hypertension, menstrual disorders, obesity, gastric ulcers, headaches, sexual inhibition, insomnia, depression, stress, respiratory problems, AIDS, multiple sclerosis and drug dependency (Selvam et al., 2009; Gupta and Patel, 2013; Murata et al., 2014).

About 96 volatile compounds were identified in ripe fruit of *M. citrifolia* and more than 200 compounds were isolated from several parts of the plant, of which as hexanoic acid, octanoic and asperuloside acid, alcohols, esters, ketones and lactones are few of them. Nonetheless, the phytochemical composition is not complete yet (Potterat and Hamburger, 2007; Assi et al., 2015). Although folk uses of *M. citrifolia* fruits are an earlier report, still there is a lack of consistent data regarding hepatotoxic, genotoxic, mutagenic, and cytotoxic effects. Thus, studies addressing DNA damage in eukaryotic cells are important to undersand the risk of cellular injury. This study evaluated the possible biochemical changes and hepatotoxic, genotoxic and mutagenic effects of aqueous extract of the fruit of *M. citrifolia* (AEMC) in liver, bone marrow and peripheral blood cells in Wistar rats.

MATERIALS AND METHODS

Plant and extract preparation

Fruits of *M. citrifolia* were collected in 2014 in the municipality of Altos, Piauí, Brazil, (05° 02'20" S– latitude, 42° 27'39" O– longitude) at 187 m above sea level. The botanical identification was held at the Center for Environmental Sciences of Tropic Ecotonal Northeast, Teresina, Piauí (voucher number: 21644). After collection, the fruits were dried in a forced air oven for 8 days at a maximum temperature of 45°C (±1°C). Then, they followed by course grinding and were preserved in an amber glass.

HPLC analysis

High performance liquid chromatograph (HPLC) analysis was performed in AEMC to determine the presence of flavonoids and phenolic compounds. Briefly, Waters 2695 liquid chromatograph equipped with autosampler and a variable wavelength UV/VIS detector (Waters 2487 Detector Dual Absorbance, 190 to 700 nm). Columns: Waters Spherisorb ODS2 (5 µm, 4.6×250 mm). The mobile phases consisted of acetonitrile (A) HPLC grade purchased and 0.1% H_3PO_4 aqueous solution (B, filtered using a Millipore system). The following were the gradient conditions: 5% (A); 15% (A) for 10 min; 35% (A) for 40 min; 100% (A) for 15 min and 5% (A) for 5 min. Total run time was 70 min at a flow rate of 1.8 ml/min. Injection volumes were 20 µl. The AEMC was solubilized in acetonitrile (30%, HPLC grade and 0.1% H_3PO_4 aqueous solution, 70%).

Animals and treatment

A total of 50 Wistar adult *Rattus norvegicus* of both sexes weighing 200 to 250 g were purchased from the Faculty of Health, Life Sciences and Technology of Piauí (NOVAFAPI), Teresina, Piauí, Brazil. The animals were kept in plastic cages (6 animals/cage) with maintenance of diet (Purina, Brazil) and water *ad libitum* in air-conditioned temperature of 25°C with a photoperiod of 12 h light/dark cycle. The investigational protocols were approved by the local Ethical Committee on Animal Research at NOVAFAPI (Process No. #0039/10) and are in accordance with the national (*Colégio Brasileiro de Experimentação Animal* – COBEA) and international standard for the care and use of experimental laboratory animals (EEC Directive, 1986).

Animals (10 per group: 5 males and 5 females) were randomly distributed into the following groups: negative control (NC; untreated animals); positive control (Cyclophosphamide 25 mg/kg, i.p.) and three for AEMC (2.5, 5 and 10 mg/kg, by gavage). Animals of the positive control received cyclophosphamide (CPA) for 2 days and were sacrificed on the 3rd day. After 14 days of treatment, the rats were intraperitoneally anaesthetized with a solution composed of ketamine and xylazine at a dose of 40 and 5 mg/kg, respectively. Then, blood (5 ml) from the portal vein was collected to evaluate biochemical parameters. For the micronucleus test, blood was collected from the caudal vein. Liver tissue samples were collected for histopathological analysis and comet assay. In addition, bone marrow samples from both femurs were collected for micronucleus test and comet assay.

Biochemical parameters

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) were measured by using commercially available assay kits (Labtest Diagnóstica S.A., Brazil).

Liver histopathological analysis

For histological analysis, the livers of the remaining animals of each group were fixed in 10% formalin for 72 h. To examine

*Corresponding author. E-mail: marcus-alencar@msn.com.

morphological changes by light microscopy (Olympus, Tokyo, Japan) at a magnification of 200 and 400X, small pieces were processed, embedded in paraffin and 3 to 5 µm thick sections were prepared and stained with Hematoxylin and Eosin (H&E).

Comet assay

The alkaline comet assay was performed by the method earlier described by Speit and Rothfuss (2012). Briefly, 10 µl of the cell suspension (hepatocytes and bone marrow mononuclear cells) was mixed with a thin agarose layer low melting point (0.75%) (90 µl) and placed on pre-covered slides with normal melting point agarose (1.5%). The slides were immersed in lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA and 10 mmol/L Tris, pH 10 with 1% Triton X-100 and 10% of DMSO) up to 72 h at 4°C. The slides were then incubated in alkaline buffer (300 mmol/L NaOH and 1 mmol/L EDTA; pH >13) for 20 min followed by exposition to an electrical current 300 mA and 25 V (0.90 V/cm) for 15 min (electrophoresis). Finally, the slides were neutralized with Tris buffer 0.4 M, pH 7.5 and stained with silver solution. The results were expressed in index of damage (ID) and frequency of damage (FD). The ID was calculated from the visual assessment of the damage classes (0 to 4), extracting an index that expresses the overall damage suffered by the cells (100 cells/slide in duplicate). However, in injured cells, the DNA migrates from the core to the anode during electrophoresis, showing a similar comet tail. From these images, the cells were sorted between classes 1 (minimal damage) and 4 (maximum damage), 0 denoted for intact nucleus. The FD was calculated based on the equation: $FD = 100 - N_0$, where FD is the frequency of damage; N_0: number of tailless cells.

Micronucleus frequency assay

The micronucleus test was carried out according to Mavournin et al. (1990) and Kasamoto et al. (2013). Briefly, the collected bone marrow was mixed with 0.3 ml of fetal bovine serum on the slide previously coded. Additionally, peripheral blood was collected from the tails of live animals. The smears were performed by extender blade and after 30 min of drying, the slides were fixed in methanol for 10 min. Then, the slides were stained with Giemsa (Merck) in phosphate buffer 0.2 mol/L, pH 5.8. The counting of normochromatic erythrocytes (NCE), polychromatic erythrocytes (PCE) and micronuclei in polychromatic erythrocytes (MNPCE) were carried out by photomicrography at 100X. In each animal, 2000 PCE were analyzed. The ratio of PCE/NCE was determined by evaluating the frequency of PCE in 500 erythrocytes per animal.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) (n=10). In order to determine the differences, data were compared by one-way analysis of variance (ANOVA) followed by the Tukey test (p<0.05).

RESULTS

Identification of compounds by HPLC analysis

Qualitative HPLC analysis of AEMC is as shown in Figure 1a. The correlation of chromatographic peaks was achieved by comparing experimental retention times (t_R) with reference standards (Figure 1b). All chromatographic operations were carried out in triplicate at ambient temperature. According to the chromatogram obtained for AEMC, gallic acid, chlorogenic acid, caffeic acid, ellagic acid, rosmarinic acid and flavonoid like-rutin with the following retention time (t_R) were found: 3,4,5-trihydroxybenzoic acid (1) t_R: 5.0 min; (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5 tri hydroxycyclohexane-1-carboxylic acid (2) t_R: 14.9 min; (E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid (3) t_R: 15.1 min; 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R, 4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R, 4R,5R, 6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl] oxychromen-4-one (4) t_R: 32.5 min; 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde] chromene-5,10-dione (5) t_R: 35.0 min; (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid (6) t_R: 37.5 min.

In vivo hepatotoxicity

Hepatotoxicity of AEMC in rats was observed by an increase (p<0.05) of the values of AST, ALT and ALP, especially at the highest dose (10 mg/kg) in female (174.8 ± 50.7, 221.4 ± 24.6 and 174.7 ± 14.3 U/L) and male (156.5 ± 21.6, 183.7 ± 21.5 and 147.3 ± 17.8 U/L) when compared with the negative control [(female: 32.1 ± 13.6, 41.1 ± 9.8 and 53.5 ± 2.7 U/L) and male (33.2 ± 14.8, 49.9 ± 12.0 and 59.7 ± 4.7 U/L), respectively]. Furthermore, high levels of ALP were found in all AEMC-treated animals (Table 1) and male animals also showed GGT increasing at the dose of 10 mg/kg (1.2 ± 0.2 U/L) in comparison with the untreated group (0.9 ± 0.1 U/L) (p<0.05). Similarly, histological analysis of livers also revelaed signs of injury, corroborating the alteration in serum enzymes, as morphological changes in both genders at 10 mg/kg, which showed histoarchitecture preservation with inflammatory infiltrating cells in the perivascular space (Figure 2a), nuclear fragmentation of hepatocytes (suggestive of apoptosis) (Figure 2b), microvacuolization (Figure 2c), cellular swelling (Figure 2d), points of inflammatoy necrosis (Figure 2e) and discrete microvesicular steatosis (Figure 2f), all findings indicative of hepatotoxicity.

DNA damage in liver and bone marrow cells

AEMC caused significant DNA damage (p<0.05) in hepatocytes of rats in all doses studied as described in Figure 3 (ID) and in Table 2 (FD). Once again, higher doses were more toxic for both genders, mainly at 10 mg/kg (FD: 78.1 ± 4.5 and 70.4 ± 7.3%; ID: 107.6 ± 14.2 and 136.0 ± 26.9 for male and female, respectively) when compared with NC (FD: 19.1 ± 6.9 and 20.6 ± 9.5%; ID: 36.8 ± 12.2). Similar results were observed in bone marrow, though, only higher doses (5 and 10 mg/kg) have demonstrated genotoxic activity (Figure 3 [ID] and Table 2 [FD]). There was increase with the frequency of

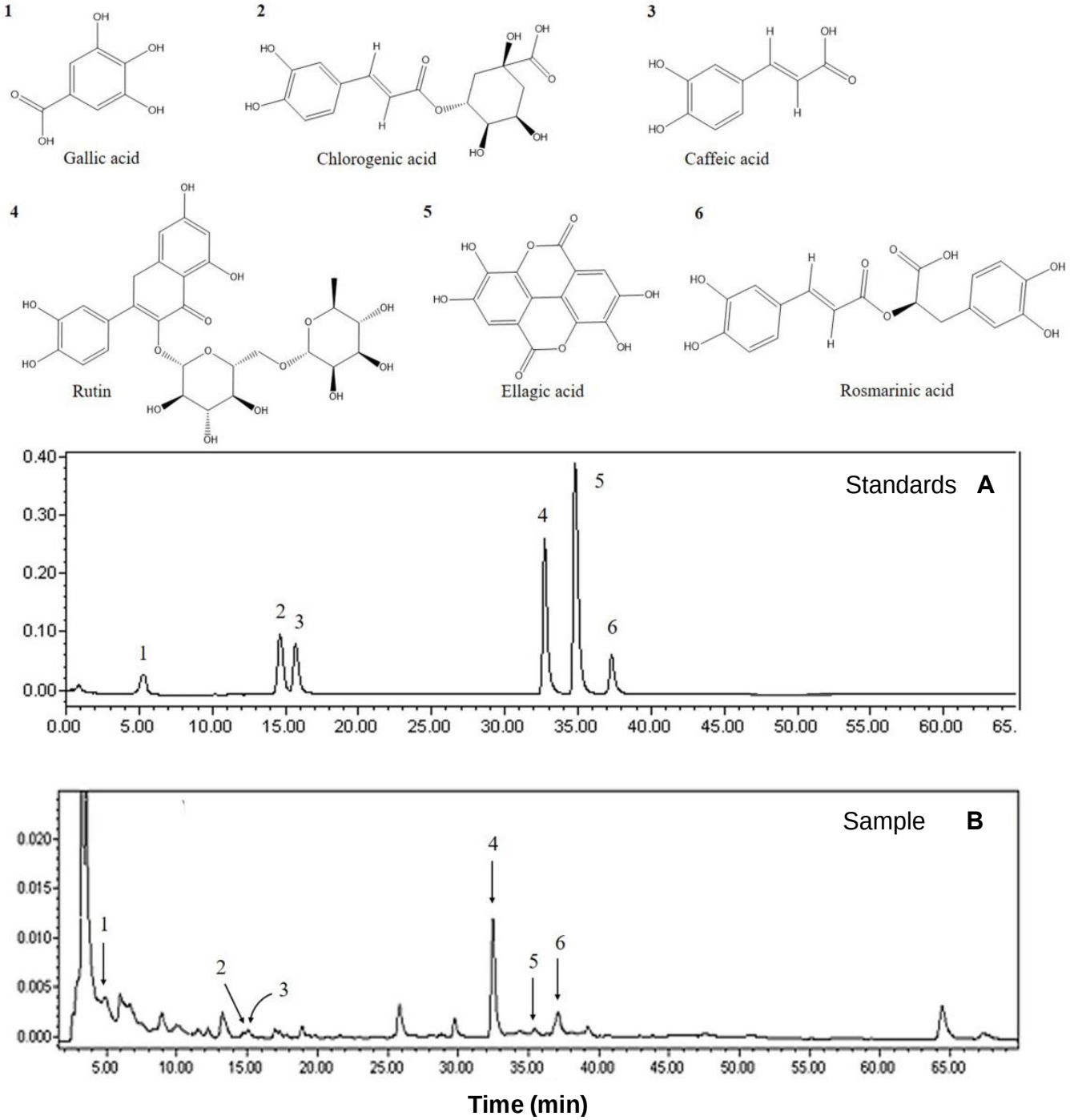


Figure 1. HPLC chromatogram of a standard mixture (A) and aqueous extract of the fruit of *M. citrifolia* (AEMC) (A). 3,4,5-trihydroxybenzoic acid (**1**) t_R: 5.0 min; (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid (**2**) t_R: 14.9 min; (E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid (**3**) t_R: 15.1 min; 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one (**4**) t_R: 32.5 min; 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione (**5**) t_R: 35.0 min; (2R)-3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid (**6**) t_R: 37.5 min.

damage at 5 mg/kg in bone marrow cells of males only (44.6 ± 4.4) and at 10 mg/kg it occurred in both genders (78.4 ± 5.7 and 68.1 ± 8.6, for male and female, respectively) (p<0.05). On the other hand, CPA, positive control, caused increased in FD and ID levels in an independent way of gender.

Table 1. Biochemical markers evaluated in rats treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC).

Marker	Treatment									
	Negative control		CPA 25 mg/kg		AEMC					
					2.5 mg/kg		5 mg/kg		10 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
ALT (U/L)	32.1 ± 13.6	33.2 ± 14.8	263 ± 83.4*	267.1 ± 53.2*	80.8 ± 11.1	81.1 ± 28.2	129 ± 22.3*	107.1 ± 15.4*	156.5 ± 21.6*	174.8 ± 50.7*
AST (U/L)	41.1 ± 9.8	49.9 ± 12.0	368 ± 47.5*	316.4 ± 78.5*	112.0 ± 23.0*	90.7 ± 8.93	116.7 ± 44.4*	83.4 ± 14.4	183.7 ± 21.5*	221.4 ± 24.6*
GGT (U/L)	0.9 ± 0.1	1.0 ± 0.1	1.3 ± 0.2*	1.3 ± 0.3*	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	1.2 ± 0.2*	1.2 ± 0.9
ALP (U/L)	53.5 ± 2.7	59.7 ± 4.7	163.3 ± 18.8*	155.9 ± 11.1*	120.9 ± 12.4*	116.7 ± 10.5*	113.2 ± 5.6*	123.5 ± 20.8*	147.3 ± 17.8*	174.7 ± 14.3*

Values are mean ± SD (n=10 animals/group). CPA: Cyclophosphamide; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyltransferase; ALP: alkaline phosphatase. ANOVA followed by Tukey test. *p<0.05 compared to the negative control.

Table 2. Frequency of DNA damage (FD) evaluated by alkaline comet assay in bone marrow cells and hepatocytes of rats treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC).

Group	Bone marrow (%)		Liver (%)	
	Male	Female	Male	Female
Negative control	12.1 ± 5.7	17.6 ± 3.2	19.1 ± 6.9	20.6 ± 9.5
CPA	29.8 ± 8.7*	74.6 ± 9.9*	62.8 ± 7.4*	73.8 ± 5.3*
AEMC 2.5 mg/kg	18.4 ± 8.3	12.1 ±7.3	41.6 ± 4.6*	26.1 ± 4.6
AEMC 5 mg/kg	44.6 ± 4.4*	26.8 ± 5.5	37.2 ± 5.8*	61.2 ± 4.1*
AEMC 10 mg/kg	78.4 ± 5.7*	68.1 ± 8.6*	78.1 ± 4.5*	70.4 ± 7.3*

Values are mean ± SD (n=10 animals/group). CPA: Cyclophosphamide 25 mg/kg. *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

Induction of micronucleus formation

For complementary evaluation, the mutagenicity of AEMC was evaluated by the MN assay in bone marrow polychromatic erythrocytes and peripheral blood cells (Figure 4). Again, the doses 5 and 10 mg/kg showed a significant increase in MN (4.4 ± 0.8 and 7.8 ± 1.1; 7.4 ± 1.1 and 9.6 ± 1.4 for peripheral blood and bone marrow cells, respectively) (p<0.05). In addition to these results of mutagenicity by clastogenic and/or aneugenic effects, cytotoxicity was also evident, since the reduction in the PCE/NCE ratio was found in all doses tested, mainly at 10 mg/kg, which showed a significant decrease in PCE/NCE ratio for males (0.8 ± 0.1%) and females (1.0 ± 0.1%) (Figure 5).

DISCUSSION

Phytochemical analysis of methanolic extract of *M. citrifolia* indicated the presence of flavonoids, tannins, alkaloids, glycosides, saponins, carbohydrates, steroids and quercetin (Ramesh et al., 2012), corroborating our findings mainly in relation to the presence of flavonoids. Some reports describe products derived from *M. citrifolia*, especially those obtained from the fruit, has several pharmacological activities (Furusawa et al., 2003; Deng et al., 2007; Pachauri et al., 2012). Phenolic acids are consistently associated with reduced risk of cardiovascular disease, cancer and other chronic diseases by mechanisms

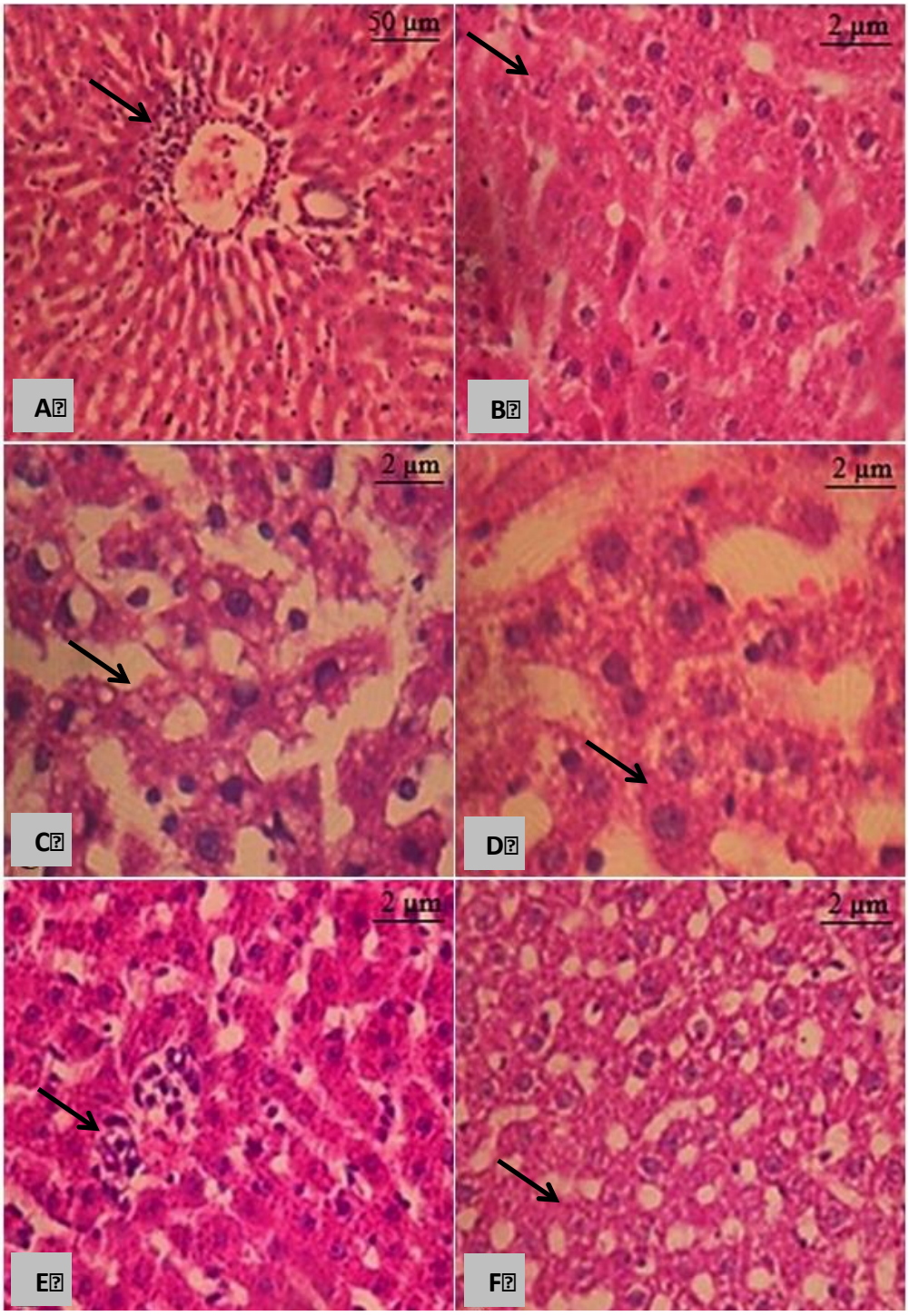


Figure 2. Liver histopathology analyzes of rats treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC) at 10 mg/kg. A: Perivascular inflammatory infiltrate (200X). B: Nuclear fragmentation (400X). C: Microvacuolization (400X). D: Cellular swelling (400X). E: Lobular necroinflammatory (200X). F: Microvesicular steatosis (200X). Hematoxylin-Eosin staining.

related to the scavenging of free radicals and as prooxidant metals (antioxidant), although there are reports for toxic effects of compounds isolated from the fruit of M. citrifolia (Ee et al., 2009; Nualsanit et al., 2012; Aziz et al., 2014). Earlier reports indicated that fruits (Millonig et al., 2005; Stadlbauer et al., 2005; Yüce

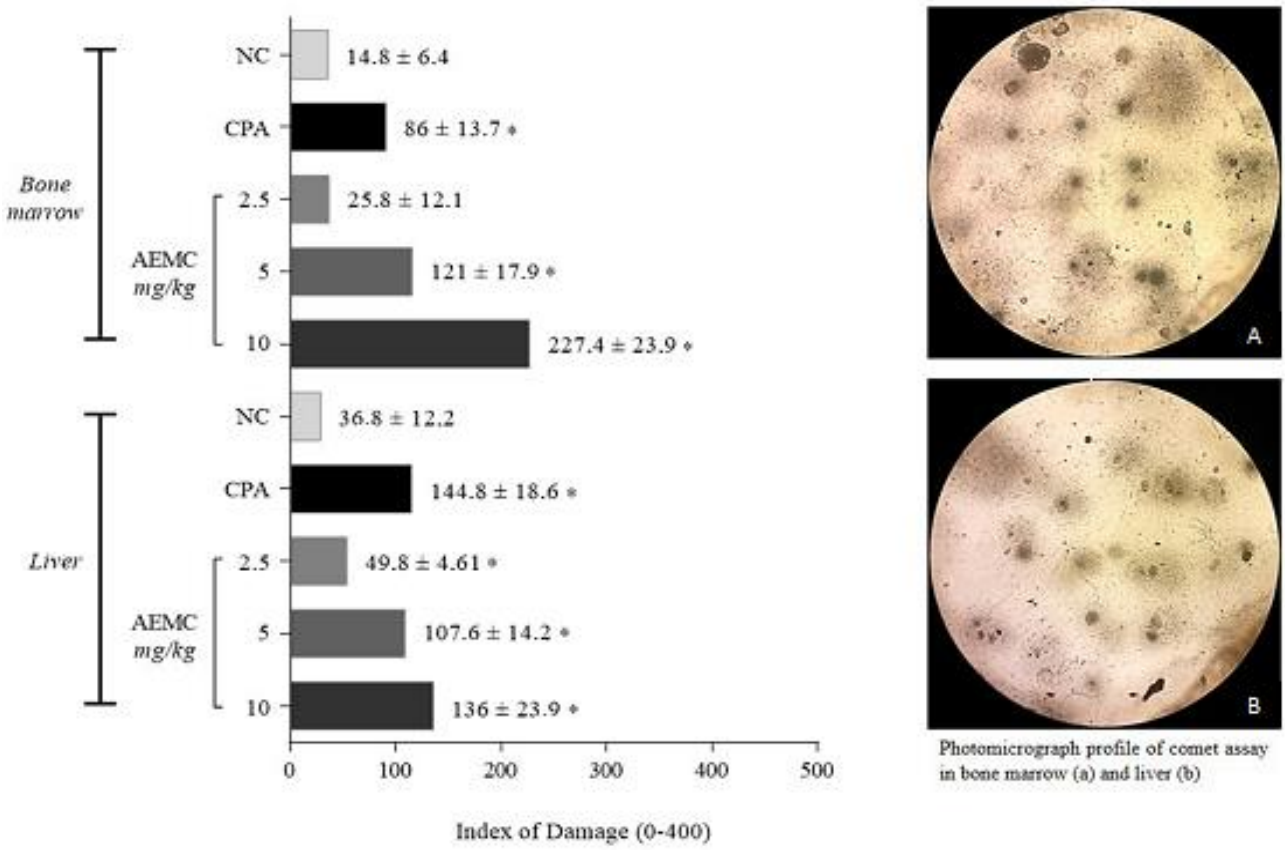


Figure 3. Genotoxicity in (A) bone marrow and (B) hepatic cells in rats treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC). NC: negative control; CPA: cyclophosphamide 25 mg/kg. Values are mean ± S.D. (n=10 animals/group). *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

et al., 2006), leaves (West et al., 2006; Lopez-Cepero et al., 2007) and juice (Sivagnanam et al., 2011; Mrzljak et al., 2013) of M. citrifolia are hepatotoxic in humans, since preparations for them are rich inanthraquinones and coumarins (scopoletin) (Ee et al., 2009), they induce generation of free radicals derived from oxygen and trigger oxidative stress. Free radicals cause depletion of intracellular reduced glutathione and mitochondrial membrane potential, thus initiating lipid peroxidation and eventually, cell death (Su et al., 2005; Bussmann et al., 2013).

Herein, using biochemistry and morphological analysis, it was shown that the AEMC at 2.5, 5 and 10 mg/kg induced hepatotoxicity in rats, as confirmed by levels of AST, ALT, ALP and GGT (this latter only at 10 mg/kg). Levels of ALT, AST and ALP were found to be approximately 2 to 6-fold higher in *M. citrifolia* treated group than those seen in untreated animals. Hepatic ‘‘leakage’’ enzymes are usually cytosolic, which gain access into circulation by leaking out of the cytoplasm either by reversible (membrane blebs) or irreversible (mitochondrial membrane damage) hepatic injury (Gores et al., 1990; Van Hoof et al., 1997). Following acute injury resulting in moderate to severe zonal necrosis by a liver toxicant, there is generally a moderate to marked increase in the serum ALT and AST activities which returns to normal within a few days indicative of resolution of injury. Although signals of tissue impairment have also been seen, since even necrosis are found with conjunctive tissue preservation, liver always presents good regeneration capacity, generally achieving a complete hepatic restoration (Hall and Cash, 2012). Although our studies have linked *M. citrifolia* consumption with liver damage, some assessments suggest it may protect liver from CCl_4-induced damage (Wang et al., 2008).

Toxicity can also be evidenced in genetic materials by *in vivo* Comet assay, a technique with applicability in different tissues and able to detect low levels of DNA damage in a small number of cells (Pourrut et al., 2015). Using this method in its alkaline version, it was possible to detect DNA single and double-strand breaks in the liver and bone marrow mononuclear murine cells, especially at AEMC 10 mg/kg. These genotoxic effects have dramatic effects on higher-order chromatin structure because of its supercoiling and tight packaging within the

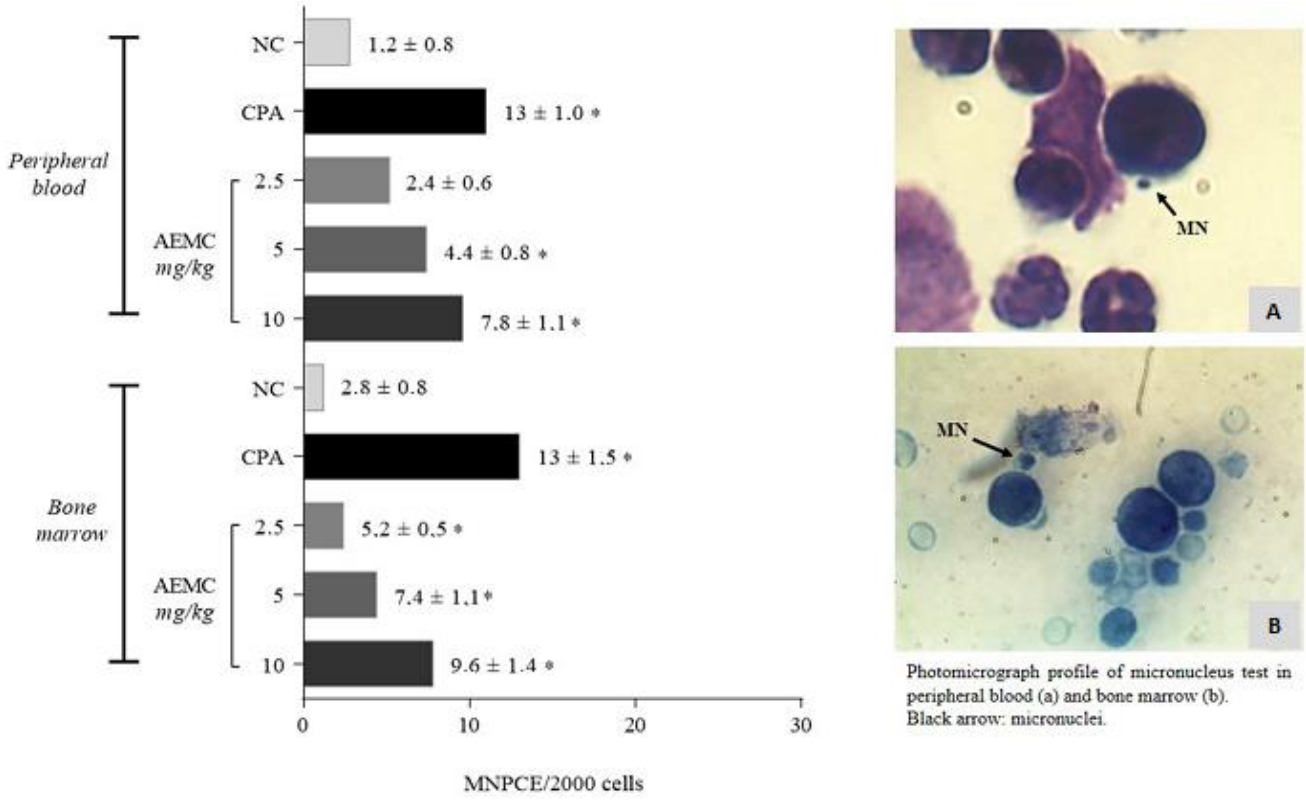
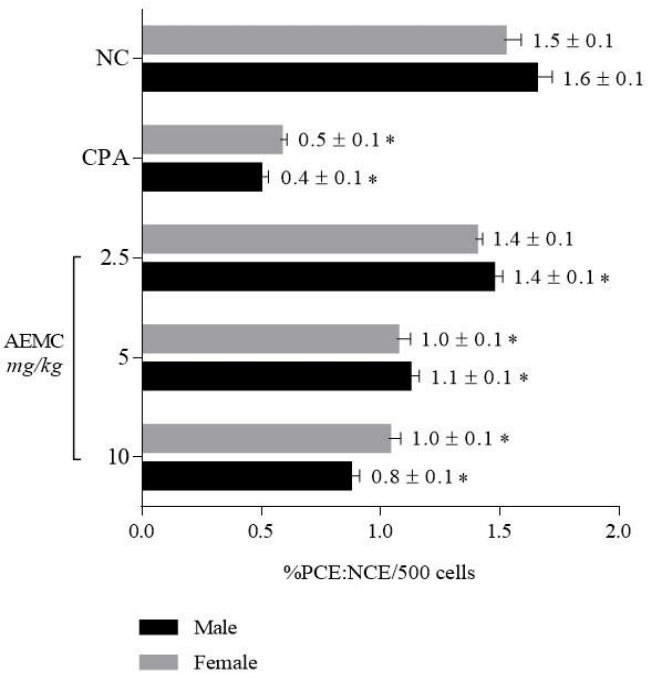


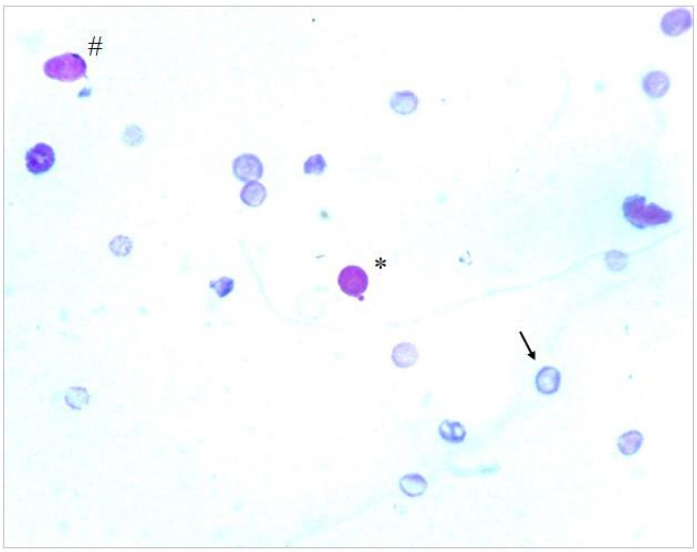
Figure 4. Mutagenicity in (A) bone marrow and (B) peripheral blood cells of rats treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC). NC: Negative control; CPA: cyclophosphamide 25 mg/kg; MN: micronuclei; MNPCE: polychromatic erythrocytes with micronuclei. Values are mean ± S.D. (n=10 animals/group). *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

nucleus (Fairbairn et al., 1995).

It should be emphasized that genotoxic damages can be repaired (Azqueta et al., 2014). However, extensive genotoxic injuries probably led to unrepaired genoma. This probably occurred at higher doses of AEMC, since chromosome breakage, chromosome rearrangement, chromosome loss, non-disjunction, gene amplification, necrosis and apoptosis, resulting in micronuclei, mainly from chromosome breaks or whole chromosomes that fail to engage with the mitotic spindle when the cell divides (Fenech et al., 2011). Notably, studies confirm that the high frequency of MNs increases the risk of developing tumors, because cancer is based on the accumulation of mutations on genetic material (Cardinale et al., 2012; Bhatia and Kumar, 2013).

Genotoxicity and mutagenicity findings can be attributed to the effects of complex mixtures of bioactive compounds present in *M. citrifolia* fruits. One of its components, caffeic acid (CA), has been related to induction of cell cycle arrest and apoptosis, protein kinases changes and inhibition of cyclooxygenase-2 (COX-2) activity (Kuo et al., 2015) and can generate hydrogen peroxide and hydroxyl radicals (Ruiz-Laguna and Pueyo, 1999). Ellagic acid (EA) has anticarcinogenic DNA injury and micronuclei figures were more usually found. In such condition, when a damage of superior intensity leads to unrepaired genomic instability at the chromosome/molecular level and such defects accumulate point mutations in the DNA, it can reflect on effects due to inhibition of tumor proliferation and angiogenesis, as well as induction of breaking connections between DNA caused by carcinogens (Zhang et al., 2014). Rutin possesses antitumor activity by inducing DNA damage to the mutant cell BRCAgene, probably by cell cycle arrest in G2/M phase and apoptotic effects on neuroblastoma cell lines (Chen et al., 2013; Maeda et al., 2014). However, this flavonoid can contribute to the growth of peritoneal tumors by the inhibition of macrophage migration in metastatic processes (Van der Bij et al., 2008). So, despite the pharmacological properties of these compounds, they probably have low selectivity on tumor cells and such compounds also attack normal tissues, which can explain, at least in part, the hepatotoxicity and mielotoxicity with AEMC. It is likely that distinct bioactive substances may jointly or independently contribute to nonselective biological effects (Ferreira et al., 2014). Drug/chemical-mediated hepatic injury is the most





Photomicrograph profile of bone marrow. Asterisk: MNPCE. Hash: PCE. Black arrow: NCE.

Figure 5. Cytotoxicity in bone marrow cells of rats treated with aqueous extract of fruit of *Morinda citrifolia* (AEMC). NC: negative control. CPA: Cyclophosphamide 25 mg/kg. MN: micronuclei; NCE: normochromatic erythrocyte; PCE: polychromatic erythrocytes; MNPCE: polychromatic erythrocytes with micronuclei. Values are mean ± SD (n=10 animals/group). *p<0.05 compared to the negative control by ANOVA followed by Tukey test.

common manifestation of drug toxicity and accounts for greater than 50% of acute liver failure cases. Hepatic damage is the largest obstacle to the development of drugs and is the major reason for withdrawal of drugs from the market (Cullen and Miller, 2006).

Conclusion

Outcomes pointed that the AEMC, at 5 and 10 mg/kg, predominantly, induces hepatotoxicity, genotoxicity and mutagenicity in liver, bone marrow and peripheral blood cells of rats. These findings suggest clastogenic and/or aneugenic effects and genetic instability activated by *M. citrifolia*, which indicates precaution regarding the consumption of medicinal formulations or folk preparations based on this plant.

Conflict of Interests

The authors have not declared any conflict of interests.

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