BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2013, **112**, 34–41 Doi: 10.1111/j.1742-7843.2012.00924.x

Investigation of Biological Activities of Dichloromethane and Ethyl Acetate Fractions of *Platonia insignis* Mart. Seed

Joaquim S. Costa Júnior[1,2], Alexandre B. F. Ferraz[1], Taciana O. Sousa[2], Romézio A. C. Silva[2], Sidney G. De Lima[3], Chistiane M. Feitosa[3], Antônia M. G. L. Citó[3], Ana A. C. Melo Cavalcante[4], Rivelilson M. Freitas[4], Angelo R. Moura Sperotto[5], Valéria F. Péres[5], Dinara J. Moura[5] and Jenifer Saffi[5,6,*]

[1]Post-Graduation Programme in Genetics and Applied Toxicology, Lutheran University of Brazil, Canoas, RS, Brazil, [2]Department of Chemistry, Federal Institute of Piaui, Teresina, PI, Brazil, [3]Department of Chemistry, Federal University of Piaui, Teresina, PI, Brazil, [4]Post-Graduation Programme in Pharmaceutics Science, Federal University of Piaui, Teresina, PI, Brazil, [5]Laboratory of Genetic Toxicology, Department of Basic Health Sciences, Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS, Brazil and [6]National Institute for Translational Research on Health and Environment in the Amazon Region, CNPq/MCT, Rio de Janeiro, RJ, Brazil

(Received 7 January 2012; Accepted 3 July 2012)

Abstract: *Platonia insignis* Mart., a native species of the Brazilian Amazon more commonly known as bacuri, is a member of the Clusiaceae family. In this study, we evaluated the chemical composition and the antioxidant and toxicity activities of the dichloromethane and ethyl acetate fractions from *P. insignis* seed ethanolic extract using different experimental models. Our results demonstrate *in vitro* antioxidant effects, by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt and 1,1-diphenyl-2-picryl-hydrazyl assays, as well as *in vivo* effects in antioxidant-defective *Saccharomyces cerevisiae* strains to both fractions. Toxicity was evaluated against the micro-crustaceous *Artemia salina* Leach. and promastigote *Leishmania amazonensis*. The dichloromethane fraction was the most active fraction evaluated on *A. salina* and promastigote *L. amazonensis* (IC_{50} = 24.89 μg/mL and 2.84 μg/mL, respectively). In addition, a slight cytotoxicity was observed in mammalian V79 cells using ethyl acetate and dichloromethane fractions with MTT assays. Both fractions displayed genotoxicity up to 25 μg/mL (dichloromethane) and 10 μg/mL (ethyl acetate) in V79 cells, as evaluated by the alkaline comet assay. Thus, in this study, we demonstrate for the first time that ethyl acetate and dichloromethane fractions from *P. insignis* seeds display antioxidant effects, a toxic effect against *A. salina* and *L. amazonensis* and induce genotoxicity in V79 mammalian cells. The observed activities can be attributed to the phenolic compounds present in these fractions and to the presence of xanthones (alpha- and gamma-mangostin).

Recently, the focus on plant research has increased worldwide, and evidence has been collected showing the immense potential of medicinal plants used in various traditional systems [1, 2].

Clusiaceae family species are important medicinal plants used in Brazilian folk medicine, in particular for treating eczemas, herpes, gastrointestinal diseases, dermatitis, schistosomiasis, leishmaniasis and malaria [3]. Furthermore, preparations obtained from these plants have shown anti-inflammatory, anti-malarial, anti-hypertensive, anti-diabetics, immunomodulatory, antiviral, anti-tumour, antidepressive, anti-allergic, anti-mutagenic and antioxidant effects [4–10]. *Platonia insignis* Mart. (Clusiaceae), which belongs to the Clusiaceae family, is a timber and fruit species native to the Brazilian Amazon, commonly known as bacuri. Its fruit has a thick skin, is 7–15 cm long and 5–15 cm in diameter, weighs 200–1000 g and contains a large amount of resin. The pulp enclosing the seeds is white, bittersweet and harbours a pleasant smell and taste [11]. Notably, this species is the only representative of the genus *Platonia* in the Brazilian flora [12]. The *P. insignis* seed oil is used to treat eczemas and herpes, and the seed decoction is used against diarrhoea [4].

Chemical studies on the *Platonia* genus have isolated several biologically active natural products, such as xanthones and phloroglucinol derivatives, which compose the major class of widely occurring secondary metabolites in the Clusiaceae family [13, 14]. These derivatives have been widely investigated for their biological activities, including antioxidant [15, 16], anti-inflammatory [17], cytotoxic and anti-microbial [18], antidepressive [13, 19], anti-HIV [20], anti-tumour [14] and antioxidant effects [21].

Recently, we have shown that an ethanolic extract from *P. insignis* has anticonvulsant and antioxidant effects in rats. The pre-administration of *P. insignis* ethanolic extract reduced lipid peroxidation levels and nitrite content after pilocarpine-induced seizures [22]. Therefore, given the reported pharmacological properties of the Clusiaceae family, we aimed to investigate the antioxidant, leishmanicidal, cytotoxic and genotoxic activities of ethyl acetate and dichloromethane fractions from *P. insignis* Mart. seed ethanolic extract. Moreover, we aimed to provide an increase in the knowledge about *P. insignis* ethanolic extract in other biological models, including pathogenic (*Leishmania amazonensis*) and non-pathogenic organisms (*Artemia salina* and *Saccharomyces cerevisiae*).

Materials and Methods

Chemicals. Yeast extract, yeast nitrogen base, Bacto peptone and Bacto agar were obtained from Difco Laboratories (Detroit, MI, USA). 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS),

Author for correspondence: Jenifer Saffi, Rua Sarmento Leite, 245, sala 29, Anexo II, 90050-170 Porto Alegre, RS, Brazil (fax + 55 51 3303 8810, e-mail jenifers@ufcspa.edu.br).

amphotericin B, dimethylsulphoxide (DMSO), quercetin, rutin, gallic acid, Trolox, methyl methanesulphonate (MMS) and MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), foetal bovine serum (FBS), trypsin–EDTA, L-glutamine, penicillin/streptomycin and trypan blue (TB) were obtained from GIBCO (Grand Island, NY, USA). Low-melting point agarose and agarose were obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade.

Fractions of ethanolic extract from Platonia insignis seeds. *Platonia insignis* fruits were collected at Barras, Piauí State, Brazil, in March 2009. A voucher specimen was identified and deposited at the 'Graziela Barroso' Herbarium of Biology Department of Federal University of Piauí, Brazil (Voucher No.: ICN TEPB27164). The fractions were prepared from *P. insignis* seeds as previously described [23]. Briefly, dried and crushed *P. insignis* seeds were extracted with hexane in a soxhlet extraction apparatus for 8 hr to remove the lipophilic constituents. Next, the seeds underwent a second extraction with absolute ethanol for 8 hr in the soxhlet extraction apparatus. The ethanolic extract was filtered and concentrated to dryness in a rotary evaporator under reduced pressure at 40°C. Then, the crude *P. insignis* ethanol extract was dissolved in distilled water and fractionated with dichloromethane and ethyl acetate. The crude ethanol extract yield and the dichloromethane and ethyl acetate fractions were 5.8, 3.4 and 0.4% w/w, respectively.

Gas chromatography/mass spectrometry (GC/MS) analysis. The ethyl acetate and dichloromethane fractions from *P. insignis* seeds were subjected to a methylation reaction with diazomethane and analysed in a GC-MS (Shimadzu, Tokyo, Japan) [24]. Fraction analysis was performed on a Shimadzu GC-17A/MS QP5050A (GC/MS system) with the following specifications: DB-5HT capillary column (30 m × 0.251 mm, 0.1 μm film thickness), helium at 1.7 mL/min. as a carrier gas, column inlet pressure of 107.8 kPa, column flow of 1.7 mL/min., linear velocity of 47.3 cm/sec., total flow of 24 mL/min., carrier flow of 24 mL/min., injector temperature of 280°C, detector temperature of 300°C and column temperature of 80°C (1 min.)–300°C at 10°C/min. (15 min.). Mass spectrometer operating conditions were 70 eV of ionization energy. Mass spectra were recorded from 43 to 650 m/z. The quantity of all identified components was investigated using a per cent relative peak area. A putative identification of the compounds was performed based on the comparison of their relative retention times and mass spectra with those of the GC/MS system WILEY229 library data. Spectra were considered coincident if the similarity index was higher than 90%.

Total phenolic compound analysis. The total phenolic (TF) amount from the *P. insignis* seed fractions was determined with the Folin–Ciocalteu reagent according to the method of Slinkard and Singleton [25] with minor modifications. Briefly, the stock fraction solution at 1 mg/mL in methanol was prepared. Samples (500 μL) were introduced into test cuvettes, and then 1.0 mL of Folin–Ciocalteu's reagent and 0.8 mL of Na_2CO_3 (7.5%) were added. Sample absorbance was measured at 765 nm using the Shimadzu UV-Vis spectrophotometer after incubating at 30°C for 1.5 hr. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight.

DPPH radical scavenging assay. This assay was performed as described by Tepe *et al.* [26] with the following modifications: 0.5 mL of various dilutions of pure antioxidants or *P. insignis* seed fractions were mixed with 1.5 mL of a 0.004% methanolic solution of DPPH. After 30 min. at 25°C, the absorbances at 517 nm, which is the wavelength of DPPH$^{\bullet}$ maximum absorbance, were recorded as A_{sample} using a Hitachi UV-2000 UV/Vis spectrophotometer. An additional experiment was performed by applying the same procedure to the methanolic solution without the test material, and the absorbance was recorded as A_{blank}. The free radical scavenging activity of each solution was calculated as per cent inhibition according to the following equation:

$$\%\ \text{Inhibition} = 100 \times \frac{(A_{blank} - A_{sample})}{A_{blank}}$$

Antioxidant activities of test compounds or extracts were expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in the initial DPPH$^{\bullet}$ concentration.

$ABTS^{\bullet+}$ radical scavenging assay. This assay was performed according to the procedure described by Re *et al.* [27] with modifications. Briefly, 5.0 mL of 7 mM ABTS was mixed with 88.0 μL of 140 mM potassium persulphate overnight in the dark to yield the $ABTS^{\bullet+}$ radical cation. Prior to use, the $ABTS^{\bullet+}$ was diluted with 50% ethanol for an initial absorbance of 0.7 at 734 nm at 30°C. Pure antioxidants or *P. insignis* fractions were dissolved and diluted with ethanol, and antioxidants or *P. insignis* fractions produced a 20–80% decrease in the absorbance of the blank solution at 734 nm after the introduction of a precise volume of each dilution into the assay. After adding 40 μL of test solution to 1960 μL of $ABTS^{\bullet+}$ solution, $A_{734} = 0.70 \pm 0.01$. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) at 1, 4 and 6 min. TEAC is defined as the mM concentration of a Trolox solution with an antioxidant activity equivalent to the activity of a 1.0 mM test solution. To obtain the TEAC values, a separate concentration response curve for standard Trolox solutions was prepared.

In vivo *antioxidant assays using* Saccharomyces cerevisiae. The *S. cerevisiae* strains used in this work are listed in table 1. The YPD (1% yeast extract, 2% glucose, 2% peptone and 2% agar)-grown stationary phase yeast cells were inoculated at 30°C for 24 hr to a density of 1×10^8 cells/mL. These cells were harvested, washed and re-suspended in sterile saline (0.9% NaCl) to a final concentration of $1–2 \times 10^7$ cells/mL. To determine the protective *P. insignis* seed fraction concentrations, the cultures were exposed to increasing concentrations and incubated under growth conditions for 1 hr in phosphate-buffered saline solution (PBS; Na_2HPO_4, and NaH_2PO_4; 20 mM; pH 7.4) at 30°C. Next, cells were appropriately diluted and plated in triplicate on solid YPD (2–3 days, 30°C) after colony-forming units were counted. To evaluate *in vivo* antioxidant effects, the cultures were concomitantly treated with increasing *P. insignis* seed fraction concentrations (50, 100, 250 and 500 μg/mL) with H_2O_2 (5 mM) and incubated at 30°C for 1 hr. Cells were appropriately diluted and plated on solid YPD. After 3 days, colony-forming units were counted. Sensitivity was expressed as percentage of survival in relation to the negative control (solvent).

In vitro *leishmanicidal activity.* The experiment to test the *in vitro* toxicity against promastigote forms of *L. amazonensis* (IFLA/BR/67/PH-8) was performed in accordance with Oliveira-Silva *et al.* [28]. Log-phase *L. amazonensis* (1×10^6 parasites/mL) were incubated with *P. insignis* seed fractions and solubilized in 0.2% DMSO at 26°C in Schneider's medium (Sigma) supplemented with 10% of FBS. Amphotericin B was used as a control. After 48 hr, parasites were collected, fixed in an isotonic solution (10.5-g citric acid, 7.0-g NaCl, 5.0 mL formalin and 1000 mL distilled water) and examined using light microscopy. The inhibitory effect of the fraction on cellular

Table 1.

Saccharomyces cerevisiae strains used in this study.

Strains	Genotype	Enzymatic defence lacking	Source
EG103 (SOD-WT)	*MATα leu2Δ0 his3-D1 trp1-289 ura3-52*	None	E. Gralla[1]
EG118 (*sod1*Δ)	Like EG103, *except SOD1::URA3*	Cu–Zn superoxide dismutase (cytosolic)	E. Gralla
EG110 (*sod2*Δ)	Like EG103, *except SOD2::TRP1*	Mn superoxide dismutase (mitochondrial)	E. Gralla
EG133 (*sod1*Δ*sod2*Δ)	Like EG103, *except SOD1::URA3 and SOD2::TRP1*	All superoxide dismutase	E. Gralla
EG223 (*ctt1*Δ)	Like EG103, except *ctt1::TRP1*	Cytosolic catalase	E. Gralla
EG213 (*sod1*Δ*ctt1*Δ)	Like EG103, except *sod1::URA3e ctt1::TRP1*	Cu-Zn superoxide dismutase and cytosolic catalase	E. Gralla

[1]The *S. cerevisiae* strains used in this work were kindly provided by Dr. E. Gralla (University of California, Los Angeles, CA, USA).

growth was estimated by cell counting using a Neubauer chamber. The concentration that inhibited 50% of the growth (IC_{50}) was determined by regression analysis.

The brine shrimp assay. The brine shrimp assay is a safe, practical and economic method for determining the bioactivity of natural compounds. The brine shrimp lethality bioassay was performed in accordance with Meyer *et al.* [29]. The growth medium was prepared with filtered seawater in a small tank divided into two compartments. Shrimp eggs were added to the covered compartment, and a lamp was placed above the open side of the tank to attract hatched shrimps through perforations in the partition wall. After 48 hr, the mature nauplii (*A. salina*) were ready for the assay. Stock solutions of ethanolic extract fractions from *P. insignis* seeds were prepared in DMSO (1%) and seawater and filtered. Appropriate volumes of stock solutions were then added to the tubes containing seawater and 10 nauplii each. Four different extract concentrations were applied in triplicate to each tube. After 24 hr of incubation under light, the numbers of dead and surviving brine shrimps were counted in each tube. The LC_{50} values were calculated from graphing drug concentration *versus* lethality percentage using a probit adjust scale.

V79 cell culture and treatment. Chinese hamster lung fibroblast cells (V79 cells) were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated FBS, 0.2 mg/mL L-glutamine, 100 UI/mL penicillin and 100 g/mL streptomycin. Cells were incubated in tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 in air and were harvested by treatment with 0.15% trypsin and 0.08% EDTA in PBS. Cells (5×10^5) were seeded in the medium and grown for 1 day prior to treatment with *P. insignis*. The ethanolic extract fractions from *P. insignis* seeds were dissolved in DMSO and added to the FBS-free medium to yield various concentrations (10, 25, 50 and 100 μg/mL). Cells were treated for 2 hr under standard conditions. The final DMSO concentration in the media never exceeded 0.2%, and the negative control was exposed to an equivalent concentration of solvent. MMS (40 μM) was used as positive control for the comet assay.

MTT assay. The MTT assay was performed according to Denizot and Lang [30]. After treatment, cells were briefly washed with PBS. A serum-free medium (0.15 mL) containing a yellow dye consisting of tetrazolium salt (MTT; 1 mg/mL) was then added, and the mixture was incubated for 3 hr at 37°C. After incubation, the supernatant was removed. The residual purple formazan product was solubilized in 0.2 mL DMSO and stirred for 15 min. The absorbance of the product was measured at 570 nm. The absorbance of the negative control was considered as corresponding to a viability of 100%, and the values of treated cells were calculated as a percentage of the control.

The alkaline comet assay. The alkaline comet assay was performed as described by Hartmann and Speit [31] and Tice *et al.* [32] with minor changes. At the end of the treatment, cells were washed with ice-cold PBS, trypsinized with 100 μL trypsin (0.15%) and re-suspended in complete medium. Next, 20 μL of cell suspension (~10^6 cells/mL) was suspended in 0.75% low-melting point agarose and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose. The slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for a minimum of 1 hr to remove cellular proteins. The slides were then placed in a horizontal electrophoresis box containing a freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH ~13.0) at 4°C for 20 min. to allow DNA unwinding. DNA electrophoresis was performed with a 300 mA and 25 V (0.90 V/cm) electric current for 20 min. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver nitrate as described by Nadin *et al.* [33] and analysed using an optical microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analysed for each test substance concentration. Cells were scored visually into five classes, according to tail size (from undamaged – 0, to maximally damaged – 4), and a damage index (DI) and damage frequency (DF) value was assigned to each comet according to its class. Visual scoring of comets is a valid evaluation method determined by international guidelines and recommendations for the comet assay [31, 32, 34]. The DI is an arbitrary score calculated for cells in different damage classes that were visually scored by measuring the DNA migration length and the amount of DNA in the tail. DF, which is the proportion of cells presenting tails after electrophoresis, was also considered in our study. The DI ranged from 0 (no tail: 100 cells × 0) to 400 (with maximum migration: 100 cells × 4), and the DF (%) was calculated based on the number of cells with tails compared with those with no tail [31].

Statistical analysis. All experiments were independently repeated at least three times. The results were expressed as the mean ± S.D. Data were analysed by one-way analysis of variance (ANOVA), and the means were compared using Dunnett's test, with $p < 0.05$ considered statistically significant.

Results and Discussion

Identification of main compounds extracted from Platonia insignis seeds.

GC/MS analysis of ethanolic extract resulted in the identification of seven compounds in the ethyl acetate fraction and six compounds in the dichloromethane fraction. The relative percentage of compounds of *P. insignis* seed fractions, based on chromatographic area counts, is presented in table 2, where compounds are listed in order of elution from the DB-5HT capillary column. The major compound in the dichloromethane fraction from *P. insignis* seeds is 1,3,5,6-tetrahydroxy-2-(2-methylbut-3-en-2-yl)-7-(3-methylbut-2-enyl)xanthen-9-one (table 2). In the ethyl acetate fraction, the predominant compounds are

alpha-mangostin (40.74%) and 1,3,5,6-tetrahydroxy-2-(2-methylbut-3-en-2-yl)-7-(3-methylbut-2-enyl)xanthen-9-one (40.11%) (table 2).

Gamma-mangostin and alpha-mangostin are xanthones that have been isolated from various parts of the mangosteen tree (*Garcinia mangostana*). These mangosteen molecules, together with a variety of other xanthones, have been investigated for biological properties including antioxidant and anti-inflammatory activities [35]. Moreover, these natural xanthones have been reported to mediate a wide range of biological effects, such as anti-tumour, anti-thrombotic, anti-microbial activities and neuropharmacological properties [36].

TF content.

The TF contents of all fractions were determined according to the Folin–Ciocalteu method and were expressed as GAE, as shown in table 3. The results in this table demonstrate that the ethyl acetate and dichloromethane fractions contained high amounts of TF. However, there is no overt difference in this content between the two fractions.

Table 2.
Main compounds detected in *Platonia insignis* seed fractions.

	Retention time (GC/MS)	Relative area (%)
Dichloromethane fraction		
Trimethyl citrate	10.842	11.32
Hexadecanoic acid methyl ester	16.423	9.35
6-Octadecenoic acid methyl ester	18.143	12.28
11,14-Eicosadienoic acid, methyl ester	18.358	5.86
1-hydroxy-3,5,6-trimethoxy-xanthen-9-one	25.191	13.37
1,3,5,6-tetrahydroxy-2-(2-methylbut-3-en-2-yl)-7-(3-methylbut-2-enyl)xanthen-9-one (gamma-mangostin)	29.321	47.82
Ethyl acetate fraction		
Hexadecanoic acid methyl ester	16.312	5.60
Heptadecanoic acid methyl ester	16.417	4.58
10-Octadecenoic acid methyl Ester	18.091	5.26
9-Octadecenoic acid methyl ester	18.137	3.71
1,3,6-trihydroxy-7-methoxy-2,8-bis (3-methylbut-2-enyl)xanthen-9-one (alpha-mangostin)	28.067	40.74
1,3,5,6-tetrahydroxy-2-(2-methylbut-3-en-2-yl)-7-(3-methylbut-2-enyl)xanthen-9-one (gamma-mangostin)	29.349	40.11

Percentages are the mean of three runs and were obtained from electronic integration measurements using a selective mass detector.
GC/MS, gas chromatography/mass spectrometry.

Table 3.
Total phenolic (TF) contents in *Platonia insignis* seeds.

Samples	TF content (mg of gallic acid equivalent/g of dry weight)
Dichloromethane fraction	115.48 ± 5.79
Ethyl acetate fraction	119.58 ± 1.92

Data are expressed as the mean ± standard error.

The search for antioxidants from natural sources has garnered extensive attention, and efforts have been taken to identify compounds that can act against reactive oxygen species (ROS) to replace synthetic ones [37]. ROS is a well-known attacker of almost all cell components, such as DNA, protein and lipid membrane [38] and has been implicated in many degenerative life cycle events, including ageing, necrosis and apoptotic cell death [39]. The identification of xanthone derivates and phenolic compounds led us to further investigate the *in vitro* and *in vivo* antioxidant potential of *P. insignis* seed fractions.

In vitro *antioxidant capacity.*

Antioxidant capacity was determined by the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays as shown in table 4. With the DPPH$^{\bullet}$ method, the dichloromethane fraction displayed a better effect (IC_{50} = 90.90 μg/mL of DPPH$^{\bullet}$) than the ethyl acetate fraction (IC_{50} = 141.80 μg/mL of DPPH$^{\bullet}$). Evaluation with the ABTS$^{\bullet}$ method revealed that the *P. insignis* seed fraction TEAC ranged from 0.89 to 2.55. Notably, whereas the TF content did not vary greatly (table 3), the radical scavenging activities varied considerably between the fractions (table 4).

In vivo *antioxidant assays.*

To evaluate the antioxidant capacity in *S. cerevisiae* strains, the cytotoxicity of the ethyl acetate and dichloromethane fractions in wild-type and isogenic strains lacking antioxidant defences were tested (data not shown). Based on the results of this preliminary test, non-cytotoxic fraction concentrations (ranging from 50 to 500 μg/mL) were chosen for the subsequent experiments to verify the protective activity against oxidative stress. The results of a growth inhibition assay revealed that both dichloromethane and ethyl acetate fractions effectively protected *S. cerevisiae* strains against H_2O_2 (fig. 1), confirming that *P. insignis* seed fractions harbour ROS quenching ability. However, this antioxidant effect on SOD strains was not dose dependent. The *P. insignis* ethyl acetate fraction was more effective than the dichloromethane fraction against H_2O_2. Whereas the ethyl acetate fraction increased cell survival in the *sod2*Δ, *sod1*Δ*sod2*Δ and *sod1*Δ*ctt1*Δ mutants in concentrations up to 50 μg/mL, the dichloromethane fraction significantly protected only the single mutant *ctt1*Δ and the double mutant *sod1*Δ*ctt1*Δ at a higher tested concentration (fig. 1A,B).

Rufino *et al.* [40] calculated antioxidant capacities of polyphenolic extracts from 18 fresh and dry native, non-traditional fruits from Brazil using ABTS, 2,2-diphenyl-1-picrylhydrazyl radical (DDPH), ferric reducing antioxidant power and ß-carotene bleaching methods between this *P. insignis*, revealing a considerable antioxidant capacity with all methods. In another study, Rufino *et al.* [41] demonstrated that *P. insignis* harbours a high potential as a natural antioxidant source. In support of this finding, ethanolic extract from pre-treated *P. insignis* significantly reduces the lipid peroxidation levels and nitrite content after pilocarpine-induced seizures [22].

Table 4.

Radical scavenging activities of *Platonia insignis* fractions.

Samples	DPPH$^{\bullet}$	ABTS$^{\bullet+}$ (TEAC, mM Trolox)		
	IC_{50} (μg/mL)	1 min.	4 min.	6 min.
Dichloromethane fraction	90.90 ± 1.09	1.95 ± 0.16	2.40 ± 0.07	2.55 ± 0.05
Ethyl acetate fraction	141.80 ± 2.14	0.89 ± 0.04	0.98 ± 0.14	1.1 ± 0.06
Gallic acid	21.98 ± 1.12	8.52 ± 0.62	12.55 ± 0.25	13.40 ± 0.27
Quercetin	36.95 ± 7.01	4.18 ± 0.35	6.22 ± 0.66	6.44 ± 0.30
Rutin	38.57 ± 2.10	1.96 ± 0.04	2.19 ± 0.07	2.29 ± 0.07

Data are expressed as the mean ± standard error.

DPPH, 1,1-diphenyl-2-picryl-hydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; TEAC, Trolox equivalent antioxidant capacity.

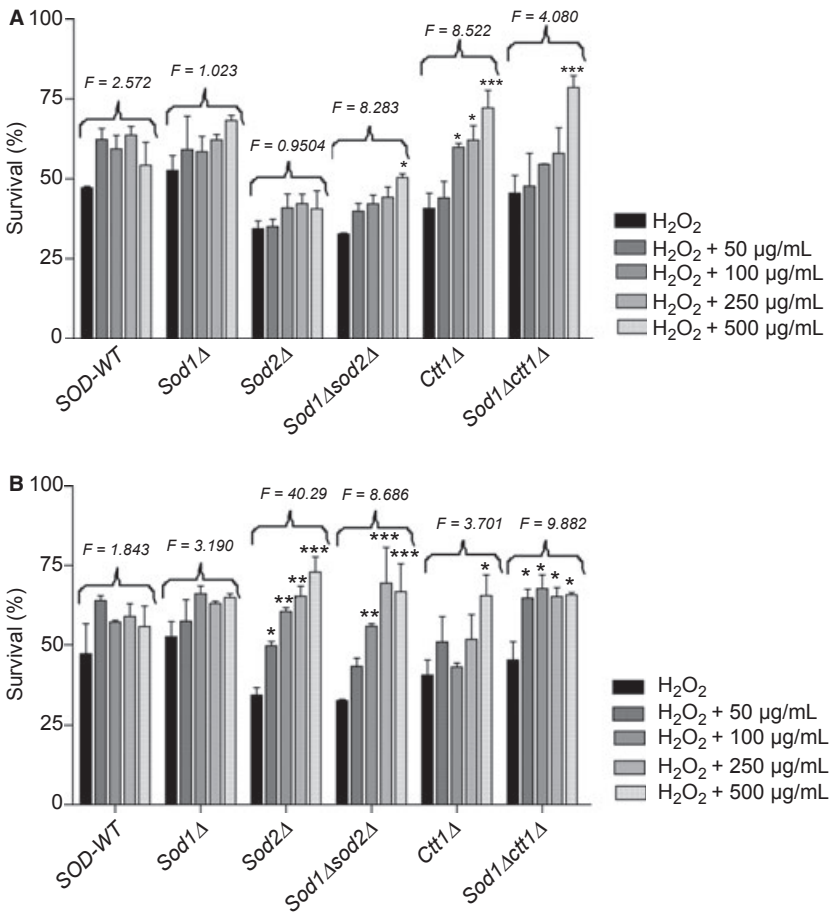

Fig. 1. Effect of co-treatments with *Platonia insignis* fractions and hydrogen peroxide (H_2O_2) on EG103 (WT) and mutant isogenic strain survival in stationary phase after 1 hr of incubation at 30°C. (A) Dichloromethane fraction results. (B) Ethyl acetate fraction results. Percentage survival is expressed relative to the untreated control culture (100%). Values shown are the mean of at least three determinations. Data are significant in relation to oxidant-treated samples to each strain at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$/one-way ANOVA and Dunnett's multiple comparison test. Nine degrees of freedom and six independent analyses were used for the dichloromethane fraction, and 14 degrees of freedom and six independent analyses were used for the ethyl acetate fraction.

As previously mentioned, experimental studies have demonstrated that *G. mangostana* extracts display a wide spectrum of biological activities, including antioxidant effects [42]. Xanthones have been isolated from pericarp, whole fruit, heartwood and leaves [43]. Our results suggest that the presence of gamma- and alpha-mangostin in ethyl acetate fractions, representing 80.84% of the major components detected in *P. insignis* seed fractions, along with the 47.82% of gamma-mangostin present in dichloromethane fractions, may contribute to the free radical scavenging assays (DPPH and ABTS) and protective potential against H_2O_2-induced toxicity in *S. cerevisiae* strains.

Table 5.
Toxicity against *Artemia salina* and promastigote anti-leishmania activity of *Platonia insignis* seed fractions.

	IC_{50} (μg/mL)[1,2] *Leishmania amazonensis*	IC_{50} (μg/mL)[1] *A. salina*
Fractions	48 h	24 h
Dichloromethane fraction	2.84	24.89
Ethyl acetate fraction	26.20	129.0

[1]The IC_{50} values obtained from a minimum of three separate experiments performed in triplicate are shown (95% confidence limits).
[2]Positive control: amphotericin B (IC_{50} 0.04 μg/mL).

In vitro *leishmanicidal activity.*
The *in vitro* toxicity assays were performed to determine the inhibitory concentration at 50% (IC_{50}) of the *P. insignis* fractions using the promastigote form of *L. amazonensis*. After a 48-hr incubation, the evaluated fractions exhibited an IC_{50} of 2.84 μg/mL for the dichloromethane fraction and 26.20 μg/mL for the ethyl acetate fraction. The dichloromethane fractions were more efficient against the promastigote forms of *L. amazonensis*. The IC_{50} value for amphotericin B was 0.04 μg/mL (table 5).

Toxicity against Artemia salina *Leach.*
To assess the toxicity towards *A. salina*, IC_{50} values for the fractions were determined (table 5). The control samples with solvents (seawater and DMSO) did not yield significant brine shrimp mortality. Both fractions were cytotoxic against brine shrimps. The dichloromethane fraction displayed a reasonably high toxicity in this assay (IC_{50} = 24.89 μg/mL), whereas the ethyl acetate fraction displayed a lower cytotoxicity (IC_{50} = 129.0 μg/mL). In accordance with McLaughlin [44], compounds with IC_{50} < 100 μg/mL in the brine shrimp lethality assay are considered active and potentially cytotoxic against tumour cell lines. Consistently, our results suggest that the dichloromethane fraction could be further investigated for anti-tumour activity.

Leishmaniasis is a public health problem in developing countries, where 350 million people are at risk of infection. Systematic studies in different parts of the world searching for anti-protozoal activity of medicinal plants have been reported [45]. In the present study, fractions from *P. insignis* seeds displayed inhibitory effects against the promastigote form (table 5). Although the leishmanicidal effect did not result in an IC_{50} as low as that of amphotericin B, the results obtained in this study, particularly for the dichloromethane fraction, encourage the development of new compounds with potential anti-leishmania properties. Nevertheless, further studies are required to characterize the active component of *P. insignis* against leishmania using purified compounds.

V79 cell cytotoxicity measured by an MTT assay.
The MTT assay was used to evaluate the cytotoxicity of the dichloromethane and ethyl acetate fractions in V79 cells. The results revealed that both fractions are slightly cytotoxic at concentrations up to 100 μg/mL. However, this increase in sensitivity is not significant when compared with the negative control (fig. 2).

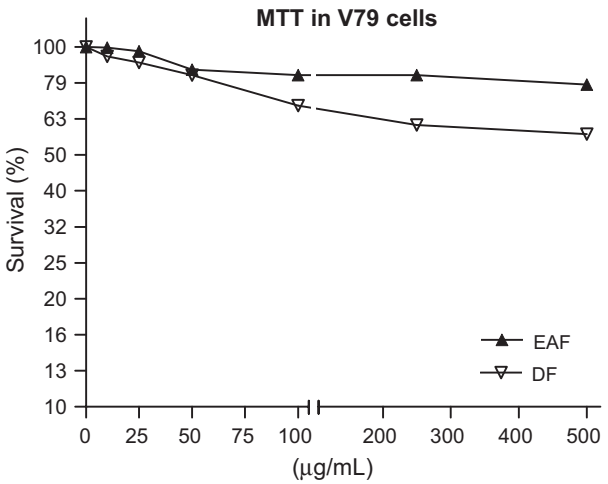

Fig. 2. Survival of V79 cells after exposure to *Platonia insignis* seed dichloromethane (DF) and ethyl acetate fractions (EAFs) after 2 hr measured by the MTT assay. The results are expressed as a mean percentage in treated cells compared with control (solvent) ± standard deviation of three independent experiments performed in triplicate. DF, damage frequency.

Table 6.
Induction of DNA strand breaks from *Platonia insignis* seed fractions in V79 cells as evaluated by the comet assay after 2 hr of treatment at 37°C.

Agent	Treatment (μg/mL)	DI	DF (%)
Alkaline conditions (pH ~ 13.0)			
NC[1]		14.33 ± 4.41	14.00 ± 4.64[2]
MMS[3]	40	120.30 ± 13.69***	76.00 ± 6.75***
Dichloromethane fraction	10	42.50 ± 12.60***	28.50 ± 2.73
	25	50.00 ± 12.05***	27.50 ± 1.64
	50	69.00 ± 10.95***	37.00 ± 4.38***
	100	143.0 ± 5.48***	48.50 ± 8.22***
Ethyl acetate fraction	10	28.00 ± 6.57***	12.00 ± 1.09
	25	57.00 ± 8.49***	22.00 ± 2.82*
	50	61.00 ± 0.0***	31.00 ± 2.19***
	100	103.0 ± 7.66***	40.00 ± 3.28***

DI: Dichloromethane fraction (*F = 104.3*) and ethyl acetate fraction (*F = 121.5*). DF: Dichloromethane fraction (*F = 11.88*) and ethyl acetate fraction (*F = 59.71*). The degree of freedom used for six independent analyses was 19.
DI, damage index; DF, damage frequency; MMS, methyl methanesulphonate.
[1]Negative control (solvent).
[2]Mean value and standard deviation obtained from an average of 100 cells per experiment from three experiments per concentration for each fraction.
[3]Positive control.
*Significant difference compared to the negative control treatment (dimethylsulphoxide 0.2%) at $p < 0.05$; ***$p < 0.001$/one-way ANOVA and Dunnett's multiple comparison test.

V79 cell genotoxicity by a comet assay.

The induction of genotoxicity by *P. insignis* in V79 cells using an alkaline version of the comet assay is shown in table 6. The dichloromethane and ethyl acetate fractions clearly drove significant increases in DI and DF values compared with the values obtained for the control groups at concentrations of up to 25 and 10 μg/mL, respectively. In addition, the increase in damage score occurred in a dose-related manner.

The use of plants in therapies is a worldwide phenomenon. Currently, drugs derived from plants are being investigated for the possible presence of cytotoxic, mutagenic and genotoxic substances, as well as other biological activities. The detection and evaluation of the cytotoxic, mutagenic and carcinogenic effects of plant compounds is crucial for minimizing the possible risks of these agents, especially when they are part of a long-term treatment. Many plants occasionally consumed by human beings can have toxic effects [46].

Platonia insignis seed fractions do not induce significant cytotoxicity in V79 cells (fig. 2). However, a genotoxic effect was observed. The alkaline comet assay, a validated method for detecting DNA damage in cells [47], revealed that the ethyl acetate and dichloromethane fractions induced an increase in DNA damage in a dose-dependent manner (table 6). Matsumoto *et al.* [48] examined the effect of 6 xanthones (α, β and γ-mangostins, mangostinone, garcinone E and 2-isoprenyl-1,7-dihydroxy-3-methoxy xanthone) on cell growth inhibition of the human leukaemia cell line HL60. Although all xanthones showed a significant inhibition effect, α, β and γ-mangostins were more effective. As previously mentioned, the most abundant compound identified in dichloromethane and ethyl acetate fractions from *P. insignis* seeds in this study was α-mangostin, which can be attributed to the genotoxicity in V79 cells as well as the toxicity against *A. salina* and *L. amazonensis*. In addition, the increase in DNA damage induced by ethyl acetate and dichloromethane fractions can justify the anti-parasite activity displayed by these fractions.

Conclusions

Our results indicate that the ethyl acetate and dichloromethane fractions from the *P. insignis* seed ethanolic extract present antioxidant activities *in vitro*, measured by ABTS and DPPH assays, and *in vivo*, measured by protective effects against the cytotoxicity induced by H_2O_2 in *S. cerevisiae* strains. The antioxidant effect may be attributed to the polyphenols present in these fractions, as well as to the presence of alpha- and gamma-mangostin. Our results also show that the *P. insignis* seed fraction displayed a leishmanicidal effect and genotoxicity in V79 cells, and paradoxically, alpha- and gamma-mangostin are the putative compounds responsible for these actions. Further studies designed to isolate, identify and characterize the constituents of these fractions may provide a greater understanding of the mechanisms governing their antioxidant, leishmanicidal and genotoxic effects.

Acknowledgements

The authors are grateful to the Brazilian agencies Fundação de Amparo a Pesquisa do Estado do Piauí (FAPEPI), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Instituto Federal do Piauí (IFPI), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Instituto Nacional de Pesquisa Translacional em Saúde e Ambiente na Amazônia (INPeTAm/CNPq/MCT), Brazil.

Conflict of Interest

The authors declare no conflict of interest.

References

1 Halberstein RA. Medicinal plants: historical and cross-cultural usage patterns. Ann Epidemiol 2005;**15**:686–99.
2 Krishnaiah D, Sarbatly R, Nithyanandam R. A review of the antioxidant potential of medicinal plant species. Food Bioprod Process 2010;**89**:217–33.
3 Alves TMA, Silva AF, Brandão M, Grandi TSM, Smânia EFA, Smânia Júnior A *et al.* Biological screening of Brazilian medicinal plants. Mem Inst Oswaldo Cruz 2000;**95**:367–73.
4 Agra MF, Freitas PF, Barbosa-Filho JM. Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. Rev Bras Farmacogn 2007;**17**:114–40.
5 Bilanda DC, Dimo T, Dzeufiet Djomeni PD, Bella NMT, Aboubakar OBF, Nguelefack TB *et al.* Antihypertensive and antioxidant effects of *Allanblackia floribunda* Oliv. (Clusiaceae) aqueous extract in alcohol-and sucrose-induced hypertensive rats. J Ethnopharmacol 2010;**128**:634–40.
6 Gurib-Fakim A. Medicinal plants: traditions of yesterday and drugs of tomorrow. Mol Aspects Med 2006;**27**:1–93.
7 Hosni K, Msaâda K, Taarit MB, Hammami M, Marzouk B. Bioactive components of three *Hypericum* species from Tunisia: a comparative study. Ind Crops Prod 2010;**31**:158–63.
8 Lizcano LJ, Bakkali F, Begona RL. Antioxidant activity and polyphenol content of aqueous extracts from Colombian Amazonian plants with medicinal use. Food Chem 2010;**119**:1566–70.
9 Ngoupayo J, Tabopda TK, Ali MS. Antimicrobial and immunomodulatory properties of prenylated xanthones from twigs of *Garcinia staudtii*. Bioorg Med Chem 2009;**17**:5688–95.
10 Tchamadeu MC, Dzeufiet PDD, Nouga CC, Azebaze AGB, Allard J, Girolami JP *et al.* Hypoglycaemic effects of *Mammea africana* (Guttiferae) in diabetic rats. J Ethnopharmacol 2010; **127**:368–72.
11 Alves S, Jennings WG. Volatile composition of certain Amazonian fruits. Food Chem 1979;**4**:149–59.
12 Engler HGA. In: Martius CFR (ed). Flora Brasiliensis. 1888; **XII**, pars. I (fasc. CII):468–9.
13 Ciochina R, Grossman RB. Polycyclic polyprenylated acylphloroglucinols. Chem Rev 2006;**106**:3963–86.
14 Zhang LJ, Chiou CT, Cheng JJ, Huang HC, Kuo LM, Liao CC *et al.* Cytotoxic polyisoprenyl benzophenonoids from *Garcinia subelliptica*. J Nat Prod 2010;**73**:557–62.
15 Baggett S, Protiva P, Mazzola EP, Yang H, Ressler ET, Basile MJ *et al.* Bioactive benzophenones from *Garcinia xanthochymus* fruits. J Nat Prod 2005;**68**:354–60.
16 Cuesta-Rubio O, Frontana-Uribe BA, Ramirez-Apan T, Cardenas J. Polyisoprenylated benzophenones in Cuban propolis; biological activity of nemorosone. Z Naturforsc C 2002;**57**:372–8.

17 Weng JR, Tsao LT, Wang JP, Wu RR, Lin CN. Anti-inflammatory phloroglucinols and terpenoids from *Garcinia subelliptica*. J Nat Prod 2004;**67**:1796–9.
18 Xu G, Kan WLT, Zhou Y, Song JZ, Han QB, Qiao CF *et al.* Cytotoxic acylphloroglucinol derivatives from the twigs of *Garcinia cowa*. J Nat Prod 2010;**73**:104–8.
19 Verotta L. Are acylphloroglucinols lead structures for the treatment of degenerative diseases? Phytochem Rev 2002;**1**:389–407.
20 Piccinelli AL, Cuesta-Rubio O, Chica MB, Mahmood N, Pagano B, Pavone M *et al.* Structural revision of clusianone and 7-epi-clusianone and anti-HIV activity of polyisoprenylated benzophenones. Tetrahedron 2005;**61**:8206–11.
21 Lin KW, Huang AM, Tu HY, Weng JR, Hour TC, Wei BL *et al.* Phloroglucinols inhibit chemical mediators and xanthine oxidase, and protect cisplatin-induced cell death by reducing reactive oxygen species in normal human urothelial and bladder cancer cells. J Agric Food Chem 2009;**57**:8782–7.
22 Costa Júnior JS, Freitas RM, Citó AMGL, Henriques JAP, Saffi J. Evaluation of effects of ethanolic extract (EE) from *Platonia insignis* Mart. on pilocarpine-induced seizures. J Biol Sci 2010;**10**:747–53.
23 Bentes MHS, Serruya H, Rocha Filho GN, Godoy RLO, Cabral JAS, Maia JGS. Estudo químico das sementes de bacuri. Acta Amaz 1986;**16**:363–8.
24 De Lima SG, Neto JMM, Cito AMGL, Da Costa JGM, Am Reis F. Monoterpenes, sesquiterpenes and fatty acids from *Julocroton triqueter* (Euphorbiaceae) from Ceara-Brazil. J Chil Chem Soc 2009;**54**:55–7.
25 Slinkard K, Singleton VL. Total phenol analysis: automation and comparison with manual methods. Am J Enol Vitic 1977;**28**:49.
26 Tepe B, Sokmen M, Sokmen A, Daferera D, Polissiou M. Antimicrobial and antioxidative activity of the essential oil and various extracts of *Cyclotrichium origanifolium* (Labill.) Manden. & Scheng. J Food Eng 2005;**69**:335–42.
27 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;**26**:1231–7.
28 Oliveira-Silva F, Morais-Teixeira E, Rabello A. Antileishmanial activity of azithromycin against *Leishmania* (*Leishmania*) *amazonensis*, *Leishmania* (*Viannia*) *braziliensis*, and *Leishmania* (*Leishmania*) *chagasi*. Am J Trop Med Hyg 2008;**78**:745.
29 Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med 1982;**45**:31–4.
30 Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;**89**:271–7.
31 Hartmann A, Speit G. The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). Toxicol Lett 1997;**90**:183–8.
32 Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H *et al.* Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;**35**:206–21.
33 Nadin SB, Vargas–Roig LM, Ciocca DR. A silver staining method for single-cell gel assay. J Histochem Cytochem 2001;**49**:1183.
34 Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR *et al.* Fourth International Workgroup on Genotoxicity testing: results of the *in vivo* Comet assay workgroup. Mutat Res 2007;**627**:31–5.
35 Jung HA, Su BN, Keller WJ, Mehta RG, Kinghorn AD. Antioxidant xanthones from the pericarp of *Garcinia mangostana* (Mangosteen). J Agric Food Chem 2006;**54**:2077–82.
36 Ha WY, Wu PK, Kok TW, Leung KW, Mak NK, Yue PYK *et al.* Involvement of protein kinase C and E2F-5 in euxanthone-induced neurite differentiation of neuroblastoma. Int J Biochem Cell Biol 2006;**38**:1393–401.
37 Singh G, Maurya S, Delampasona MP, Catalan CAN. A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. Food Chem Toxicol 2007;**45**:1650–61.
38 Izawa S, Inoue Y, Kimura A. Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. FEBS Lett 1995;**368**:73–6.
39 Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. Free Radical Biol Med 1999;**26**: 463–71.
40 Rufino MSM, Alves RE, Brito ES, Pérez-Jiménez J, Saura-Calixto F, Mancini-Filho J. Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. Food Chem 2010;**121**:996–1002.
41 Rufino MSM, Alves RE, Fernandes FAN, Brito ES. Free radical scavenging behavior of ten exotic tropical fruits extracts. Food Res Int 2011;**44**:2072–5.
42 Obolskiy D, Pischel I, Siriwatanametanon N, Heinrich M. *Garcinia mangostana* L.: a phytochemical and pharmacological review. Phytother Res 2009;**23**:1047–65.
43 Pedraza-Chaverri J, Cárdenas-Rodríguez N, Orozco-Ibarra M, Pérez-Rojas JM. Medicinal properties of mangosteen (*Garcinia mangostana*). Food Chem Toxicol 2008;**46**:3227–39.
44 McLaughlin JL. Crown gall tumours on potato discs and brine shrimp lethality: two simple bioassays for higher plant screening and fractionation. Meth Plant Biochem 1991;**6**:1–32.
45 Braga FG, Bouzada MLM, Fabri RL, de OM. Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil. J Ethnopharmacol 2007;**111**:396–402.
46 Varanda EA, Pozetti GL, Lourenco MV, Vilegas W, Raddi MSG. Genotoxicity of *Brosimum gaudichaudii* measured by the Salmonella/microsome assay and chromosomal aberrations in CHO cells. J Ethnopharmacol 2002;**81**:257–64.
47 Moller P, Möller L, Godschalk RWL, Jones GDD. Assessment and reduction of comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group. Mutagenesis 2010;**25**:109.
48 Matsumoto K, Akao Y, Kobayashi E, Ohguchi K, Ito T, Iinuma M *et al.* Induction of apoptosis by xanthones from mangosteen in human leukemia cell lines. J Nat Prod 2003;**66**:1124–7.