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Chemical characterisation and toxicity assessment *in vitro* and *in vivo* of the hydroethanolic extract of *Terminalia argentea* Mart. leaves

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ABSTRACT

Ethnopharmacological relevance: *Terminalia argentea* Mart. (Combretaceae), known mainly as “capitão”, is a native tree, not endemic, that occurs in the Amazon, Caatinga, Cerrado and Atlantic Forest in Brazil. Leaf infusion is popularly mentioned by riverine communities that inhabit the microregion of Northern Araguaia (Mato Grosso, Brazil) for the treatment of gastric ulcer, bronchitis and haemorrhage. Considering the wide medicinal use, lack of studies that evaluate the safety of use and the scarcity of phytochemical studies of *T. argentea* leaves, this work was carried out with the objective of evaluating the toxicity of the hydroethanolic extract of the leaves of *T. argentea* Mart. (HETa) in experimental models *in vivo* and *in vitro*, as well as to advance the phytochemical analysis of HETa.

Materials and methods: HETa was prepared by macerating the leaf powder in hydroethanolic solution. Phytochemical characterisation was carried out by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and mass spectrometry through direct flow infusion coupled with electrospray ionization and ion-trap analyzer (DFI-ESI-IT-MS analyses) The contents of phenols, flavonoids and phytosterols were analysed by colorimetric methods. Cytotoxicity was assessed by the Alamar blue assay on Chinese hamster ovary epithelial cells (CHO-K1) and human gastric adenocarcinoma cells (AGS). *In vitro* genotoxicity of HETa (10, 30 or 100 µg/mL) was assessed by micronucleus (MN) and comet tests using CHO-K1 cells. The acute toxicity assessment was performed by oral administration of HETa in single dose Swiss mice (males and females) up to 2000 mg/kg and sub-chronic toxicity by daily oral administration of HETa (50, 200 and 800 mg/kg) in Wistar rats for 30 days. The parameters related to the clinical and toxicological observations were determined every 6 days and at the end of the treatment the blood was collected for biochemical and haematological analysis, and some organs were removed for macroscopic and histopathological analysis.

Results: Preliminary phytochemistry and TLC analysis of HETa revealed the presence of phenolic compounds (18.8%), flavonoids (10.8%), saponins, tannins and phytosterols (19%). The HPLC data revealed the presence of gallic acid, rutin, ellagic acid, catechin, quercetin and kaempferol. In the analysis by DFI-ESI-IT-MS, the presence of gallic acid, rutin, ellagic acid and quercetin was confirmed and identified caffeic acid, quinic acid, galloylmucic acid, quercetin xyloside, quercetin rhamnoside, quercetin glucoside, caffeoyl ellagic acid, quercetin galloyl xyloside, terminalin, quercetin galloyl glucose, corilagin, quercetin digalloyl xyloside, quercetin digalloyl glucoside, punicalin and punicalagin. HETa showed no cytotoxic effect on CHO-K1 and AGS cells. In the MN assay, HETa increased the number of MNs and nuclear buds (NBUDs) in binucleate cells at the three concentrations tested and the nucleoplasmic bridges (NPBs) number at 30 µg/mL. In the comet test, HETa (10 and 100 µg/mL) alone showed a genotoxic effect on CHO-K1 cells. In pre-treatment, HETa at all concentrations tested prevented DNA damage induced by H_2O_2. In co-treatment with H_2O_2, HETa showed genotoxic effects at the

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three concentrations, and post-treatment DNA damage in exposed CHO-K1 cells to H_2O_2 was repaired in 22.5% with 10 μg/mL HETa. In the acute toxicity test, the HETa did not cause death in the mice, being verified only by piloerection and reversible in 2 h in males and in 4 days in females. No macroscopic changes were observed in the analysed organs. In the sub-chronic toxicity test, the HETa did not cause death in the rats after 30 days and the few changes were: absolute (10^3/mm^3) and relative (%) values of basophils increased by 477.8% and 423% ($p < 0.001$), respectively, with 50 mg/kg; reduction in feed intake (23.6%, $p < 0.01$) only on day 18; total cholesterol concentration (13.1%, $p < 0.05$) and relative heart weight (13.2% %, $p < 0.05$) at a dose of 800 mg/kg. These effects were not dose-dependent nor followed by clinical signs and symptoms of intoxication, nor of macroscopic and histopathological changes in the organs of animals treated with HETa.

Conclusions: The results demonstrated that HETa had no cytotoxic *in vitro* effects for CHO-K1 and AGS cells. In *in vitro* genotoxicity assays, the HETa induced different responses, according to concentration and experimental condition. In the MN test the HETa presented genotoxic potential by increasing the number of MNs, NBUDs and NPBs. In the comet assay, HETa was genotoxic by itself and in the co-treatment protocol with H_2O_2. In pre-treatment or post-treatment protocols with H_2O_2, HETa presented an antigenotoxic effect by preventing or repairing, respectively, the genotoxicity induced by H_2O_2. In the *in vivo* models, HETa was shown to be relatively safe after acute administration in mice [no-observed-adverse effect level (NOAEL) of 2000 mg/kg] and sub-chronic in rats (NOAEL of 800 mg/kg), confirming the riverine information that it is non-toxic in the dosage used. Phytochemical analysis of HETa revealed the presence of phenolic compounds, flavonoids, saponins, tannins and phytosterols. Among the flavonoids and tannins, we highlight gallic acid, rutin, ellagic acid, quercetin, caffeic acid, quinic acid, corilagin, punicalin and punicalagin. Thus, it can be stated that HETa has a good safety margin for therapeutic use.

1. Introduction

The research and development of a new drug involves multi-disciplinary approaches to ensure its effectiveness, safety and quality (Izzo et al., 2016). The fact that medicinal plants are natural does not automatically guarantee they are safe. For this reason, experimental models as *in vitro* or *in vivo* toxicity, even though they have limitations, have been used to characterise the potential adverse effects of medicinal plant derivatives and the obtained results (ICH, 2009; Izzo et al., 2016).

Regarding to the *in vitro* assays, the guidelines established by drug regulatory agencies recommend the use of cells for the evaluation of the cytotoxic, genotoxic and carcinogenic potential of a test compound (OECD, 2016a, 2016b, 2016c). There are several methods and models for testing the cytotoxicity of a given product. One of the most used is the Alamar blue test with Chinese hamster ovary epithelial cells (CHO-K1), which assesses the intrinsic capacity of a product to cause metabolic alterations in the cells in the culture, culminating, or not, in cell death (Rampersad, 2012). All the *in vitro* assays lead to determine the 50% inhibitory concentration(IC_{50}) and estimate the initial doses for *in vivo* acute oral toxicity tests, thereby reducing the number of animals to be used or, for relatively toxic substances, reducing the number of animals that die or should be sacrificed due to severe toxicity (OECD, 2008a, 2016c).

In vitro and *in vivo* genotoxicity assays are brief and effective in determining whether the new compound can interact with the DNA. The battery of tests for assessing chromosomal damage (genotoxicity) covers the cytogenetic and gene mutation tests in bacteria (ICH, 2012). Of the cytogenetic tests, the micronucleus (MN) test is the most used. Micronuclei are DNA fragments separated from the main nucleus resulting from aneugen or clastogen chromosome damage, indicating mutagenic potential of the new compound (OECD, 2016a).

Another test that is widely used for assessing genotoxicity is the comet assay, which identifies the extent of DNA damage and migration by the appearance of each nucleoid. It is different from other mutagenicity assays, because it is more specific and can detect both DNA damage and repair in a single cell (Collins, 2004; OECD, 2016b).

In vitro toxicology assays are recognised and well established; however, *in vivo* assays are the greatest source of information on the safety and efficacy of drugs (Timbrell, 2009). *In vivo* preclinical toxicological assays include, among others, assessment of single dose (acute) and repeated dose (sub-chronic and chronic) toxicity. The acute toxicity test is the most elementary *in vivo* test among preclinical toxicological assays and consists in administering one or more doses in two species of mammals (both sexes) followed by observation of signs or symptoms of toxicity over a period of 14 days, particularly animal mortality. They reveal species-, dose- and organ-specific toxic effects and, therefore, must be performed prior to Phase I of clinical research (OECD, 2008a).

When there is indication of continuous use of a drug for two weeks or more within a period of one year, the repeated dose toxicity test is performed on rodents and non-rodents. This test better characterises the toxicological profile of a product by assessing several parameters—behavioural, physiological, haematological, biochemical, anatomical and histopathological in target organs as well as their durations and reversibility, also indicating the non-observed effect level (NOEL) and non-observed adverse effect level (NOAEL) that support clinical research phases I, II and III (Lewis et al., 2002; OECD, 2008b; ICH, 2009).

Terminalia argentea Mart., (*T. argentea*), Combretaceae, popularly known in Brazil as “capitão”, “capitão-do-campo” or “pau-de-bicho”(Guarim-Neto and Morais, 2003; Lorenzi, 2008; Ribeiro et al., 2017), is a native tree 8–10 m high, not endemic, that can be found from north to south in Brazil in the phytogeographic domains of the Amazon, Caatinga, Cerrado and Atlantic Forest. The heterotypic synonyms are *Myrobalanus argentea* Kuntze, *T. biscutella* Eichler and *T. modesta* Eichler (www.theplantlist.org).

T. argentea adapts very well to poor soils, and it is used in programmes to recover degraded areas and, for its exuberant beauty, in urban gardens. In the construction sector, its wood is used due to its hardness and resistance, and the ash obtained from its burning is useful in the tanning of leather and preparation of soap (Lorenzi, 2008).

Ribeiro et al. (2017) reported that riverine communities living in the North Araguaia microregion of Mato Grosso, Brazil using medicinal infusions made with *T. argentea* leaves for treating gastric ulcers, bronchitis and haemorrhages; the macerated bark for treating ulcers and flu with fever; the bark decoction for diarrhoea, inflammation, wounds, cramps, cancer and as a tranquiliser and diuretic; the flower infusion for anxiety and finally the macerated roots for rheumatism and body pains.

Species of the genus *Terminalia* are known as a rich source of secondary metabolites, such as tannins (gallic acid and simple gallate esters; chebulic acid; chebulic and non-chebulic ellagitannins; ellagic acid and ellagic acid derivatives and glycosides), flavonoids (quercetin, kaempferol, rutin, luteolin, apigenin, vitexin, isovitexin, catechin, gallocatechin, epicatechin, epigallocatechin and leucocyanidin among

others), phenolic acids (caffeic acid, ferulic acid, vanilic acid and coumaric acid among others), triterpenes (ursolic acid, asiatic acid, oleanolic acid, arjunic acid, arjunolic acid, β-sitosterol, stigmasterol and terminalin among others), triterpene glycosides (arjunetin, chebuloside, terminoside, sericoside, bellericoside and daucosterol) and lignans (isoguaiacin, termilignan, thannilignan and anolignan among others) (Fahmy et al., 2015). Garcez et al. (2003) identified β-amirin and oleanolic acid in the ethanolic extract of the leaves of *T. argentea*, and, in the trunk bark, triterpenoids (tormentic acid β-D-glucopyranosyl ester and arjunetin), lignans (isoguaiacin) and flavones (7,3´-dihydroxy-4´-methoxyflavan, 7,4´-dihydroxy-3´-methoxyflavan, and catechin).

No pharmacological studies involving *T. argentea* were found in the databases consulted. However, in the polar and apolar extracts of leaves from other species of the *Terminalia* genus (*T. chebula*, *T. catappa*, *T. prunioides, T. brachystemma, T. sericea, T. gazensis, T. mollis, T. sambesiaca*, *T. triflora, T. arjuna, T. muelleri, T. ferdinandiana* and *T. glaucescens*), antioxidant, antifungal (Masoko et al., 2005), antibacterial, antiviral, anti-inflammatory, analgesic, antidiabetic, anticancer (Cock, 2015), wound healing (Fahmy et al., 2015), anti-*Helicobacter pylori* and antiulcer properties have been described (Silva et al., 2015).

There are no studies, which described the toxicological effects of *T. argentea*. However, *in vitro* cytotoxic activities are reported for the acetone extracts of *T. calamansanai* (Chen et al., 2009), ethanol extracts of *T. chebula* (Ravi Shankara et al., 2016) and hexanic extract with supercritical CO_2 in *T. catappa* (Ko et al., 2003). Genotoxicity studies with aqueous extract of *T. catappa* on CHO-K1 report anti-clastogenic effect in MN assay (Liu et al., 1996), antimutagenic in the *hgprt* gene mutation assay and antigenotoxic in the comet assay (Chen et al., 2000). In the Ames test, a mutagenic effect with the hydroethanolic extract (Mininel et al., 2014), and an antimutagenic effect with the hexane extract and supercritical CO_2 (Ko et al., 2003) in the *T. catappa* leaves were reported. *In vivo* toxicological studies indicated absence of acute toxicity to the methanolic extract of the leaves of *T. arjuna*, *T. citrina* and *T. coriacea* in rodents (Biswas et al., 2011; Das et al., 2015; Ali Khan et al., 2017), with the first two being devoid of sub-chronic toxicity (Biswas et al., 2011; Das et al., 2015). Oelrichs et al. (1994) reported intoxication of sheep and cattle due to the ingestion of *T. oblongata* leaves.

Considering the economic importance, the wide medicinal use by traditional populations of Mato Grosso, the absence of studies that assess the safety of use and the scarcity of phytochemical studies of the leaves of *T. argentea*, this work was carried out with the objective of assessing the toxicity of the hydroethanolic extract of the leaves of *T. argentea* Mart. (HETa) with *in vivo* and *in vitro* experimental models as well as advancing HETa phytochemical analysis.

2. Material and methods

2.1. Animals

Mus musculus, Swiss-Webster male and female mice (25–30 g), and *Rattus norvegicus,* Wistar female rats weighing 180–200 g, were collected from the Central Animal House of the Universidade Federal de Mato Grosso (UFMT) and placed in polypropylene cages at 24 ± 1 °C in a light/dark 12 h cycle with access to Purina® feed and water *ad libitum*. The experimental protocol was performed according to International Guiding Principles for Biomedical Research Involving Animals (CIOMS/ICLAS, 2012) and after approval (n° 23108.030953/2014-7) by UFMT's Ethics Committee for the Use of Animals (CEUA).

2.2. Botanical material

The *T. argentea* leaves (2.0 kg) were collected in Bom Jesus do Araguaia (12°17′S 51°23′W) in April 2014 at the end of the rainy season in Mato Grosso. Witness samples containing fertile plant material can be found at the UFMT Central Herbarium (voucher specimen n°. 40.753) and the taxonomic identification of the species was made by the Herbarium curator, Prof. Germano Guarim Neto, Ph.D. Authorisations for access to associated traditional knowledge (n° 135/2013) and genetic heritage (n° 199/2014) were granted by the Genetic Heritage Management Council of the Ministry of the Environment (CG*en*/MMA), Brazil.

2.3. Drugs, dyes, cell culture medium and reagents

Ellagic acid, formic acid, gallic acid, normal melting point agarose (NMP), low melting point agarose (LMP), trypan blue, catechin, cytochalasin B, dimethylsulfoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), doxorubicin, ethylenediamine tetra-acetic acid (EDTA), stigmasterol, kaempferol, luteolin, methanol Chromasolv LC–MS-grade, morin, naringin, penicillin, quercetin, rutin, streptomycin, trypsin, Tris-HCl, Trizma® base and Triton® X-100 were purchased from Sigma-Aldrich Co. (USA). Ultrapure water was produced using a Milli-Q system (Millipore, USA). Other reagents used were Alamar blue® (Invitrogen, USA), ketamine and xylazine (Syntec, Brazil), haematoxylin and eosin (HE) (Easypath, Brazil), GelRed™(Biotium, USA) and foetal bovine serum (SBF) (Cultilab, Brazil). All drugs and reagents presented analytical purity and were dissolved in an appropriate vehicle immediately prior to use.

2.4. Cell lineages

For the cytotoxicity assay, CHO-K1 (code: 0067) and human gastric adenocarcinoma (AGS, code: 0311) cells obtained from the Cell Bank of Rio de Janeiro, Brazil, were used. CHO-K1 cells were also used in the genotoxicity assays (MN and comet). After thawing, the cells were kept in DMEM supplemented with 10% SBF, penicillin (100 U/mL) and streptomycin (100 µg/mL)at 37 °C in a 5% CO_2 and 90% humidity atmosphere. The average cell cycle time was 12 h under these conditions and for the bioassays the cell lineage was used after the fourth passage. Prior to each experiment, cell viability was determined using the trypan blue dye exclusion test (Strober, 2001).

2.5. Preparing the extract

The collected material was cleaned, dried at room temperature and minced in a knife mill with no. 50 tamis (Tecnal TE-625, Brazil). The powder obtained was macerated in 70% hydroethanolic solution (1:5, p/v) and stirred once a day for 7 days at room temperature, filtered and concentrated in a rotary evaporator (model 801, Fisatom, Brazil) under reduced pressure of 600 mmHg and at a temperature of 40 °C. Residual solvent was eliminated in a hot air circulating oven (Marconi MA037, Brazil) at 40 °C, and HETa was frozen at −80 °C in a bio freezer (Indrel® Ultra Freezer IULT 335D, Brazil), lyophilised (Scientific LJJ02, Brazil), packed in an amber bottle and kept in a freezer at −30 °C (Brastemp 350 L, Brazil).

2.6. Chemical analysis

2.6.1. Preliminary phytochemical analysis

The preliminary phytochemical analysis was carried out according to the methodology described by Matos (2009), based on chemical reactions in staining, precipitation and foaming as well as fluorescence evidenced, in the case of coumarins, in the UV chamber (model VA50, Prodicil, Brazil).

2.6.2. Thin-layer chromatography (TLC)

Phytochemical constituents of HETa were identified by the TLC method according to Wagner and Bladt (2001) using silica gel plates 0.20 mm Kieselgel 60 Alugram® Xtra Sil G (Macherey-Nagel GmbH & Co., Germany) for the stationary phase; a mixture of benzene, ethyl

acetate, formic acid and methanol (60/30/10/3 v/v) as the mobile phase; and NP/PEG (1% 2-aminoethyldiphenylborinate diluted in methanol and 5% polyethylene glycol diluted in ethanol) as revealing. The visualisation was carried out in a UV camera at 365 nm. Rutin, naringin, gallic acid, catechin, morin, luteolin, quercetin and kaempferol were used as reference standards.

2.6.3. High-performance liquid chromatography (HPLC)

The HPLC system was developed by Shimadzu® chromatograph (LC-10 Avp series, Japan) equipped with a (LC-10 CE) pump, (DGU-14A) degasser, UV–vis (SPD-0A) detector, column oven (CTO-10A), manual injector Rheodyne (loop 20 µL) and CLASS(LC-10A) integrator. The separation was carried out by a gradient system using a reverse-phase Phenomenex Luna 5 µM C18 (2) (250 × 4.6 mm^2) column with direct-connect C18 Phenomenex Security Guard Cartridges (4 × 3.0 mm^2) filled with similar material as the main column. Phases were mobile phase A = 2% formic acid in Milli-Q water and mobile phase B = 2% formic acid in methanol. Program gradients are 0.01–0.10 min, 2.5% B; 0.10–5 min, 25% B; 5–10 min, 45% B; 10–16 min, 45% B; 16–20 min, 80% B; 20–25 min, 80% B; 25–30 min, 80% B; 30–35 min, 100% B and 35–36 min, 100% B. Flow rate is 1 mL/min, temperature is 35 °C, UV detection was done at 280 nm and elution time 37 min. The compounds were identified by comparing the retention times of samples and authentic standards such as gallic acid, rutin, catechin, quercetin and ellagic acid. The concentrations of compounds were expressed as percentage on a peak area of the total in matrix compounds.

2.6.4. Mass spectrometry

2.6.4.1. Clean-up of the extract. The solid phase extraction (SPE) cartridges (500 mg, Macherey-Nagel, Chromabond C18 ec, Germany) was first preconditioned by the consecutive passing of 5 mL of methanol and then 5 mL of methanol/water 8:2 (v/v). The extract obtained was solubilised in methanol/water 8:2 (v/v), loaded to the cartridge and first eluted with 5 mL of methanol/water 8:2 (v/v), followed by 5 mL of pure methanol. The methanol/water 8:2 (v/v) fraction obtained was transferred into a clean tube and dried under nitrogen gas at room temperature. The sample was redissolved in methanol to a concentration of 10 ppm and analysed by mass spectrometry.

2.6.4.2. Electrospray Ionisation Mass Spectrometry (ESI-MS^n) analysis. Direct flow infusion of the samples was performed on a Thermo Scientific LTQ XL linear ion trap analyser equipped with an electrospray ionisation (ESI) source, both in negative mode (Thermo, San Jose, USA). It was used with a metallic capillary tube at 280 °C, spray voltage of 5.00 kV, capillary voltage of −35 V, tube lens of −200 V and a 15 µL/min flow. Full scan analysis was recorded in *m/z* range from 100 to 1200. Multiple-stage fragmentations (ESI-MS^n) were performed using the collision-induced dissociation (CID) method against helium for ion activation. The first event was a full-scan mass spectrum to acquire data on ions in that *m/z* range. The second scan event was an MS/MS experiment performed by using a data-dependent scan on the deprotonated molecules from the compounds of interest at a collision energy of 30% and an activation time of 30 ms. The product ions were then submitted to further fragmentation in the same conditions, until no more fragments were observed.

2.6.5. Quantitative phytochemical analysis

The quantification of the total phenols was performed by the Folin–Ciocalteu method, as described by Amorim et al. (2012) using gallic acid as a standard. Methanolic solutions (0.2 mL) of the HETa (1 mg/mL, w/v) or standard (0.1–1.0 mg/mL w/v) aqueous solution were mixed with the Folin–Ciocalteu reagent (0.5 mL of 10%, v/v), sodium carbonate (1 mL of 75%, w/v) and 8.3 mL of Milli-Q water, gently agitated and kept for 30 min in the dark. The absorbance was measured at 760 nm in a UV–vis spectrophotometer (Biochrom® Biowave II^+, United Kingdom) equipped with quartz cells of 1 cm path length calibrated with Milli-Q water. Total phenols were determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of the gallic acid standard (y = 0.0997x + 0.004, adjusted R^2 = 0.9956), expressed as mg gallic acid equivalents (GAE) per gram of lyophilised extract (mg GAE/g HETa).

The quantification of total flavonoids was performed according to the description of Peixoto-Sobrinho et al. (2008) with modifications, using rutin as the standard. The reactions were performed by mixing 0.5 mL of methanolic solutions of the HETa (1 mg/mL w/v) or standard (1–10 mg/mL w/v) with an aqueous solution of 0.5 mL of 60% acetic acid, 2 mL methanolic solution of 20% pyridine (v/v), 1 mL of 5% aluminum chloride (w/v) and 6 mL of Milli-Q water. This reaction mixture was gently stirred and kept for 30 min in the dark, and its absorbance was measured at 420 nm in a spectrophotometer. The total flavonoid contents were determined by interpolating the absorbance of the samples against a calibration curve constructed with different concentrations of the rutin standard (y = 0.0215x – 0.0020, adjusted R^2 = 0.9978) and expressed as milligrams of rutin equivalents (RE) per gram of lyophilised extract (mg RE/g HETa).

The content of phytosterol was determined by sulfate–phosphate–ferric method (SPF) described by Lin et al. (2009) with modifications. SPF chromogenic reagent was prepared as follows: 2.5 g $FeCl_3 \cdot 6H_2O$ was dissolved in 25 mL phosphoric acid, then 10 mL was taken out and redissolved in 100 mL sulfuric acid before the experiment. Ethanolic solutions (1 mL) of the HETa (0.2 mg/mL, w/v) or standard (0.02–0.1 mg/mL w/v) were mixed with 1 mL SPF chromogenic reagent and homogenised. It was stirred and kept away from light for 1 h. Subsequently, the absorbance at 550 nm was measured in a spectrophotometer calibrated with ethanol. The phytosterol content was determined by interpolation of the absorbances of the samples against a calibration curve (y = 77.858x + 0.0442, adjusted R^2 = 0.9926) constructed with various concentrations of the stigmasterol standard solution, expressed as milligrams of phytosterol equivalents (PE) per gram of lyophilised extract (mg PE/g HETa).

All assays were performed in triplicate.

2.7. Cytotoxicity assay

This experiment was performed using Alamar blue assay with slight modification (Nakayama et al., 1997). CHO-K1 and AGS cells were plated (1 × 10^5 cells/mL) into 96-well microplates containing 200 µL of DMEM and incubated overnight (37 °C, 5% CO_2). Afterwards, they were treated with HETa (3.125–400 µg/mL) or doxorubicin, the standard drug for this assay, at the same concentrations. As negative control, only culture medium was used. The treatments were removed after 24 or 72 h of incubation and 200 µL of 10% Alamar blue (resazurin) were added to each well and incubated for 6 h. The conversion of resazurin to resorufin by the cells was measured at 540 nm (oxidised state) and 620 nm (reduced state) in a microplate-reader spectrophotometer (Multiskan EX, Thermo Scientific, USA). The results were expressed as inhibitory concentration (IC_{50}), and the values considered to be non-cytotoxic were IC_{50} > 30 µg/mL for the extract and IC_{50} > 4 µg/mL for pure substances (Suffness and Pezzuto, 1990).

2.8. Genotoxicity assay

The MN and comet assays were performed with CHO-K1 cells plated in 6-well microplates at a density of 1 × 10^6 cells/well (MN) or 5 × 10^5 cells/well (comet) and incubated overnight at 37 °C in 5% CO_2. For the MN assay, performed in duplicate, the cells were pre-treated with the vehicle (DMEM and DMSO 1%), HETa (10, 30 or 100 µg/mL) or doxorubicin (0.03 µg/mL). Three treatment protocols were used for the comet assay, which was also performed in duplicate: 1) pre-treatment of the cells with HETa (10, 30 or 100 µg/mL) for 24 h and subsequent induction of DNA damage with H_2O_2 (50 µM) for 4 h; 2) co-treatment of

the cells with HETa (10, 30 or 100 μg/mL) and H_2O_2 (50 μM) for 4 h; 3) post-treatment of cells (previously incubated with H_2O_2 for 4 h) with HETa for 24 h. In each of the protocols, a vehicle group (DMEM and DMSO 1% not exposed to H_2O_2 50 μM) and a positive control group (DMEM and DMSO 1% exposed only to H_2O_2 50 μM) were used.

2.8.1. MN assay with cytokinesis block

A modified version of the technique described by Fenech (2007) was used for the MN assay. After 24 h of pre-treatment, the cells were treated with cytochalasin B (4.5 μg/mL) and incubated for another 24 h. After trypsinisation, the cells were centrifuged (Fanem Excelsa II, Brazil) at 400 × *g* at 4 °C; the supernatant was discarded and 5 mL of 1% sodium citrate were added, and the cells were resuspended. After 15 s, 5 mL of fixative solution (methanol/acetic acid 3:1, v/v) and four drops of formaldehyde were added, and the cell suspension centrifuged at 400 × *g* for 5 min. The supernatant was discarded, and the pellet fixed in methanol/acetic acid (3:1, v/v) two more times, without the addition of formaldehyde. After the third fixation step, part of the supernatant was discarded and approximately 1 mL was kept. The cell suspension was dripped onto previously cleaned and chilled slides; after drying, they were stained with panoptic dye according to manufacturer's instructions.

Microscopic analysis was performed under a 40x optical microscope (Nikon, Eclipse E200). For each cell culture, 1000 binucleate cells were analysed for the presence of MN, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs), according to criteria previously established by Fenech (2007). Another 500 viable cells were analysed to establish the cytokinesis-block proliferation index (CBPI) and the replication index (RI) given by the formulas: CBPI = [(No. mononucleate cells) + (2 x No. binucleate cells) + (3 x No. multinucleate cells)]/N, where *N* = total number of cells; and RI = [(No. binucleate cells) + (2 x No. multinucleate cells)]/N_T ÷ [(No. binucleate cells) + (2 x No. multinucleate cells)]/N_C, where *T* refers to treatment data and *C* to control (vehicle) data (OECD, 2016a). The CBPI value is equal to 1.0 if all viable cells fail to divide during the cytokinesis block and, therefore, are all mononucleated; 2.0 if all cells complete a nuclear division and, therefore, are all binucleated; and > 2.0 if a substantial part of viable cells complete more than one nuclear division and are multinucleated (Fenech, 2007).

2.8.2. Comet assay

A modified version of the Collins (2004) technique was used for the comet assay. After the treatment protocols, the cells were removed with 1% trypsin, centrifuged at 400 × *g* for 5 min, the supernatant discarded, the pellet resuspended and the viable cells counted by the addition of trypan blue. The cell suspension was adjusted to at least 2×10^4 cells in 200 μL of 0.5% LMP agarose gel at 37 °C. Samples (80 μL) were dripped onto slides covered with a thin layer of NMP agarose gel with coverslip and kept in a refrigerator at 4 °C for 5 min. After the coverslips were removed, the slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris) at 4 °C overnight, placed in a running buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min, electrophoresed (25 V, 30 mA) for 30 min, incubated in neutralising solution (0.4 M Tris, pH 7.5) for 15 min and fixed in 96 °C ethanol for 5 min. At the time of reading, the slides were stained with 50 μL GelRed™diluted 10x in phosphate buffered saline (PBS) and the images were captured (magnification 400 ×) using a fluorescence microscope coupled to a photographic camera (AxioScope. A1, Carl Zeiss, Germany). The genetic damage was identified by the presence of a tail similar to a comet made up of DNA fragments, quantified by the ratio of tail intensity to total comet intensity, multiplied by 100 and expressed in % of DNA in the tail. One hundred nucleoids were counted per slide (two slides per sample), photographed and assessed using TriTek Comet Score™ Freeware (USA).

2.9. Acute toxicity (single doses)

Six male adult mice and six female adult mice weighing 25–30 g were treated with HETa by orogastric gavage (p.o.) with doses of 1000 or 2000 mg/kg or with vehicle (distilled water, 10 mL/kg) and observed individually in open fields at 0, 15 and 30 min; 1, 2, 4 and 8 h; and once daily for 14 days. The results of general behavioural observations were noted in a table adapted from Malone (1983). The body weight (g) of each animal was obtained before and after 14 days of the treatments to determine variations in weight gain (g). At the end of the period, the animals were euthanised by increasing intraperitoneal (ip.) anaesthesia with xylazine/ketamine (10/100 mg/kg). The organs (heart, lung, liver, stomach, spleen and kidneys) were removed, autopsied and weighed to determine the relative weight (%) [(weight of the organ/body weight) x 100].

2.10. Sub-chronic toxicity (repeated doses)

Sub-chronic toxicity was assessed through single and daily exposure of the rats to the vehicle (distilled water, 1 mL/100 g, p.o.) or HETa (50, 200 or 800 mg/kg, p.o.) for a period of 30 days (n = 6/group), according to the method described by Chan et al. (1982). The animals were kept in individual metabolic cages (Model 41800, Ugo Basile, Italy). The excreted faeces and urine as well as body weight and ingested amount of feed and water were determined every 3 days and grouped every 6 days (D_6, D_{12}, D_{18}, D_{24} and D_{30}). Any signs of toxicity, such as changes in skin, hair, mucous membranes and eyes, circulatory, gastrointestinal, respiratory, central and peripheral nervous systems, somatic-motor activity and behavioural manifestations in general, were recorded. At the end of the treatment, the animals were anaesthetised with xylazine/ketamine (10/100 mg/kg, ip.) and blood from the inferior vena cava was collected for haematological and biochemical analyses. The animals were then euthanised after deepening of the anaesthesia, and the main organs were removed, autopsied, weighed for relative weight determination [(weight of the organ/body weight) x 100] and stored in 4% paraformaldehyde for histopathological analysis.

2.10.1. Haematological and biochemical analyses

Blood, collected in Vacutainer® tubes containing EDTA, was used for the haematological tests (haematocrit, haemoglobin, platelets, erythrocytes, total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils) on an automatic cell counter (Cell Dyn 3700, Abbott Laboratories, EUA).

For the biochemical analysis, the blood was collected without anticoagulant, centrifuged at 3000 × *g* (4 °C, 10 min). The serum was separated and the biochemical parameters glucose, urea, creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total cholesterol, triglycerides, total and gamma-glutamyltranspeptidase (GGT) were quantified by colorimetric assays using Labtest® kits.

2.10.2. Histopathological analyses

After 24 h of immersion in 4% paraformaldehyde in PBS, the organs were processed in an automated tissue processor (MTP 100, Slee Mainz, Germany). The samples were then embedded in paraffin, frozen and cut with a microtome HIRAX M60 (Carl Zeiss, Germany). For the preparation of the slides, 3-μm sections of each organ were deparaffinised, rehydrated and stained with HE. With the optic microscope (Axio Scope. A1, Carl Zeiss, Germany) increased to 100 ×, the organs were analysed for the presence of necrosis/degeneration, leukocyte infiltrate and vascularisation (Jubb et al., 1993).

2.11. Data analysis

The results of the parametric tests were expressed as mean ± standard error (S.E.M.). One-way analysis of variance (ANOVA) was

used for comparing the means; when there was statistical significance, it was followed by the Student-Newman-Keuls test. Non-parametric data were expressed as median and quartiles (Q_1; Q_3), and the Kruskal-Wallis ANOVA was used for comparisons between the medians, followed, when there was statistical significance, by Dunn's multiple comparison test. The value of $p < 0.05$ was considered the critical level for rejection of the null hypothesis. GraphPad Prism version 6.07 (USA) was used for data tabulation and analysis. The IC_{50} was determined by the linear regression curve, relating the percentage of inhibition *versus* the logarithm of the tested concentrations and assuming a confidence interval of 99% ($p < 0.01$) for the resulting curve.

3. Results

3.1. Preliminary phytochemical analysis and TLC

HETa showed positive reaction in the following tests: ferric chloride, Shinoda, alkali hydroxides and aluminum chloride, foaming, gelatin precipitation, alkaloids and metal salts and the Lierbermann-Burchard test, indicating the presence of phenolic compounds, flavonoids, saponins, tannins and phytosterols, respectively. The presence of alkaloids and coumarins in the HETa was not detected by the Dragendorff's reagent and fluorescence emission in alkaline medium, respectively.

The analysis of HETa by TLC revealed the presence of gallic acid and flavonoids (Fig. 1).

3.2. HPLC analysis

The HPLC analysis (Fig. 2) confirmed the presence of phenolic compounds detected in the preliminary phytochemical analysis and by the TLC. At a working time of 37 min, a matrix of phenolic compounds was detected at intervals between 10 and 41 min. The data revealed the presence of gallic acid (10.10 min), rutin (24.88 min), ellagic acid (25.47 min), catechin (26.10 min), quercetin (27.77 min) and kaempferol (28.41 min), representing 0.47; 7.98; 6.98; 7.19; 10.76% and 1.42%, respectively, of the total content of compounds available in the sample, based on the retention time of authentic standards.

3.3. DFI-ESI-IT-MS^n analyses

In order to obtain a fingerprint about the chemical composition of *T. argentea*, methanol/water (8:2 v/v) fractions from leaves were directly injected into the ESI source of the ion trap (Fig. 3). After MS/MS fragmentation, it is possible to identify the compounds in Table 1.

3.4. Quantitative phytochemical analysis

The contents of total phenols, total flavonoids and phytosterols in the HETa were 187.89 ± 1.53 mg GAE/g HETa, 108.0 ± 0.05 mg RE/g HETa and 189.75 ± 3.23 mg PE/g HETa, respectively.

3.5. Cytotoxicity assay

The treatment with HETa for 24 and 72 h did not affect the viability of CHO-K1 ($IC_{50} > 200$ µg/mL and $IC_{50} > 200$ µg/mL, respectively) and AGS ($IC_{50} > 400$ µg/mL and $IC_{50} > 400$ µg/mL, respectively) cells, and so it is non-cytotoxic at the concentrations tested. Doxorubicin (positive control) was non-cytotoxic for CHO-K1 at 24 h ($IC_{50} > 58$ µg/mL) and cytotoxic at 72 h ($IC_{50} = 0.30 \pm 0.04$ µg/mL). For the AGS cells, doxorubicin did not show cytotoxicity at neither 24 nor 72 h ($IC_{50} > 58$ µg/mL and $IC_{50} = 8.40 \pm 3.22$ µg/mL, respectively).

3.6. Genotoxicity assay

3.6.1. MN test

In the vehicle group, MNs, NPBs and NBUDs values were 24.8 ± 2.6, 7.8 ± 2.3 and 6.00 ± 0.7%, respectively (Table 2). Compared to the vehicle, the treatment of CHO-K1 cells with HETa (10, 30 and 100 µg/mL) increased the number of binucleate cells with MN in 266%, 274% and 225% ($p < 0.01$, $p < 0.05$ and $p < 0.05$) and with NBUDs in 428%, 478% and 428% ($p < 0.05$), respectively. The number of binucleated cells with NPBs only increased significantly in the group treated with 30 µg/mL of HETa (487%, $p < 0.05$). Doxorubicin, standard for this assay, promoted an increase in the number of MNs (366%, $p < 0.001$), NPBs (483%, $p < 0.05$) and NBUDs (512%, $p < 0.01$) when compared to the vehicle. The CBPI of the vehicle group was 1.63, and the treatment of the cells with 10 and 30 µg/mL of HETa increased this index by 6.1% and 6.7%, respectively. Conversely, at the highest concentration (100 µg/mL), HETa reduced CBPI by 7.4% as well as 0.03 µg/mL of doxorubicin (29.4%).

3.6.2. Comet assay

In the pre-treatment protocol, CHO-K1 cells incubated only with medium (vehicle) had a % of tail DNA of 0.0002090 (0.0001550; 0.7319) (Table 3). Incubation of the cells for 4 h with 50 µM of H_2O_2 increased DNA damage by about 3360 times ($p < 0.001$). Pre-treatment of the cells with HETa prevented DNA damage ($p < 0.001$) induced by H_2O_2 in all concentrations, reaching its highest effect (4277 times) at a concentration of 30 µg/mL. Pre-treatment of the cells with HETa (10 and 100 µg/mL), without incubation with H_2O_2, increased the genetic damage by 203 ($p < 0.01$) and 1160 times ($p < 0.001$), respectively, when compared to the vehicle.

In the co-treatment protocol, cell incubation for 4 h with 50 µM of H_2O_2 increased DNA damage by 1.4 times ($p < 0.001$) when compared to the vehicle (Table 3). Co-treatment of the cells with HETa resulted in an increase in H_2O_2-induced genotoxicity ($p < 0.001$) at all concentrations, reaching its highest effect (3.8 times) at a concentration of 10 µg/mL.

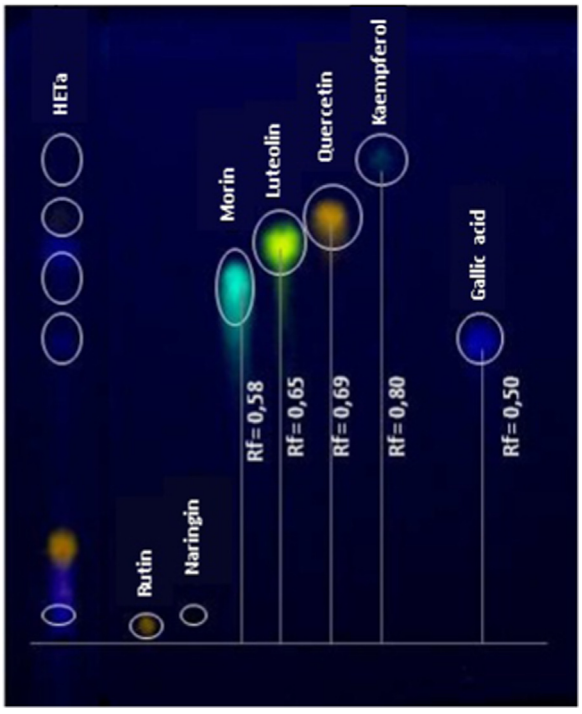

Fig. 1. Thin layer chromatography (TLC) of the hydroethanolic extract of *Terminalia argentea* leaves (HETa). Rf = retention factor. Standards: rutin, naringin, morin, luteolin, quercetin and kaempferol.

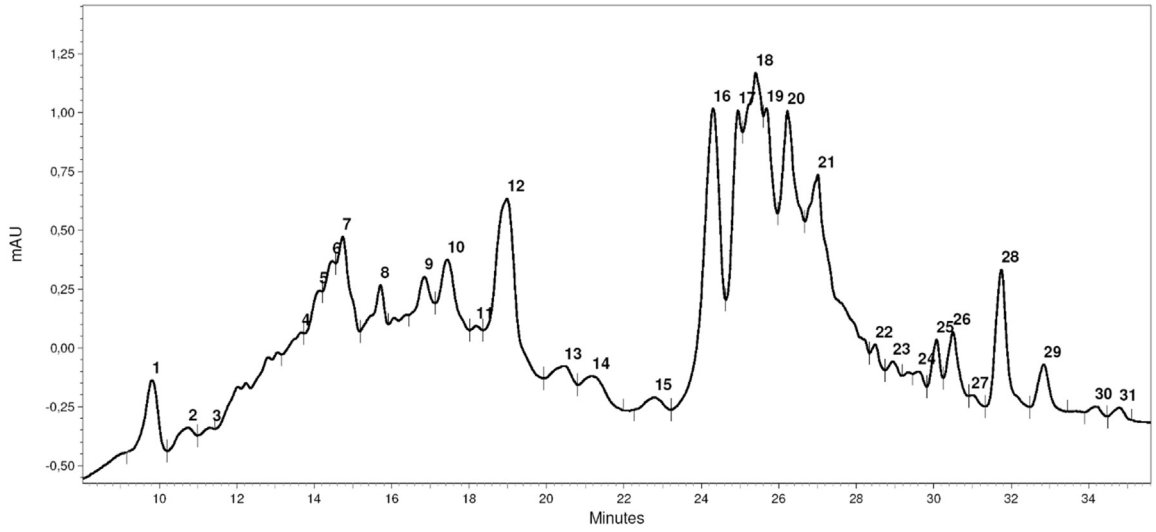

Fig. 2. Fingerprint of the hydroethanolic extract from leaves of *Terminalia argentea* by high- performance liquid chromatography (HPLC) detected at 280 nm. Peak 2: gallic acid; 16: rutin; 18: ellagic acid; 20: catechin; 21: quercetin and 22: kaempferol.

In the post-treatment protocol, cell incubation for 4 h with 50 μM of H_2O_2 increased DNA damage by 2.3 times ($p < 0.001$) when compared to the vehicle (Table 3). Only at a concentration of 10 μg/mL, the post-treatment of the cells with HETa was able to reverse the damage ($p < 0.001$) induced byH_2O_2.

3.7. Acute toxicity

Single oral administration of HETa at doses of up to 2000 mg/kg caused reversible piloerection in both male (reversible in up to 2 h) and female (reversible in up to 4 days) mice. This was observed in 1/6 of the females for the two doses tested, and in 5/6 and 4/6 males, for doses of 1000 and 2000 mg/kg, respectively. No deaths occurred with the doses tested (Table 4).

Single oral administration of HETa doses of 1000 and 2000 mg/kg did not change the weight gain of the mice in the 14-day period nor did it alter the relative weight of the analysed organs. No macroscopic changes were observed in the analysed organs.

3.8. Sub-chronic toxicity test (repeated doses)

Table 5 shows the effect of a 30-day daily treatment with vehicle or HETa (mg/kg, p.o.) on the rats' body weight and weight gain, feed and water intake and excretion of faeces and urine accumulated every 6 days. There was no statistically significant difference in accumulated weight gain (g), water consumption, and faeces and urine during the 30-day treatment period with HETa for the three doses (50, 200 and 800 mg/kg). In animals treated with 800 mg/kg of HETa, feed intake was only altered at D_{18}, 23.6% lower ($p < 0.01$) than the vehicle group (Table 5). No change in feed intake was observed in animals treated with HETa doses of 50 and 200 mg/kg.

Table 6 compares the groups regarding several biochemical and

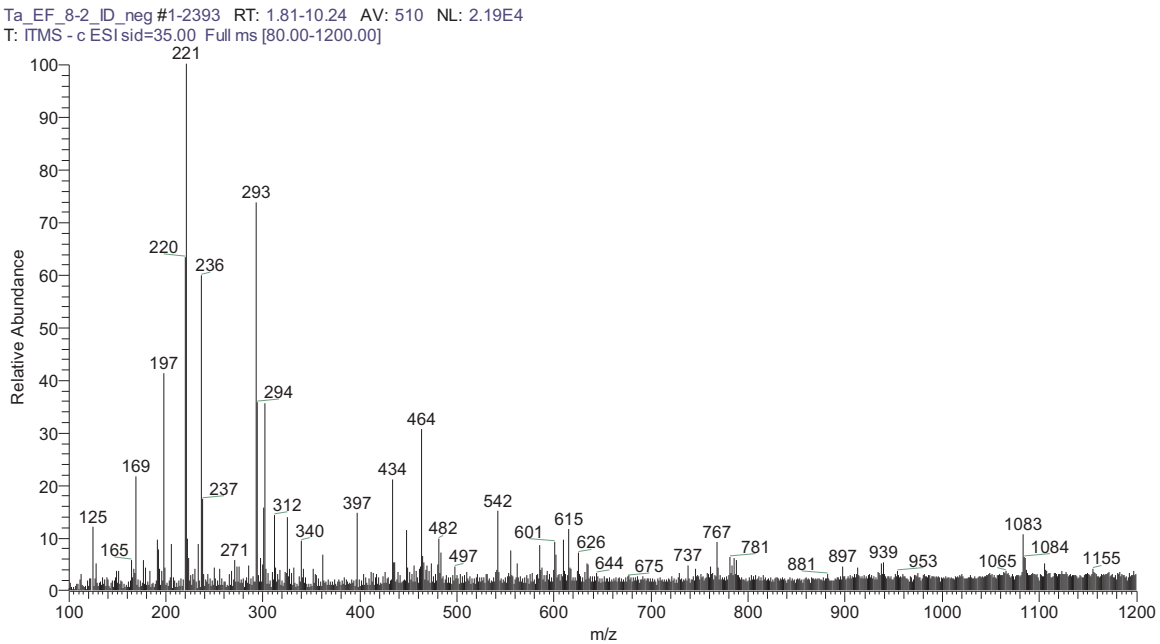

Fig. 3. Full scan mass spectrometry through direct flow infusion coupled with electrospray ionization and ion-trap analyzer (DFI-ESI-IT-MS) of the hydroethanolic extract of Terminalia argentea leaves (HETa), negative mode.

Table 1
Compounds identified in the hydroethanolic extract of *Terminalia argentea* leaves (HETa). In bold: precursor ions of the tandem mass spectrometry (MS/MS) experiments.

No.	[M-H]$^-$	MS^2 Fragments	MS^3 Fragments	Compound
1	169	125	–	Gallic acid
2	179	151. 135	–	Caffeic acid
3	191	173. 147. 111. 99. 85	–	Quinic acid
4	301	179. 151	–	Quercetin
5	301	257. 229	–	Ellagic acid
6	361	343. 293. 236	–	Galloylmucic acid
7	433	**301**	179. 151	Quercetin xyloside
8	447	**301**	179. 151	Quercetin rhamnoside
9	463	**301**	179. 151	Quercetin glucoside
10	481	463. 437. 409. **301**	257. 229	Caffeoyl ellagic acid
11	585	433.**301**	179. 151	Quercetin galloyl xyloside
12	601	543. 527. 411. 299	–	Terminalin
13	609	591.**463**. 441. 301	301	Rutin
14	615	463.**301**	179. 151	Quercetin galloyl glucose
15	633	615. 489. **301**	257. 229	Corilagin
16	737	719. **585**. 391.**301**	193. 151	Quercetin digalloyl xyloside
17	767	**615**. 465. 313.**301**	179. 151	Quercetin digalloyl glucoside
18	781	763. 721. **601**. 449	299. 271	Punicalin
19	1083	1065.**781**. 721. 601. 549	721. 601. 575. 449	Punicalagin

haematological parameters. HETa doses of 50, 200 and 800 mg/kg did not alter plasma levels of glucose, urea, creatinine, uric acid, AST, ALT, alkaline phosphatase, triglycerides, total proteins, albumin, globulin and GGT. HETa at 800 mg/kg reduced total plasma cholesterol levels by about 13% ($p < 0.001$) when compared to the vehicle. At doses of 50 and 200 mg/kg, there were no changes in total cholesterol levels.

As to the haematological parameters, HETa at 50 mg/kg increased the absolute (10^3/mm^3) and relative (%) values of basophils by 477.8% and 423%, respectively ($p < 0.001$), but this was not significantly different ($p > 0.05$) in animals treated with HETa doses of 200 and 800 mg/kg. In the other haematological parameters, no change was significantly different from the vehicle group (Table 6).

No macroscopic changes were observed in the heart, lung, liver, stomach, spleen, kidneys and brains of the animals treated with HETa (50, 200 and 800 mg/kg) and, on microscopic examination, the sub-chronic treatment did not cause deleterious effects to the normal histological architecture of the analysed organs. However, the relative weight of the heart in animals treated with HETa at doses of 800 mg/kg was 13.2% lower than in the vehicle group ($p < 0.05$), and there was no statistically significant difference at doses of 50 and 200 mg/kg ($p > 0.05$). The relative weight of the other organs was not altered in the animals treated for 30 days with HETa at doses of 50, 200 and 800 mg/kg (Table 7).

4. Discussion

Homemade preparations of *T. argentea* leaves are widely used in central Brazil for several diseases, such as bronchitis, haemorrhages and ulcers (Ribeiro et al., 2017). As a vital step in the investigation of a new drug, this study carried out the HETa pre-clinical toxicity studies regarding cytotoxicity, genotoxicity and acute and sub-chronic toxicity.

The cytotoxicity of the extract was assessed by the Alamar blue assay in CHO-K1 and AGS cells. This assay can be used to analyse cell viability and to track cytotoxicity, and the results serve as the basis for planning other tests (OECD, 2008a; Rampersad, 2012). Treating the cells with HETa for 24 and 72 h did not affect the viability of CHO-K1 ($IC_{50} > 200$ μg/mL) and AGS ($IC_{50} > 400$ μg/mL) cells. Since IC_{50} values were higher than 30 μg/mL, established by the U.S. National Cancer Institute (NCI) as the cytotoxicity threshold for extracts (Suffness and Pezzuto, 1990), the data indicate that HETa is not cytotoxic to these normal and tumour cell lineages. In previous studies, there are no reports of cytotoxicity studies with *T. argentea*. In extracts from the leaves of other species of the genus, cytotoxicity is reported in *T. calamansanai* acetone extracts (Lih-Geeng et al., 2009), *T. catappa* supercritical CO_2 (Ko et al., 2003) and *T. chebula* ethanolic extracts (Ravi Shankara et al., 2016); however, they use different methodologies, with a value of $IC_{50} > 200$ μg/mL (Ravi Shankara et al., 2016) or uncalculated IC_{50} values (Ko et al., 2003; Lih-Geeng et al., 2009).

Although HETa has been shown to be non-cytotoxic *in vitro*, a single *in vitro* toxicity study cannot be used as conclusive evidence that an agent is not toxic. Therefore, this study assessed the genotoxicity of HETa using the MN and comet assays in CHO-K1 cells. The MN assay is considered to be one of the most efficient cytogenetic tests to assess chromosomal breaks and/or losses in mitotic cells (Fenech, 2007). Treatment of CHO-K1 cells with HETa increased the number of MN and NBUDs in binucleated cells at the three concentrations tested (10, 30 and 100 μg/mL), the number of NPBs with the lowest concentration and reduced the CBPI, indicating that the extract presented genotoxic and cytotoxic potential, in the latter case, when in high concentrations.

Caution is required when extrapolating the genotoxicity results obtained with HETa using only the MN assay. The comet assay, recognised for detecting DNA damage in both mitosis and interphase cells, is recommended along with the MN assay, characterising the best test battery to characterise the mutagenic potential of a given compound (Araldi et al., 2015). In the comet assay, the genotoxic or anti-genotoxic effect of HETa was assessed in the absence or presence of H_2O_2 as an inducing agent, respectively. Cell treatments with HETa (10 and 100 μg/mL) or HETa (10, 30 and 100 μg/mL) and H_2O_2 (50 μM) increased genetic damage, while pre-treatment (10, 30 and 100 μg/mL) and post-treatment (10 μg/mL) with HETa prevented and repaired DNA damage, respectively. In previous works, there are no genotoxicity studies with *T. argentea* and, therefore, there is no data to compare to the results obtained with the HETa. However, in aqueous extracts of *T. catappa* leaves, an anticlastogenic activity was demonstrated in a mitomycin C induced MN assay done on CHO-K1 cells (Liu et al., 1996), antigenotoxic in a comet assay and antimutagenic in a bleomycin-

Table 2
Effect of the hydroethanolic extract of *Terminalia argentea* leaves (HETa) on the number of micronuclei, nucleoplasmic bridges and nuclear buds on binucleated Chinese hamster ovary epithelial (CHO-K1) cells and on the cytokinesis-block proliferation index (CBPI) and replication index (RI).

Treatment	μg/mL	MN (‰)	NPBs (‰)	NBUDs (‰)	CBPI	RI (%)	Cytotoxicity (%)
Vehicle	–	24.8 ± 2.6	7.8 ± 2.3	6.0 ± 0.7	1.63	100	0
Doxorubicin	0.03	90.7 ± 1.8**	37.7 ± 2.2*	30.7 ± 2.8**	1.15	23.1	+ 76.9
HETa	10	66.0 ± 17.4**	15.0 ± 5.5	25.7 ± 8.7*	1.73	115.9	−15.9
	30	68.0 ± 7.2*	38.0 ± 13.5 *	28.7 ± 1.2*	1.74	117.5	−17.5
	100	55.7 ± 5.0*	19.0 ± 0.0	25.7 ± 4.3*	1.51	80.7	+ 19.3

MN (‰): micronucleus (MN) in 1000 binucleate cells. NPBs (‰): nucleoplasmic bridges (NPBs) in 1000 binucleate cells. NBUDs (‰): nuclear buds (NBUDs) in 1000 binucleate cells. Cytotoxicity (%) = 100–100 [$CBPI_{Treatment}$-1 / $CBPI_{Vehicle}$-1]. (+) increase; (-) decrease. Results expressed as mean ± S.E.M. in duplicate. One- way ANOVA followed by the Student-Newman-Keuls test. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ *versus* vehicle.

Table 3
In vitro genotoxicity evaluation of the hydroethanolic extract of *Terminalia argentea* leaves (HETa) by comet assay in Chinese hamster ovary epithelial (CHO-K1) cells.

Group	% of DNA tail		
	Pre-treatment	Co-treatment	Post-treatment
Vehicle	0.0002090 (0.0001550; 0.7319)	0.00038 (0.00029; 0.00069)	0.00038 (0.00031; 0.00054)
H_2O_2 50 μM	0.7031 (0.0002061; 4.291)***	0.00053 (0.00036; 0.0017)***	0.00089 (0.00065; 0.0017)***
10 μg/mL	0.04244 (0.0001668; 0.9368)**	–	–
HETa 30 μg/mL	0.0002233 (0.0001498; 0.1390)	–	–
100 μg/mL	0.2424 (0.0001639; 1.501)***	–	–
10 μg/mL + H_2O_2	0.0001737 (0.0001411; 0.09343)†††	0.0020 (0.00075; 13.74)†††	0.00069 (0.00057; 0.00085)†††
HETa 30 μg/mL + H_2O_2	0.0001644 (0.0001416; 0.1027)†††	0.0012 (0.00071; 1.91)†††	0.00084 (0.00063; 0.0015)
100 μg/mL + H_2O_2	0.0001748 (0.0001449; 0.3040)†††	0.00070 (0.00057; 0.0011)†††	0.00081 (0.00065; 0.66)

(–): unrealized. Results expressed as median (Q1; Q3). Kruskal-Wallis analysis followed by the Dunn test. *$p < 0.05$ and ***$p < 0.001$ *versus* vehicle; ††† $p < 0.001$ *versus* H_2O_2.

Table 4
Effects of acute oral administration in single doses of the hydroethanolic extract of *Terminalia argentea* leaves (HETa) on the general behavioural activities in mice observed for 14 days.

Group		Dose (mg/kg)	Clinical signs and symptoms	Deaths / Survivors
Male	Vehicle	–	No alterations	0/6
	HETa	1000	Piloerection (5/6 - reversible from 2nd h)	0/6
		2000	Piloerection (4/6 - reversible from 2nd h)	0/6
Female	Vehicle	–	No alterations	0/6
	HETa	1000	Piloerection (1/6 - reversible from 4th day)	0/6
		2000	Piloerection (1/6 - reversible from 4th day)	0/6

induced hgprt gene mutation assay (Chen et al., 2000). With the Ames test, a hydroethanolic extract of *T. catappa* leaves presented a mutagenic effect (Mininel et al., 2014), whereas the hexane and supercritical CO_2 extracts were antimutagenic (Ko et al., 2003).

The fact that HETa caused DNA damage by itself in the comet assay corroborates the genotoxicity results found in the MN assay and reinforces that HETa is genotoxic, at least *in vitro*. In addition, HETa was also genotoxic in a co-treatment protocol in the H_2O_2-induced comet assay. Paradoxically, the extract presented an antigenotoxic effect in pre- and post-treatment protocols with H_2O_2, an apparently conflicting result, but it can be explained by the phytochemical complexity of the extract and the antioxidant and pro-oxidant characteristics of the phenolic compounds present in HETa.

The HETa UV spectrophotometric quantification revealed significant amounts of phenols (18.8%), flavonoids (10.8%) and phytosterols (19%), with quercetin, rutin, catechin and ellagic acid as the major components, as confirmed by HPLC analysis. The ESI-MS^n

Table 5
Effect of subchronic oral administration of the hydroethanolic extract of *Terminalia argentea* leaves (HETa) on body weight, accumulated weight gain, consumption of water and feed and excretion of faeces and urine from rats.

Group/Analysed parameters	Period of treatment (days)					
	D_0	D_6	D_{12}	D_{18}	D_{24}	D_{30}
Vehicle						
Body weight (e)	164.2 ± 3.4	187.5 ± 4.2	200.1 ± 4.0	215.3 ± 4.5	226.8 ± 4.8	237.9 ± 5.3
Cumulative weight gain (g)	0.0	23.3 ± 2.1	35.9 ± 2.6	51.1 ± 2.7	62.6 ± 3.3	73.7 ± 4.1
Water consumption (mL)	0.0	63.5 ± 2.6	59.3 ± 2.1	61.6 ± 2.7	57.8 ± 2.3	64.1 ± 2.8
Feed consumption (g)	0.0	36.1 ± 1.0	35.6 ± 0.9	36.0 ± 0.8	35.2 ± 1.3	34.5 ± 1.2
Faeces (g)	0.0	19.4 ± 1.0	20.4 ± 1.1	23.8 ± 1.0	21.1 ± 1.0	18.0 ± 0.7
Urine (mL)	0.0	24.6 ± 1.5	23.6 ± 1.9	24.4 ± 1.7	25.3 ± 1.9	25.2 ± 1.6
HETa 50 mg/kg						
Body weight (e)	160.3 ± 5.8	189.5 ± 8.3	205.7 ± 6.6	222.6 ± 5.0	233.1 ± 5.6	245.5 ± 6.4
Cumulative weight gain (g)	0.0	29.2 ± 4.5	45.3 ± 3.4	62.3 ± 4.4	72.8 ± 3.8	85.1 ± 4.3
Water consumption (mL)	0.0	53.5 ± 4.9	58.5 ± 3.5	52.7 ± 3.3	56.0 ± 3.5	58.7 ± 6.7
Feed consumption (g)	0.0	34.1 ± 2.7	38.4 ± 1.7	36.3 ± 1.7	38.0 ± 1.7	36.9 ± 1.7
Faeces (g)	0.0	20.0 ± 2.2	22.0 ± 1.5	21.1 ± 0.9	23.1 ± 1.1	20.7 ± 1.4
Urine (mL)	0.0	20.2 ± 2.1	20.3 ± 2.3	22.0 ± 2.4	23.0 ± 2.4	21.3 ± 2.8
HETa 200 mg/kg						
Body weight (e)	163.7 ± 3.6	185.0 ± 3.5	200.9 ± 3.1	214.1 ± 3.2	221.6 ± 2.5	231.9 ± 2.2
Cumulative weight gain (g)	0.0	21.4 ± 3.7	37.2 ± 3.3	50.4 ± 4.5	58.0 ± 4.6	68.2 ± 4.3
Water consumption (mL)	0.0	60.2 ± 3.8	55.0 ± 1.9	56.3 ± 1.8	51.1 ± 2.9	53.4 ± 3.0
Feed consumption (g)	0.0	35.0 ± 2.3	35.0 ± 0.7	36.0 ± 1.1	32.4 ± 1.4	33.0 ± 1.1
Faeces (g)	0.0	19.6 ± 1.3	19.2 ± 1.0	22.5 ± 0.7	19.9 ± 1.0	18.3 ± 1.2
Urine (mL)	0.0	26.3 ± 3.3	22.0 ± 0.9	21.9 ± 1.5	22.9 ± 2.2	21.5 ± 1.5
HETa 800 mg/kg						
Body weight (e)	158.7 ± 5.5	185.3 ± 6.1	193.3 ± 5.3	206.3 ± 5.6	217.7 ± 5.8	228.4 ± 4.6
Cumulative weight gain (g)	0.0	26.6 ± 2.6	34.7 ± 3.9	47.6 ± 4.3	59.0 ± 2.8	69.8 ± 3.9
Water consumption (mL)	0.0	59.2 ± 4.2	52.6 ± 4.4	57.6 ± 3.6	62.3 ± 6.0	55.3 ± 2.5
Feed consumption (g)	0.0	37.4 ± 1.9	31.2 ± 2.6	27.5 ± 3.4**	32.0 ± 2.5	31.0 ± 2.0
Faeces (g)	0.0	20.6 ± 1.3	18.7 ± 1.5	22.9 ± 2.5	20.5 ± 0.8	19.3 ± 1.8
Urine (mL)	0.0	25.2 ± 2.0	23.0 ± 1.9	26.3 ± 3.0	25.6 ± 3.0	26.0 ± 2.5

Results were expressed as mean ± S.E.M. of 6 animals per group. One-way ANOVA followed by the Student-Newman-Keuls test. ** $p < 0.01$ *versus* vehicle.

Table 6
Effect of oral subchronic administration of the hydroethanolic extract of *Terminalia argentea* leaves (HETa) on some biochemical and haematological parameters in rats at the end of 30 days.

Parameters	Vehicle	HETa (mg/kg)			Reference values[a]
		50	200	800	
Biochemicals					
Glucose (mg/dL)	184.5 ± 6.3	178.2 ± 9.9	178.0 ± 8.6	178.1 ± 9.1	76–175
Urea (mg/dL)	45.0 ± 2.7	39.5 ± 4.6	47.0 ± 2.4	45.3 ± 5.2	13.2–27.1
Creatinine (mg/dL)	0.40 ± 0.01	0.47 ± 0.01	0.41 ± 0.03	0.46 ± 0.02	0.2–0.6
Uric acid (mg/L)	1.6 ± 0.1	1.6 ± 0.2	1.7 ± 0.4	2.1 ± 0.4	–
AST (IU/L)	107.3 ± 6.4	98.8 ± 8.6	103.6 ± 5.4	97.3 ± 5.3	65–203
ALT (IU/L)	60.8 ± 2.3	53.7 ± 1.7	62.7 ± 2.6	59.3 ± 2.2	16–48
Alkaline phosphatase (IU/L)	348.7 ± 34.1	366.7 ± 24.5	348.9 ± 40.7	350.3 ± 54.6	26–147
Total cholesterol (mg/dL)	125.4 ± 3.0	112.3 ± 4.7	120.4 ± 2.8	109.0 ± 4.6 *	24–73
Triglycerides (mg/dL)	93.3 ± 13.3	81.2 ± 9.8	106.0 ± 17.1	82.5 ± 6.0	14–46
Total protein (mg /dL)	6.8 ± 0.1	6.5 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	5.5–7.7
Albumin (g/dL)	3.7 ± 0.1	4.1 ± 0.1	3.6 ± 0.2	3.8 ± 0.2	3.6–5.5
Globulin	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.2	2.4 ± 0.1	1.5–2.4
GGT	1.7 ± 0.3	2.0 ± 0.4	2.0 ± 0.0	2.3 ± 0.5	–
Haematological					
Blood cells (10^6/mm^3)	7.7 ± 0.1	7.6 ± 0.1	7.6 ± 0.2	7.6 ± 0.2	7.1–9.0
Haemoglobin (g/dL)	15.4 ± 0.1	14.9 ± 0.3	14.9 ± 0.3	15.0 ± 0.3	13.7–16.8
Haematocrit (%)	43.3 ± 0.5	43.1 ± 0.9	41.6 ± 0.9	42.4 ± 0.9	37.9–49.9
MCV (fL)	56.0 ± 0.5	56.7 ± 0.6	54.9 ± 0.7	56.0 ± 0.7	49.9–58.3
MCH (pg)	18.8 ± 0.4	18.2 ± 0.8	18.5 ± 0.5	19.0 ± 0.7	17.8–20.9
MCHC (g/dL)	33.7 ± 0.9	32.1 ± 1.2	33.8 ± 1.2	34.0 ± 1.1	33.3–38.1
Platelets (10^3/mm^3)	989.3 ± 49.9	861.5 ± 48.0	1041.0 ± 84.5	966.6 ± 40.1	680-1.200
Total leukocytes (10^3/mm^3)	6.9 ± 0.3	7.6 ± 0.6	8.1 ± 0.9	7.8 ± 0.5	1.1–7.5
Neutrophil					
Relative (%)	27.6 ± 3.1	22.8 ± 1.5	33.2 ± 2.8	28.1 ± 3.1	7.1–33.2
Absolute (10^3/mm^3)	1.9 ± 0.3	1.7 ± 0.1	2.6 ± 0.2	2.2 ± 0.3	0.2–1.5
Lymphocyte					
Relative (%)	70.6 ± 3.1	75.3 ± 1.5	64.9 ± 2.6	69.2 ± 2.9	62.2–90
Absolute (10^3/mm^3)	4.8 ± 0.2	5.7 ± 0.5	5.3 ± 0.4	5.3 ± 0.4	0.8–5.7
Eosinophil					
Relative (%)	0.80 ± 0.24	0.78 ± 0.18	0.91 ± 0.36	1.10 ± 0.28	0.5–4.5
Absolute (10^3/mm^3)	0.05 ± 0.02	0.06 ± 0.01	0.08 ± 0.04	0.08 ± 0.02	0.01–0.2
Basophil					
Relative (%)	0.13 ± 0.05	0.68 ± 0.13***	0.12 ± 0.08	0.20 ± 0.08	0–0.8
Absolute (10^3/mm^3)	0.009 ± 0.003	0.052 ± 0.012***	0.010 ± 0.007	0.014 ± 0.005	0–0.03
Monocyte					
Relative (%)	0.87 ± 0.25	0.50 ± 0.09	0.88 ± 0.20	1.41 ± 0.54	0.8–3.9
Absolute (10^3/mm^3)	0.06 ± 0.02	0.04 ± 0.00	0.07 ± 0.02	0.12 ± 0.05	0.02–0.16

AST = aspartate aminotransferase; ALT = alanine aminotransferase;GGT = gamma-glutamyl transferase; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration. [a] Reference values for 8–16-week-old Wistar rats (Giknis and Clifford, 2008). The values represent the mean ± S.E.M. of 6 animals per group. One-way ANOVA followed by the Student-Newman-Keuls test. * $p < 0.05$ and *** $p < 0.001$ versus vehicle.

Table 7
Effect of oral subchronic administration of the hydroethanolic extract from *Terminalia argentea* leaves (HETa) on the relative weight of some organs of rats at the end of 30 days.

Organs	Vehicle	HETa (mg/kg)		
		50	200	800
Heart	0.38 ± 0.01	0.36 ± 0.01	0.35 ± 0.01	0.33 ± 0.00*
Lung	0.58 ± 0.03	0.59 ± 0.02	0.60 ± 0.04	0.53 ± 0.03
Spleen	0.25 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.27 ± 0.01
Stomach	0.53 ± 0.01	0.51 ± 0.02	0.53 ± 0.02	0.55 ± 0.02
Liver	3.96 ± 0.06	4.28 ± 0.14	3.96 ± 0.11	4.09 ± 0.12
Kidney L	0.37 ± 0.01	0.40 ± 0.02	0.35 ± 0.01	0.38 ± 0.02
Kidney R	0.39 ± 0.01	0.43 ± 0.02	0.37 ± 0.01	0.36 ± 0.01
Brain	0.73 ± 0.01	0.74 ± 0.02	0.73 ± 0.01	0.73 ± 0.04

L = left; R = right. The values represent the mean ± S.E.M. of 6 animals per group. There was one-way ANOVA followed by the Student-Newman-Keuls test. *$p < 0.05$ *versus* vehicle.

identified phenolic (ellagic acid, caffeic acid, gallic acid and galloylmucic acid), tannins (corilagin, punicalagin, punicalin and terminalin), flavonoids (rutin, quercetin and glycosylated derivatives of quercetin) and cyclic polyol (quinic acid) compounds.

Hydrogen peroxide, used in the comet assay as a positive control, induces genotoxic effects particularly by producing reactive oxygen species (ROS); therefore, antioxidant compounds can presumably modulate the genotoxicity induced by H_2O_2 (Labieniec et al., 2003). Except for quinic acid, the other compounds identified in HETa have several hydroxyl groups in their chemical structure, making them ideal antioxidants for reactions with ROS and as metal chelators (Barreiros et al., 2006). This explains the HETa *in vitro* data, which showed antioxidant activity with 1,1-diphenyl-2-picrylhydrazylassay (DPPH), with IC_{50} = 6.25 µg/mL (unpublished data). This antigenotoxic effect by antioxidant substances was described in punicalagin in comet assays (Chen et al., 2000) and in rutin and quercetin in comet and MN assays (Barcelos et al., 2011). In the case of quercetin, its antioxidant effect effectively reduces H_2O_2-induced DNA damage, which explains its use as a standard drug in comet assays (Živković et al., 2017).

This suggests that, under pre-treatment conditions, HETa compounds, particularly phenolic and flavonoid compounds, increase the antioxidant capacity of the cells, making them more resistant to oxidative stress and protecting them from DNA damage induced by H_2O_2. On the other hand, the HETa antigenotoxic effect during post-treatment could be attributed to the elimination of free radicals and/or stimulation of repair of the damage caused by H_2O_2 to DNA.

In contrast, the same antioxidant compounds, particularly flavonoids, may exhibit pro-oxidant behaviour under certain conditions and accelerate DNA damage (Labieniec et al., 2003). These pro-oxidant and genotoxic effects are reported for gallic acid, ellagic acid, quercetin, caffeic acid, catechin and kaempferol in various genotoxicity models (Labieniec et al., 2003; Utesch et al., 2008; Weisburg et al., 2013; Léon-González et al., 2015).

Hydrogen peroxide diffuses easily through cell membranes, such as the nucleus membrane, and reacts with transition metals like Cu^{1+} and Fe^{2+}, generating hydroxyl radicals ($HO^{\bullet}$) inside, a reaction known as Fenton's reaction (Labieniec et al., 2003; Barreiros et al., 2006). In *in vitro* assays, the HETa showed a Fe^{3+} reducing-effect comparable to ascorbic acid using the ferric ion reducing power (FRAP) method (unpublished data). This suggests that the genotoxic effect of HETa alone in the comet assay and simultaneous treatment with H_2O_2, as well as in the MN assay, can be explained in part by its pro-oxidant effect, since compounds capable of reducing metals like Fe^{3+} and Cu^{2+} are potentially pro-oxidants, increasing the availability of Fe^{2+} and Cu^{1+} that participate in Fenton's reagent and generating more $HO^{\bullet}$ radicals (Barreiros et al., 2006).

As the exact mechanism is unknown, further studies should be carried out to clarify the mechanisms underlying the antigenotoxic and genotoxic effects of HETa *in vitro*. In addition, *in vitro* substance behaviour can be altered when subjected to the metabolism of a living organism, and the use of *in vivo* genotoxicity assays may be useful in interpreting the biological relevance of such data.

The systemic action of a compound in living organisms requires its absorption, distribution, metabolism and excretion, in addition to undergoing hormonal, neural and intercellular communication interferences. Therefore, *in vitro* bioassays may not be fully predictive of adverse effects *in vivo* (Rampersad, 2012). As homemade preparations with *T. argentea* leaves have been used extensively by Brazilian population in the fight against several diseases, we proceeded with the preclinical safety assessment of this also in animals.

The acute toxicity assay represents the initial *in vivo* preclinical study that predicts the toxic mode of action of a substance or compound, as well to assist in the selection of appropriate doses for other animal studies (OECD, 2008a). Administration of HETa (1000 and 2000 mg/kg) did not alter weight gain or cause death in mice. No changes were observed at a macroscopic level or to relative weight in the analysed organs. The clinical signs that emerged were reversible piloerection in both sexes. Therefore, these data indicate that, at the doses tested, HETa is safe after acute administration, even in high doses.

In order to assess the effect of cumulative and non-lethal doses of HETa (50, 200 and 800 mg/kg) a sub-chronic toxicity test was performed in rats. This test is recognised to correlate better with toxicological effects in humans (Olson et al., 2000). Three geometric doses of HETa were administered for 30 days, assessing the behavioural, physiological (weight gain, water and feed intake, and faeces and urine), haematological, biochemical, anatomical and histopathological changes in target organs. In this assay, no deaths or clinical signs and symptoms of toxicity were observed in the animals at any of the doses tested, except for absolute and relative basophil counts, relative heart weight, total cholesterol concentration and feed intake at the 18th day.

Haematological data translated from animal studies has a higher predictive value for human toxicity, so they are important and relevant for assessing changes in the haematological system as well as providing information on the haematopoietic system and immune responses (Olson et al., 2000). The haematological profile of HETa-treated rats was not significantly different from the vehicle group, except for the absolute and relative basophil counts, which were higher only at a lower dose. This effect was not followed by clinical or histopathological alterations, it was not dose-dependent and it was within the physiological range (Giknis and Clifford, 2008), indicating little relevance for this finding.

Comparison of organ weights between the treated and untreated groups of animals is conventionally used in the toxicological evaluation of drugs, chemicals, biologicals and food additives, and it is a sensitive indicator of changes that can be chemically induced to the organs (Michael et al., 2007). Liver and kidney weights are considered as the most useful and sensitive in predicting toxicity, in addition to correlating well with histopathological changes (Michael et al., 2007). Despite the well-known renal and hepatic toxicity of terminalin (Oelrichs et al., 1994), its presence in the HETa did not alter the weight of the kidneys and liver, nor cause histopathological changes in these organs.

The relative heart weight of the animals treated with 800 mg/kg HETa decreased by 13.2%. Heart weight has limited value in toxicity studies (Michael et al., 2007); in addition, this effect was not dose-dependent and only verified at a high dose, and no clinical or histopathological changes were observed, suggesting that other physiological factors may be associated with this finding. As for the other organs, HETa did not cause significant changes in the relative weights, and the same was observed for histopathological analyses.

Biochemical tests are widely used in toxicological studies and are decisive for assessing organ function or damage in animals (Boehm et al., 2007). Of the biochemical parameters analysed, there was alteration only in total cholesterol, which was reduced with the administration of HETa at a higher dose. Hyperlipidaemia is known as a major risk factor for the development of cardiovascular diseases including atherosclerosis, coronary disease and stroke (Reddy et al., 2015). Polyphenols such as quercetin, kaempferol and catechin are important antioxidants associated with decreased total cholesterol levels, inhibition of low density lipoprotein (LDL) peroxidation and increased levels of high density lipoprotein (HDL)(Kris-Etherton et al., 2002; Barbagallo et al., 2015). Also, phytosterols can reduce cholesterol bowel absorption and LDL levels (Barbagallo et al., 2015). This suggests that these substances may be involved in the hypocholesterol action of HETa. In addition, studies with other *Terminalia* species have shown hypocholesterol effect and consequent prevention of atherosclerosis in both animal and human models with *T. arjuna*(Sawale et al., 2015), *T. pallida* (Shaik et al., 2012), *T. chebula* (Reddy et al., 2015) and *T. bellerica* (Latha and Daisy, 2011).

Regarding feed intake, this was lower only on the 18th day of observation of the animals that received the highest dose of HETa, therefore, a transient and possibly unrelated event to treatment.

In general, the few clinical signs and symptoms observed after acute administration and the few changes observed in some biochemical and haematological parameters in the sub-chronic toxicity test do not seem to indicate toxicity to HETa and demonstrate that HETa has a good safety margin after acute and sub-chronic administration. These findings are consistent with the *in vivo* toxicity results reported for the methanolic extracts of *T. arjuna, T. citrina* and *T. coriacea* leaves in mice and rats (Biswas et al., 2011; Das et al., 2015; Ali Khan et al., 2017).

The NOAEL dose, which consists of the highest level of exposure to a substance where there is no statistically or biologically increased frequency or severity of adverse effects (Lewis et al., 2002), for HETa was 2000 mg/kg in mice and 800 mg/kg in rats. The riverine communities of the North Araguaia microregion prepare the infusion of *T. argentea* with approximately 20 g of dried leaves in 1 L of boiled water, corresponding to 3.7 mg/mL in extractive terms. Considering the dose of 1 shallow cup of tea (± 200 mL) three times a day, it can be inferred that an adult weighing 60 kg consumes 2220 mg of HETa daily, equivalent to a daily dose of 37 mg/kg. Thus, the maximum doses tested in mice (2000 mg/kg) and rats (800 mg/kg) were about 54.1 and 21.6 times higher, respectively, than ingested by humans.

5. Conclusion

The results show that HETa is not cytotoxic *in vitro* for CHO-K1 and AGS cells. In *in vitro* genotoxicity assays, HETa induced different responses, according to concentration and experimental condition. In the

MN assay, HETa presented genotoxic potential by increasing the number of MN, NPBs and NBUDs. In the comet assay, HETa was genotoxic by itself and in co-treatment protocol with H_2O_2. In pre-treatment or post-treatment protocols with H_2O_2, HETa presented an antigenotoxic effect by preventing or repairing, respectively, the genotoxicity induced by H_2O_2. In the *in vivo* models, HETa was shown to be relatively safe after acute administration in mice (NOAEL of 2000 mg/kg) and sub-chronic in rats (NOAEL of 800 mg/kg), confirming the knowledge of the riverine population that it is not toxic in the dosage used. Phytochemical analysis of HETa revealed the presence of phenolic compounds, flavonoids, saponins, tannins and phytosterols. Among the flavonoids and tannins, we highlight gallic acid, rutin, ellagic acid, quercetin, caffeic acid, quinic acid, corilagin, punicalin and punicalagin. Thus, it can be stated that HETa has a good margin of safety for therapeutic use.

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Disclosure of interest

The authors declare that they have no conflicts of interest.

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