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Journal of Medicinal Plants Research

Full Length Research Paper

Influence of extraction method on antibacterial activity of ethanolic extracts of *Ocimum gratissimum* L.

Lenise de Lima Silva[1], Clarissa Giesel Heldwein[1], Luiz Gustavo Brenner Reetz[2], Rosmari Hörner[2], Diogo Pompéu de Moraes[3], Fábio Andrei Duarte[3], Érico Marlon de Moraes Flores[3], Renato Zanella[3], Ana Maria Soares Pereira[4] and Berta Maria Heinzmann[1,5]*

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The aim of this study was to define whether novel extraction methods such as microwave and ultrasound could obtain the most effective ethanolic extracts of *Ocimum gratissimum* as antibacterial agents. These extracts were compared with respect to extractive yield, eugenol content, antibacterial activity and brine-shrimp (*Artemia salina*) toxicity with extracts obtained by the classical procedures of maceration and Soxhlet. Significant differences among the extracts were observed in all analyses. Soxhlet extraction gave the highest yield (19.5%). Maceration and microwave extracts yielded the highest eugenol contents (11.6 and 11.8%, respectively). The bactericidal activity of the extracts was correlated with eugenol content (r_s = 0.894). Maceration gave the extract with the broadest spectrum of activity. Ultrasound methods yielded an efficient extract for use as a topical antiseptic (minimum inhibitory concentration (MIC) = 0.66 to 1.32 mg/ml for *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA)). The most active extracts to treat vancomycin-resistant enterococci (VRE) infections were obtained by Soxhlet and microwave (MIC = 5.28 mg/ml). The extract obtained by maceration was the most toxic for brine shrimp, followed by the extracts obtained by ultrasonic horn, ultrasonic cleaning bath and Soxhlet /microwave. In conclusion, the antibacterial results showed that the extractive methodology can be chosen according to the intended use.

Key words: *Ocimum gratissimum*, microwave-assisted extraction (MAE), ultrasonic cleaning bath (UCB), ultrasonic horn (UH), antibacterial activity, toxicity, *Artemia salina*.

INTRODUCTION

With modern extraction methods such as ultrasound and microwave-assisted extraction, plant extracts can be obtained with higher yields and efficacy, less environmental impact and lower costs (Wang and Weller, 2006; Chen et al., 2007). Conventional extraction methods such as maceration and Soxhlet are based on

the choice of solvent coupled with the use of heat and/or agitation, whereas ultrasound and microwave methodologies disrupt cell walls, improving mass transfer (Wang and Weller, 2006).

Ocimum gratissimum L. (Lamiaceae), known as tree basil, is widely used medicinally as a topical antiseptic and for the treatment of conjunctivitis, bronchitis and diarrhea, as well as a food flavoring (Onajobi, 1986; Silva et al., 2006). Antimicrobial activity was confirmed for the essential oil (Nakamura et al., 1999), ethanolic (Nweze and Eze, 2009; Passos et al., 2009), methanolic (Braga et al., 2007) and aqueous extracts (Junaid et al., 2006; Passos et al., 2009). This activity has also been correlated with a high content of eugenol (Nakamura et al., 1999).

To date, studies with *O. gratissimum* were performed only with extracts obtained by conventional methods (Junaid et al., 2006; Nweze and Eze, 2009; Passos et al., 2009). In some cases, extracts obtained with different plant materials (fresh or dried) or different solvents (water, hexane, methanol, ethanol or hydroalcoholic mixture) were compared (Junaid et al., 2006; Passos et al., 2009). The aim of this study was to define whether novel extraction methods such as microwave and ultrasound could provide extracts of *O. gratissimum* that are more effective as antibacterial agents. Ethanolic extracts obtained by these methods were compared to those obtained with the classical procedures of maceration and Soxhlet , with respect to extractive yield, eugenol content, antibacterial activity, and toxicity to *Artemia salina*.

MATERIALS AND METHODS

Plant

Aerial parts of *O. gratissimum* L. used to obtain the ethanolic extracts were grown in Jardinópolis, São Paulo, Brazil. The plant material, previously identified by Dr Lin Chan Ming, was collected in March, 2007, dried in a ventilated oven at 45°C for three days and stored in closed dark packages until the extraction processes. To obtain eugenol from hydrolate, aerial parts identified by Dr Adelino Alvarez Filho were collected in December, 2006 and March, 2007 on the central Campus of the Universidade Federal de Santa Maria (UFSM), Santa Maria, Rio Grande do Sul, Brazil. Voucher specimens were deposited in the Biotechnology Department of the Universidade de Ribeirão Preto (no. 1329) and in the Herbarium of the Botany Department, UFSM (no. SMDB 11167).

Extraction and isolation of eugenol from hydrolate

Fresh aerial parts of *O. gratissimum* were steam-distilled for 3 h in a Clevenger-type apparatus (European Pharmacopoeia, 2007). After extraction, the hydrolate was collected and submitted to liquid-liquid and the residue was stored at -4°C in amber glass bottles until purification. The residue (210.7 mg) was submitted to a 15 g silica-gel chromatography column (CC, 1 × 29.5 cm) and eluted with dichloromethane at 1 ml/min, resulting in 14 fractions of 20 ml. Fractions were pooled according to their thin-layer chromatography (TLC) profiles and concentrated under reduced pressure at 40°C. Fractions 4 to 10 (103.8 mg) was analyzed by gas chromatography with mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR). GC-MS analysis was performed on an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector, using an HP5-MS column (5% phenyl - 95% methylsiloxane, 30 m × 0.25 mm i.d. × 0.25 µm) and electron ionization-mass spectrometry (EI-MS) as detector (70 eV) according to Silva et al. (2010). NMR spectra were recorded on a Bruker DPX 400 FT-NMR at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$ with tetramethylsilane (TMS) as internal standard.

Ethanolic extractions of aerial parts

Dried aerial parts of *O. gratissimum* were extracted by maceration (MAC), Soxhlet (SOX), ultrasonic cleaning bath (UCB), ultrasonic horn (UH) and microwave-assisted extraction (MAE) with 95% ethanol in triplicate. The plant material was ground at 600 µm before extraction by the Soxhlet, ultrasonic and microwave-assisted processes. The extraction time, temperature and solvent volume/ plant material weight ratio (ml/g) of the different methods are presented in Table 1. Sonochemical procedures were carried out in a UCB (model Ultrasonic Cleaner 1440D - Series Evolution, 40 kHz and 100 W) and UH (model VC 750, Sonics, 20 kHz working frequency, 750 W). The horn was operated on a 40% cycle at 2.5 cm from the surface of the extraction solution. MAE was performed in a Multiwave 3000 microwave using a quartz closed-vessel system. The microwave energy program was up to 100°C, with ramp of 5 min until 300 W followed by 5 min of radiation. To finish, the vessels containing the extract were cooled for 30 min before opening. All extracts obtained were filtered, stored in closed amber bottles and concentrated under reduced pressure at 40°C. Afterwards, the extracts were placed in a desiccator and kept at room temperature until constant weight. Yields are expressed as a percentage of extract obtained from plant material (w/w).

Purification and quantification of eugenol in ethanolic extracts

The eugenol purification process from the ethanolic extracts of *O. gratissimum* was developed to reduce interference from other compounds with the quantitative analyses. Procedures were initially performed in a pilot CC and repeated for quantitative determination. Extracts (about 400 mg) were added to a CC (1 × 29.5 cm) containing 15 g of silica-gel 60 (Merck, 70-230 mesh) and eluted with chloroform-toluene 85:15 (v/v) at 2 ml/min. Fractions of 20 ml were obtained and monitored in pilot columns by TLC (silica gel F254, chloroform-toluene 85:15 v/v, detection: anisaldehyde-H_2SO_4) to detect the presence of eugenol. In the quantitative procedure, fractions that contained eugenol according to the pilot-column results (MAC: fractions 6-13; SOX: fractions 5-15; UH: fractions 5-14; UCB: fractions 5-12; MAE: fractions 3-13) were concentrated under reduced pressure at 40°C. The resulting residue was diluted to 50 ml in a volumetric flask using the same eluent and the eugenol content was determined by gas chromatography with flame ionization detection (GC-FID). To determine the reproducibility of the CC procedure, fractions without partition with hexane. The organic phase was evaporated at 40°C eugenol were compared

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by TLC with the corresponding fractions of the pilot column. The eugenol was quantified by analyzing the area below the curve (N = 2). Eugenol previously obtained from the hydrolate of *O. gratissimum* (diluted in dichloromethane at 1.038 mg/ml) was used as external standard. GC-FID analysis was performed on a Varian gas chromatograph Model 3800 coupled to a flame ionization detector, using the Star Workstation 6.6 system for data acquisition and a CPSil 5CB column (100% methyl silicone, 30 m × 0.25 mm i. d. × 0.10 µm film thickness). Operating conditions: injection volume 1 µL; injector temperature 220°C; oven temperature program 40 to 260°C; 40°C for 4 min; ramp rate 4°C/min; detector temperature 310°C. The eugenol content was expressed as the percentage of eugenol in relation to extract (w/w).

Antibacterial activity

The antibacterial activity of the extracts was assayed by the broth microdilution method as established by M7-A6 (Clinical and Laboratory Standards Institute (NCCLS), 2003) for bacteria. The microorganisms tested included ATCC and clinical isolates from the University Hospital of Santa Maria (Table 2). Extracts were solubilized in 95% ethanol and serial dilutions were performed in culture medium to obtain concentrations of 10.550 to 0.020 mg/ml for *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 51299 and of 2.637 to 0.005 mg/ml for the other strains. Each extract was assayed three times, in triplicate. Ampicillin was used as the reference antimicrobial control according to the antimicrobial resistance profile. Negative (100 µl of Muller-Hinton broth), positive (100 µl of Muller-Hinton broth and 100 µl of inoculum) and product controls (180 µl of Muller-Hinton broth and 20 µl of stock solution) were also performed for each test. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the sample that prevented visible growth. Minimum bactericidal concentrations (MBCs) were defined as the lowest concentration yielding negative subcultures.

Brine-shrimp lethality test (BSL)

The BSL was carried out by using nauplii of brine shrimp *Artemia salina* (Leach), according to the methodology described by Silva et al. (2010). Extracts of *O. gratisssimum* (60 mg) were dissolved in 95% ethanol (1 ml) and diluted in artificial sea water to obtain final concentrations of 1000, 600, 300, and 150 µg/ml. Plates were maintained at 22 to 29°C for 24 h and the number of survivors was counted. Negative controls were tested with 95% ethanol diluted 1:100 in artificial sea water. All assays were performed in two repetitions (N = 3 per repetition).

Statistical analysis

Data are presented as the mean ± standard error of mean (SEM). To verify the homogeneity of variances, all data were submitted to a Levene test. Arcsine transformation was used for data on extractive yield and eugenol composition before statistical analysis. Results were analyzed by one-way analysis of variance (ANOVA) and Tukey test or Kruskal-Wallis and Mann-Whitney tests, when appropriate. Correlations between results were evaluated by Spearman's rank (r_s). A difference of $P < 0.05$ was considered to be statistically significant.

RESULTS

The structure of eugenol isolated from hydrolate of *O. gratissimum* was determined by comparison of its mass spectrum, retention index and NMR spectra with data reported in the literature (Adams, 2001; Carrasco et al., 2008; SDBS, 2008). The ethanolic extracts of *O. gratissimum* differed significantly in extractive yield and eugenol content (Table 1). The largest yield was obtained by Soxhlet (19.5 %), which also required a longer extraction time compared with the ultrasound and microwave-assisted methods. The highest eugenol content was obtained by microwave-assisted extraction and maceration, followed by the soxhlet and ultrasonic methods. Although there were no statistical differences in extractive yields between ultrasound methods, UCB extracted more eugenol content than UH.

All extracts tested showed bacteriostatic activity (Table 2), except against *Salmonella* sp. Most of the extracts obtained by the two ultrasound methods had similar MIC values against Gram-positive bacteria to each other and also compared with the classical maceration procedure. The exception was the extract obtained by UCB against *S. aureus* ATCC 25923, which showed the lowest MIC of all extracts. For Gram-negative bacteria, the UH extract was the most effective against extended-spectrum β-lactamase-producing *E. coli* (ESBL) and *S. choleraesuis* ATCC 10708. The extract obtained by microwave extraction was statistically similar to one or both extracts obtained by conventional and ultrasonic processes for *B. cereus* ATCC 14579, *S. aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA), ESBL, *S. flexneri* and *P. aeruginosa* ATCC 27853. Only the conventional techniques generated active extracts against the diarrheagenic pathogens tested, such as *Shigella* sp. and enteropathogenic *Escherichia coli* (EPEC). The extract obtained by maceration gave the best results against MRSA. The extract obtained by maceration showed a lower MIC against Gram-positive bacteria in comparison to the Soxhlet extract. The only exception was vancomycin-resistant *E. faecalis* ATCC 51299 (VRE), which showed more susceptibility to the extracts obtained by Soxhlet and microwave.

The extracts showed bactericidal activity assayed occurred against only some of the Gram-positive bacteria, except for the extract obtained by Soxhlet against *S. flexneri*. The eugenol content in the extracts was positively correlated (r_s = 0.894) with the number of bacterial strains with the lowest MBC for each extract (Figure 1A). On the other hand, the eugenol content and the bacteriostatic activity of each extract (through the number of bacterial strains with the lowest MIC) were not significantly correlated by Spearman's test ($P > 0.05$).

The extract obtained by maceration (LC_{50} = 331.3 µg/ml) was the most toxic for brine shrimp, followed by UH, UCB and finally Soxhlet /MAE, in decreasing order of toxicity (Table 1). Negative correlation (r_s = -0.949) between brine-shrimp toxicity and the number of bacterial strains with the lowest MIC for extracts obtained by the different processes was observed (Figure 1B).

Table 1. Experimental conditions, extract yield, eugenol composition and toxicity by brine shrimp lethality test (BSL) of ethanolic extracts of *Ocimum gratissimum* L.

Experimental condition	Extraction Methods				
	MAC	SOX	UH	UCB	MAE
Time	3×7 days	30 h	1 h	1 h	5 min
Temperature (ºC)	Ambient	78	13-44	40	41-81
Solvent volume/plant material weight ratio (ml/g)	43.8 (3×14.6)	8.6	10	10	10
Extract yield (%)	11.8±0.2[b]	19.5±0.9[a]	10.2±0.6[bc]	7.7±0.9[c]	12.1±0.9[b]
Eugenol content (%)	11.6±0.09[a]	10.0±0.05[b]	4.6±0.04[d]	7.8±0.04[c]	11.8±0.17[a]
BSL					
LC_{50} (µg/mL)	331.3	793.4	456.9	586.5	999.4
95% Confidence interval	302.1-363.1	690.9-951.8	413.9-511.4	523.9-655.4	796.3-1429.9

MAC: Maceration; SOX: Soxhlet ; UH: ultrasonic horn; UCB: ultrasonic cleaning bath; MAE: microwave-assisted extraction. Data are presented as the mean ± SEM. Different letters in the rows indicate significant differences between ethanolic extracts of *O. gratissimum* using one-way ANOVA followed by Tukey test ($P< 0.05$).

Table 2. MIC and MBC (mg/ml) of ethanolic extracts of *Ocimum gratissimum* L. obtained by different extraction methods.

Bacteria	Conc.	Extraction methods					Ampicillin
		MAC	SOX	UH	UCB	MAE	
Gram-positive							
Bacillus cereus ATCC 14579	MIC	1.32[b]	2.63[a]	1.32[bc]	1.32[c]	1.32[abc]	0.00078
	MBC	2.64[a]	>2.63	2.64[a]	1.32[b]	2.64[a]	0.03125
Bacillus cereus	MIC	0.66[a]	2.63[b]	0.66[ac]	0.66[c]	2.64[d]	0.0156
	MBC	>2.64	>2.63	>2.64	>2.64	2.64	0.5
Enterococcus faecalis ATCC 51299	MIC	10.56[a]	5.28[b]	10.58[a]	10.57[a]	5.28[b]	0.00312
	MBC	>10.56	10.57[a]	>10.58	10.57[a]	10.56[a]	>0.05
Staphylococcus aureus ATCC 25923	MIC	1.32[a]	1.32[a]	1.32[a]	0.66[b]	1.32[a]	0.00039
	MBC	2.64[a]	2.63[a]	>2.64	>2.64	>2.64	>0.05
MRSA[1]	MIC	0.66[c]	2.63[a]	1.32[abc]	1.32[b]	1.32[b]	0.03125
	MBC	>2.64	>2.63	>2.64	>2.64	>2.64	>1

Table 2. Cont'd.

Gram-negative							
Escherichia coli ESBL[2]	MIC	2.64[a]	2.63[a]	1.32[b]	2.64[a]	2.64[a]	>5
	MBC	>2.64	>2.63	>2.64	>2.64	>2.64	>5
Escherichia coli EPEC[3]	MIC	2.64[a]	2.63[a]	>2.64	>2.64	>2.64	0.0039
	MBC	>2.64	>2.63	>2.64	>2.64	>2.64	0.25
Pseudomonas aeruginosa ATCC 27853	MIC	10.56[a]	10.57[a]	10.58[a]	10.57[a]	10.56[a]	1
	MBC	>10.56	>10.57	>10.58	>10.57	>10.56	>1
Shigella sp.	MIC	2.64[a]	2.63[a]	>2.64	>2.64	>2.64	0.0039
	MBC	>2.64	>2.63	>2.64	>2.64	>2.64	0.5
Shigella flexneri	MIC	2.64[a]	2.63[a]	2.64[a]	1.32[b]	1.32[b]	0.0039
	MBC	>2.64	2.63	>2.64	>2.64	>2.64	>1
Salmonella sp.	MIC	>2.64	>2.63	>2.64	>2.64	>2.64	0.0039
	MBC	>2.64	>2.63	>2.64	>2.64	>2.64	>1
Salmonella choleraesuis ATCC 10708	MIC	2.64[a]	2.63[a]	1.32[b]	>2.64	2.64[a]	0.025
	MBC	>2.64	>2.63	>2.64	>2.64	>2.64	0.05

MAC: Maceration; SOX: Soxhlet ; UH: ultrasonic horn; UCB: ultrasonic cleaning bath; MAE: microwave-assisted extraction. Different letters in the rows indicate significant differences between ethanolic extracts of *O. gratissimum* using one-way ANOVA and Tukey tests or Kruskal-Wallis and Mann-Whitney tests ($P< 0.05$). Standard errors of the means were omitted for easy viewing (S.E.M. ≤ 0.01). [1] Methicillin-resistant *Staphylococcus aureus*; [2] Extended-spectrum β-lactamase-producing *Escherichia coli*; [3] Enteropathogenic *Escherichia coli*.

DISCUSSION

The lower eugenol content in the extracts obtained by ultrasound methods contrasts with previous reports. Terpenoids from *Salvia officinalis* L. (Salisová et al., 1997) and phenolics from *Citrus unshiu* Marc (Ma et al., 2009) were extracted with higher efficiency by ultrasonic processes compared to conventional methods. On the other hand, the higher eugenol levels in UCB compared to UH may be a consequence of the sonication conditions. According to Vinatoru (2001), high-frequency ultrasound, as used in this study in UCB, does not increase extractive yield, but rather decreases the degradation of herbal constituents. Degradation of compounds during sonication occurs in some cases, due to their dissociation in the extractive medium and subsequent recombination (Luque-Garcia and Luque de Castro, 2003). Because eugenol degradation during storage was associated with the dissociation of phenolic hydrogen (Miller et al., 1979), this reaction may have been favored during UH extraction and may have led to a smaller content of this compound in the extract. In relation to microwave results, Chen et al. (2007), using the same methodology, solvent and extraction time as in this study, also obtained an increased yield from *Ganoderma atrum* when compared to an ultrasonic bath.

The higher bacteriostatic character of the ethanolic extracts of *O. gratissimum* is consistent with previous findings using other methodologies

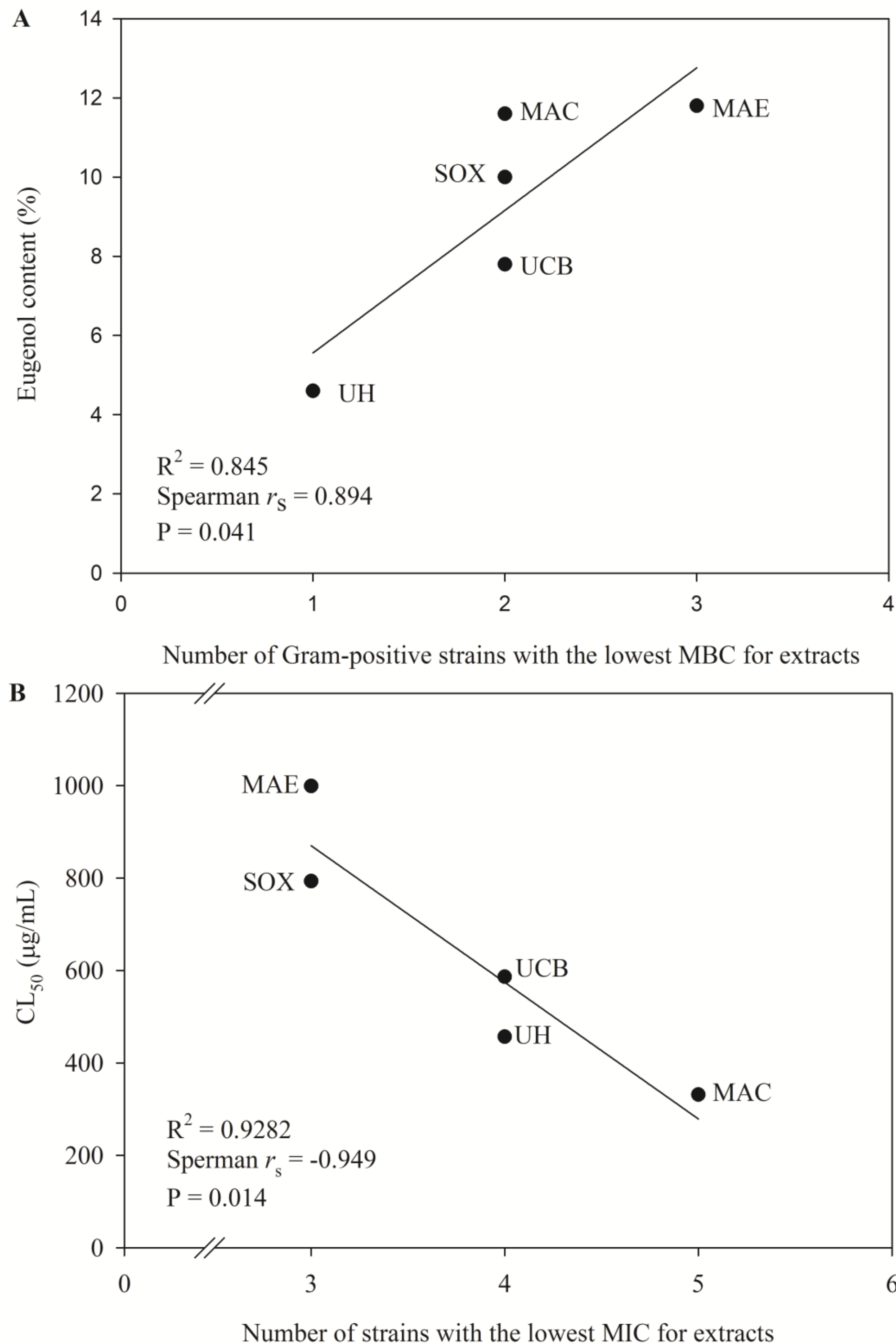

Figure 1. Correlations among results of the ethanolic extracts of *Ocimum gratissimum.* (A) Eugenol content and number of Gram-positive strains with the lowest MBC for extracts; (B) Brine shrimp toxicity and the number of bacterial strains with the lowest MIC for extracts.

(Passos et al., 2009). The differences detected in the bacteriostatic activity of the extracts seem to be a consequence of the synergistic or antagonistic action of eugenol and other constituents, such as oleanolic acid,

flavonoids and caffeic acid esters (Njoku et al., 1997; Grayer et al., 2003). This hypothesis can be partially confirmed by the absence of correlation between the eugenol content and the bacteriostatic activity. However, further studies are required in order to identify the compounds involved in the biological activity of the extracts.

The mechanism that produces the antimicrobial activity of phenolic compounds such as eugenol is not completely understood. In general, these compounds appear to exert their activity at the cytoplasmic membrane through mechanisms such as substrate complexing, membrane disruption, enzyme inactivation and metal chelation (Sikkema et al., 1995; Cowan, 1999; Gill and Holley, 2006; Di Pasqua et al., 2007).

Some bacterial species tested, such as *S. aureus*, *B. cereus*, enterococci, *P. aeruginosa*, *E. coli* and *Salmonella* sp., are common agents of nosocomial infections (Bereket et al., 2012). The development of antibiotic resistance in these pathogens, such as observed in MRSA, VRE and ESBL, and their widespread distribution in community and non-hospital health-care facilities have increased interest in finding new therapeutic alternatives (Lopes, 2005; Gaude and Hattiholli, 2013). Here, the ethanolic extracts of *O. gratissimum* showed different antimicrobial profiles according to the extraction method employed. With respect to VRE, for example, it is possible that the high temperatures used in the microwave and Soxhlet methods favored the extraction of active compounds, or reduced the extraction of constituents with an antagonist effect on antibacterial activity. On the other hand, extracts obtained by ultrasound methods were quite effective against recognized etiologic agents of wound infections, such as *S. aureus*, *B. cereus* and ESBL (Bottone, 2010; Bereket et al., 2012).

All ethanolic extracts of *O. gratissimum* demonstrated bioactivity as assessed by the BSL test (Table 1) according to the classification of Meyer et al. (1982) (LC_{50} < 1000 µg/ml). In view of the relatively low LC_{50} value detected for eugenol (LC_{50} = 186.1 µg/ml) (Silva et al., 2010), the BSL results suggest that this compound in the mixture showed lower toxicity due to a reduction of its concentration. It is also possible that the effect of other constituents could have increased the toxicity of the extracts obtained by maceration and ultrasound processes. A similar correlation between the BSL test and antibacterial results was previously suggested for species of Euphorbiaceae with antimicrobial activity (MacRae et al., 1988), but this correlation was confirmed statistically for the first time in the present study.

Conclusion

The methodology used to produce ethanolic extracts of *O. gratissimum* could be chosen according to their intended use. Ultrasound methods yield extracts that are efficient topical antiseptics. To treat nosocomial infections caused by VRE, the most active extracts could be obtained by microwave-assisted extraction.

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Conflict of interest

The authors report no declarations of interest.

REFERENCES

Adams RP (2001). Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured Publishing Corporation, Carol Stream. p. 804.

Bereket W, Hemalatha K, Getenet B, Wondwossen T, Solomon A, Zeynudin A, Kannan S (2012). Update on bacterial nosocomial infections. Eur. Rev. Med. Pharmacol. Sci. 16(8):1039-1044.

Bottone EJ (2010). *Bacillus cereus*, a volatile human pathogen. Clin. Microbiol. Rev. 23(2):382-398.

Braga FG, Bouzada MLM, Fabri RL, Matos MO, Moreira FO, Scio E, Coimbra ES (2007). Antileshmanial and antifungal activity of plants used in tradicional medicine in Brazil. J. Ethnopharmacol. 11:396-402.

Carrasco AH, Espinoza CL, Cardile V, Gallardo C, Cardona W, Lombardo L, Catalán MK, Cuellar FM, Russo A (2008). Eugenol and its synthetic analogues inhibit cell growth of human cancer cells. J. Braz. Chem. Soc. 19:543-548.

Chen Y, Xie MY, Gong XF (2007). Microwave-assisted extraction used for the isolation of total triterpenoid saponins from *Ganoderma atrum*. J. Food Eng. 81:162-170.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12:564-582.

Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D, Mauriello G (2007). Membrane toxicity of antimicrobial compounds from essential oils. J. Agric. Food Chem. 55:4863-4870.

European Pharmacopoeia (2007). European Directorate for the Quality of Medicines, Strasburg. p. 4392.

Gaude GS, Hattiholli J (2013). Rising bacterial resistance to beta-lactam antibiotics: Can there be solutions? J. NTR Univ. Health Sci. 2:4-9.

Gill AO, Holley RA (2006). Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. Int. J. Food Microbiol. 111:170-174.

Grayer RJ, Eckert MR, Veitch NC, Kite GC, Marin PD, Kokubun T, Simmonds MSJ, Paton AJ (2003). The chemotaxonomic significance of two bioactive caffeic acid esters, nepetoidins A and B, in the Lamiaceae. Phytochemistry 64:519-528.

Junaid SA, Olabode AO, Onwuliri FC, Okwori AEJ, Agina SE (2006). The antimicrobial properties of *Ocimum gratissimum* extracts on some selected bacterial gastrointestinal isolates. Afr. J. Biotechnol. 5:2315-2321.

Lopes HV (2005). CA-MRSA: a new problem for the infectologist. Rev. Panam. Infectol. 7:34-36.

Luque-Garcia JL, Luque de Castro MD (2003). Ultrasound: a powerful tool for leaching. Trends Anal. Chem. 22:41-47.

Ma YQ, Chen JC, Liu DH, Ye XQ (2009). Simultaneous extraction of phenolic compounds of citrus peel extracts: effect of ultrasound. Ultrason. Sonochem. 16:57-62.

MacRae WD, Hudson JB, Towers GHN (1988). Studies on the pharmacological activity of Amazonian Euphorbiaceae. J.

Ethnopharmacol. 22:143-172.
Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine Shrimp: A convenient general bioassay for active plant constituents. Planta Med. 45:31-34.
Miller RA, Bussell NE, Ricketts CK, Jordi H (1979) Analysis and purification of eugenol. J. Dent. Res. 58:1394-1400.
Nakamura CV, Ueda-Nakamura T, Bando E, Melo AFN, Cortez DAG, Dias Filho BP (1999). Antibacterial activity of *Ocimum gratissimum* L. essential oil. Mem. Inst. Oswaldo Cruz 94:675-678.
National Committee for Clinical Laboratory Standards (NCCLS) (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved Standard M7-A6, 6th edition. NCCLS, Wayne.
Njoku CJ, Zeng L, Asuzu IU, Oberlies NH, McLaughlin JL (1997). Oleanolic acid, a bioactive component of the leaves of *Ocimum gratissimum* (Lamiaceae). Int. J. Pharmacog. 35:134-137.
Nweze EI, Eze EE (2009). Justification for the use of *Ocimum gratissimum* L in herbal medicine and its interaction with disc antibiotics. BMC Complement. Altern. Med. 9:37.
Onajobi FD (1986). Smooth muscle contracting lipid-soluble principles in chromatographic fractions of *Ocimum gratissimum*. J. Ethnopharmacol. 18:3-11.
Passos MG, Carvalho H, Wiest JM (2009). *In vitro* inhibition and inactivation of different extraction methods in *Ocimum gratissimum* L. (“alfavacão”, “alfavaca”, “alfavaca-cravo”) - Labiatae (Lamiaceae) against foodborne bacteria of interest. Rev. Bras. Plant Med. 11:71-78.
Salisová M, Toma S, Mason TJ (1997). Comparison of conventional and ultrasonically assisted extractions of pharmaceutically active compounds from *Salvia officinalis*. Ultrasonogr. Sonochem. 4:131-134.
Sikkema J, Bont AAM, Poolman B (1995). Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59:201-222.
Silva LL, Heldwein CG, Reetz LGB, Hörner R, Mallmann CA, Heinzmann BM (2010). Chemical composition, antibacterial activity *in vitro* and brine-shrimp toxicity of the essential oil from inflorescences of *Ocimum gratissimum* L., Lamiaceae. Braz. J. Pharmacog. 20:700-705.
Silva MIG, Gondim APS, Nunes IF, Sousa FCF (2006). The use of herbal medicines in the family health care units in Maracanaú (CE). Braz. J. Pharmacog. 16:455-462.
Spectral Database for Organic Compounds (SDBS) (2008). National Institute of Advanced Industrial Science and Technology. http://riodb01.ibase.aist.go.jp/sdbs/.
Vinatoru M (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. Ultrason. Sonochem. 8:303-313.
Wang L, Weller CL (2006). Recent advances in extraction of nutraceuticals from plants. Trends Food Sci. Technol. 17:300-312.

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Full Length Research Paper

Identification of flavonol glycosides and *in vitro* photoprotective and antioxidant activities of *Triplaris gardneriana* Wedd

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***Triplaris gardneriana* belongs to the family Polygonaceae, known for producing a number of biologically important molecules. The present study was aimed at identifying and quantify its total flavonoid content and determining the antioxidant and photoprotective potential of the plant's leaves. The flavonoids present in the extract and fractions were analyzed using Liquid chromatography–mass spectrometry (LC-MS). The antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and autoxidation of β-carotene. The absorbance of the extracts was measured at different concentrations between 260 to 400 nm wavelengths. Calculation of sun protection factor (SPF) was determined using the formula developed by Mansur. The total flavonoid content was determined by the method developed by Dewanto. In the study, the following four flavonols were identified: quercetin-hexoside (2a), quercetin-pentoside (2b), quercetin-ramnobioside (2c) and myricetin-hexoside (2d). The crude ethanol extract, ethyl acetate, and methanol fractions showed higher flavonoid content and also exhibited excellent antioxidant and photoprotective activity. The SPF values were best observed for the crude ethanol extract and for the chloroform, ethyl acetate and methanol fractions. The good antioxidant and photoprotective potential can be attributed to the presence of flavonols identified for the first time in this species.**

Key words: *Triplaris gardneriana*, Polygonaceae, antioxidant activity, photoprotective activity, glycosylated flavonols, LC-MS.

INTRODUCTION

The species *Triplaris gardneriana* Wedd (Polygonaceae) is popularly known in the northeast as "Pajeu". It is used

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in popular medicine to treat bleeding hemorrhoids, coughing and bronchitis; decoction of the bark or root is used to treat gonorrhea, leucorrhea and inflammation of internal organs, as well as to stimulate the uterus of rats and as a molluscicide (Cartaxo et al., 2010). Recent studies of seed extracts showed antibacterial, antioxidant, and anticholinesterase activities (Farias et al., 2013). The plants of the family Polygonaceae are known to produce a number of important secondary metabolites such as flavonoids. We emphasized the class of flavonols occurring in 25% of the genera of the family, including *Triplaris* (Oliveira et al., 2008).

Recently, plants that have antioxidant activity have been of great interest, given the distinct roles of free radical presence in the body (Alves, 2010). Phenolic compounds such as flavonoids exhibit intense absorption in the UV region and have antioxidant action (Zuanazzi and Montanha, 2012). Such intrinsic features of these secondary metabolites allow the possibility of plant extracts that contain them in their composition to be used for their photoprotective action. This is owing to their confirmed capacity to absorb solar radiation; as an antioxidant, they may neutralize free radicals produced in the skin after exposure to sunlight (Souza et al., 2013).

In recent studies, hyphenated techniques such as Liquid chromatography–mass spectrometry (LC-MS) have been applied in the metabolomic analysis of plants, as the combination of these two techniques provides a powerful tool in the analysis of flavonoids in crude extracts by detecting ions produced by electrospray ionization (ESI) (Boulekbache-Makhlouf et al., 2012). Considering that in Brazil *T. gardneriana* presents no chemical and biological studies *in vitro*, the present study aimed, through LC-MS, to identify and quantify the total flavonoid content and determine the antioxidant and photoprotective potential of the extracts and fractions of the species' leaves.

MATERIALS AND METHODS

Plant

The leaves were collected in the city of Santa Maria of Boa Vista in the state of Pernambuco, Brazil in July, 2013, located at 349 m elevation (08° 47' 59, 00 S, 039° 50' 42, 40 W). Specialist Diogo de Oliveira Gallo confirmed the botanical identity of the plant. A voucher specimen of the plant was deposited in the Herbarium of the Federal University of San Francisco Valley (HVASF) under registration number 21221.

Obtaining and fractionation of the crude ethanol extract

The dried and powdered leaves (5,000 g) were continuously extracted three times during a 72-h period with 95% ethanol at room temperature. The ethanol solution obtained was filtered, and then the solvent was evaporated with the aid of a rotaevaporator at reduced pressure with an average temperature of 50°C, yielding 724 g of crude ethanol extract after the distillation of the solvent. This was mixed with silica gel 60 (0.04 to 0.063 mm, MACHEREY-NAGEL), subjected to a vacuum-liquid chromatography (VLC), and fractionated with hexane, chloroform, ethyl acetate and methanol (Sigma-Aldrich, USA) to yield the following four fractions: hexane (9.30 g), chloroform (42.17 g), ethyl acetate (19.29 g) and methanol (193.48 g).

Determination of total flavonoid content

The determination of total flavonoid content was determined using the methodology previously described by Dewanto et al. (2002), with adaptations. The absorbance was measured against the blank at 510 nm using a spectrophotometer (QUIMIS, Brazil) in comparison with the standard prepared similarly with known concentrations. The results were expressed as mg of catechin equivalent to a gram of extract/fractions (mgCE/g) using the calibration curve of catechin (R^2 = 0.9943). The range of the calibration curve was 50 to 1000 mg^{-1}.

LC-MS analysis

Analyses were performed by the Analytical Center of the Institute of Chemistry, University of São Paulo, using the apparatus of high-efficiency liquid chromatography coupled to ion trap mass spectrometer model Esquire 3000 plus Bruker Daltonics, equipped with electrospray ionization-ESI. The LC system consisted of two LC10AD solvent pumps, a SLC 10A system controller, a CTO-10AS column oven (Shimadzu, Japan), a 7125 Rheodyne injector with a 20 ml loop, and an UV detector (SPD 10A, Shimadzu, Japan). The samples were diluted in methanol (EM Science, USA) in 1 mg ml^{-1} concentrations, injected into the apparatus, and subjected to LC-18 Shimadzu Shim-Pack® column (250 × 4.6 mm, Japan). For elution of the column, solvent A (water: acetic acid 99:1) and solvent B (acetonitrile: acetic acid 99:1) were used with the following program: 10% B (0 to 5 min), 15% B (50 to 55 min), 20% B (65 to 75 min), 100% B (80 to 85 min), and finally 10% B (up to 90 min). The flow rate was maintained at 1.0 ml min^{-1}, and the injection volume was 10 µl. In the electrospray ionisation mass spectrometry (ESI-MS) analyses, the general conditions were: source temperature of 40°C and capillary voltage of 4.0 Kv in positive mode, data acquisition in MS. The compounds were identified according to the interpretation of their fragmentation spectra and comparison with literature data.

Determination of antioxidant activity

DPPH free radical scavenging assay

The free radical scavenging activity was measured according to the method developed by Mensor et al. (2001) with adaptations, using 2,2-diphenyl-1-picryl-hydrazyl (DPPH•). Stock solutions of 1.0 mg ml^{-1} of the extract and fractions were diluted in ethanol to final concentrations of 243, 81, 27, 9, 3 and 1 µg ml^{-1}. One ml of a 50 µg ml^{-1} DPPH ethanol solution was added to 2.5 ml of diluted sample and allowed to react at room temperature. After 30 min, the absorbance values were measured at 518 nm and converted into percentage of antioxidant activity (AA) using the following formula:

$$\%AA = [(A_C - A_A) / A_C] * 100$$

Where A_C is equivalent to absorbance of the control and A_A absorbance of the sample. Ethanol 1.0 ml with extract solution 2.5 ml were used as a blank. DPPH solution 1.0 ml with ethanol 2.5 ml was used as negative controls. The positive controls were the ascorbic acid, butylhydroxyanisole and butylhydroxytoluene. The effective concentration (EC_{50}) values were calculated by linear regression using the GraphPad Prism® 5.0 program.

Inhibition of auto oxidation of β-carotene

The ability of the extracts to prevent the oxidation of β-carotene was evaluated according to the methodology described by Wannes et al. (2010). The β-carotene 2 mg was dissolved in 10 ml of chloroform, linoleic acid 40 mg and Tween 40 (400 mg) were added to 2 ml of this solution. The chloroform was evaporated under vacuum at 40°C and 100 ml of distilled water was added; afterwards, the emulsion was vigorously shaken for two minutes. The standard compounds (ascorbic acid, butylhydroxyanisole and butylhydroxytoluene) and extracts were diluted in ethanol. The 3.0 ml emulsion was added to a tube containing 0.12 ml of the standard solutions and 1.0 mg ml^{-1} of the extracts. Absorbance was measured immediately at 470 nm and the samples were incubated in a water bath at 50°C for 120 min, when absorbance was measured again. In the negative control, extracts were replaced with an equal volume of ethanol. The antioxidant activity (%AA) was evaluated in terms of bleaching of β-carotene using the following formula:

$$\%AA = [1 - (A_0 - A_t) / (A_0^0 - A_t^0)] * 100$$

Where A_0 is the initial absorbance and A_t is the measured absorbance for the final sample. A_0^0. is the initial absorption and A_t^0) is the final absorbance measured for the negative contro. Results are expressed as percentage of antioxidant activity (%AA).

Determination of *in vitro* sun protection factor (SPF) and the maximum absorption wavelength

Determining the wavelength of maximum absorption of the extract and fractions was performed by diluting these in absolute ethanol according to the method described by Violante et al. (2009) to yield concentrations of 5, 25, 50 and 100 mg L^{-1}. Subsequently, the reading was performed in a UV-VIS spectrophotometer (QUIMIS, Brazil) at wavelengths between 260 and 400 nm, with intervals of 5 nm. Readings were taken using a quartz cell of 1.0 cm optical path and ethanol was used as a blank. Calculation of SPF was determined using the equation developed by Mansour et al. (1986):

$$SPF = CF. \sum_{290}^{320} . EE(\lambda). I(\lambda). abs(\lambda)$$

Where EE (λ) equals erythematogenic effect of radiation of wavelength; I (λ) Intensity of solar radiation at a wavelength; Abs (λ) Spectrophotometric determination of the absorbance of the solution in wavelength; CF Correction factor (= 10). The values of EE (λ). I(λ) are constants.

Statistical analysis

Data obtained in triplicate experiments were analyzed statistically using the GraphPad Prism® version 5.0 and expressed as mean ± SD. Differences were considered significant when $P < 0.05$.

RESULTS

LC-MS analysis

The LC-MS-coupled technique used in this study allowed for the identification of the presence of flavonols in the crude ethanol extract and fractions of *T. gardneriana*. This technique was used to characterize the presence of compounds with the antioxidant and photoprotective potential presented in this study. The electrospray ionisation mass spectrometry (ESI-MS) analyses were performed in positive mode, and adducts formed were predominantly as $[M+Na]^+$. Analysis through ESI-MS showed that the information of the mass spectra of some constituents present in the crude ethanol extract and fractions coincided with the mass identified as the flavonol derivatives quercetin and myricetin, based on the presence of the signals at m/z 303 and m/z 319 of aglycones, respectively. The following compounds were identified: (2a) quercetin-hexoside, (2b) quercetin-pentoside, (2c) quercetin-ramnobioside and (2d) myricetin-hexoside (Figure 2, Table 1). The chromatogram obtained from the conditions of the HPLC analysis of the crude ethanol extract is shown in Figure 1. The peak corresponding to the compound 1 (Figure 2a) was detected in the crude ethanol extract, ethyl acetate fraction and chloroform in 17.6 and 17.5 min in the methanol fraction. With the compound of m/z 465 $[M+H]^+$ and m/z 487 $[M+Na]^+$, thereby eliminating the values of the atomic mass of the protonated adduct, it was possible to get the molecular formula $C_{21}H_{20}O_{12}$ referring to 464 amu. The spectrum was presented as quercetin aglycone, with protonated fragment m/z 303 (Y^+) and sodiated m/z 325. The formation of the fragment of m/z 185 sodiated and 163 not sodiated is consistent with a hexose. The substance 2 was detected in the chromatograms of the crude ethanol extract and of the ethyl acetate and methanol fractions with retention times equal to 18.4, 18.2, and 18.3 min, respectively. The compound of mass m/z 435 $[M+H]^+$ and m/z 457 $[M+Na]^+$ showed peaks for quercetin m/z 303 (Y^+) and m/z 325 quercetin sodiated after loss of one molecule of pentose 132 amu. The fragments sodiated and not sodiated m/z 155 and 133 confirm that a pentose is linked in the aglycone. The peaks allowed us to suggest the flavonoid quercetin- pentoside (Figure 2b) mass 434 amu and molecular formula $C_{20}H_{18}O_{11}$. The peak corresponding to the compound 3 was only detected in the crude ethanol extract and in the chloroform fraction at 21.6 and 21.5 min, respectively. The substance exhibited molecular ion peak equal to m/z 617, which refers to the atomic mass of the substance added to a sodium adduct $[M+Na]^+$ and m/z 595 consistent with

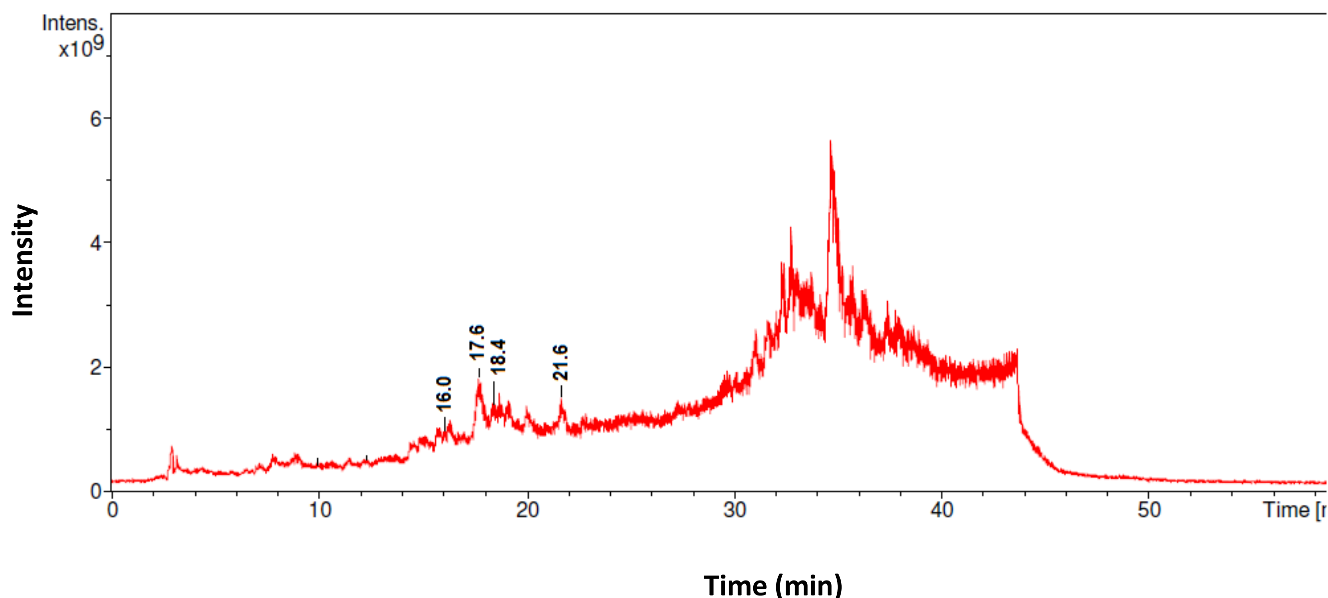

Figure 1. Chromatogram HPLC/UV-ESI-MS of the crude ethanol extract of *T. gardneriana*.

the hydrogen adduct $[M+H]^+$, coinciding with quercetin-ramnobioside (Figure 2c) with molecular formula $C_{27}H_{30}O_{15}$ and 594 amu. The fragment m/z 303 (Y^+) is consistent with quercetin aglycone with a neutral loss of 292 amu, which corresponds to two rhamnoses linked. This information has been confirmed with the fragmentation of the ions sodiated m/z 617, a small fragment of m/z 315, consistent with the disaccharide Rha-Rha, which is connected to quercetin. The fragment m/z 449 and 471 sodiated is compatible with quercetin except for a rhamnose unit. The peak m/z 169 rhamnose sodiated and 147 correspond to one rhamnose unit. Compound 4 was identified only in the crude ethanol extract and methanol fraction in 16.0 and 15.9 min, respectively. The compound showed a molecular ion peak equal to m/z 503 that refers to the atomic mass of the substance added to a sodium $[M+Na]^+$ and m/z 481 consistent adding a hydrogen $[M+H]^+$, indicating molecular formula $C_{21}H_{21}O_{13}$ and 480 amu. The spectrum showed a fragmentation of ion m/z 319 (Y^+), being compatible with myricetin, with neutral loss of 162 amu indicating the loss of a hexose unit, identified as myricetin-hexoside (Figure 2d). Figure 3 shows the mass spectra of the flavonols. All substances identified are reported for the first time in the species *T. gardneriana*.

Determination of total flavonoid content

The crude ethanol extract and the ethyl acetate and methanol fractions showed the highest content of total flavonoids 281.35 ± 6.23, 287.14 ± 2.23 and 271.35 ± 1.32, respectively (Table 2).

0

Determination of antioxidant activity

The results of the evaluation of the antioxidant activity are shown in Table 2. Data showed that the crude ethanol extract, ethyl acetate and methanol fractions exhibited excellent activity in the scavenging of DDPH with lower EC_{50} values (2.27 ± 0.19, 5.42 ± 0.84 and 3.35 ± 0.15 µg ml^{-1}), respectively. The hexane and chloroform fractions showed low antioxidant activity, with higher EC_{50} values. In the β-carotene method, the crude ethanol extract also showed better activity, 67.62 ± 2.48%, followed by chloroform fraction, 54.74 ± 1.28% (Table 2).

Photoprotective activity

The crude ethanol extract and the fractions chloroform, ethyl acetate and methanol showed spectrophotometric absorption profile within the range of UVC region (100 to 290 nm) and UVB (290 to 320 nm). However, only the chloroform and ethyl acetate fractions absorbed in the UVA region (320 to 400 nm) had maximum wavelength in this region, emphasizing the chloroform fraction, which was more efficient to range UVA as well as UVB followed by the ethyl acetate fraction. The maximum wavelength

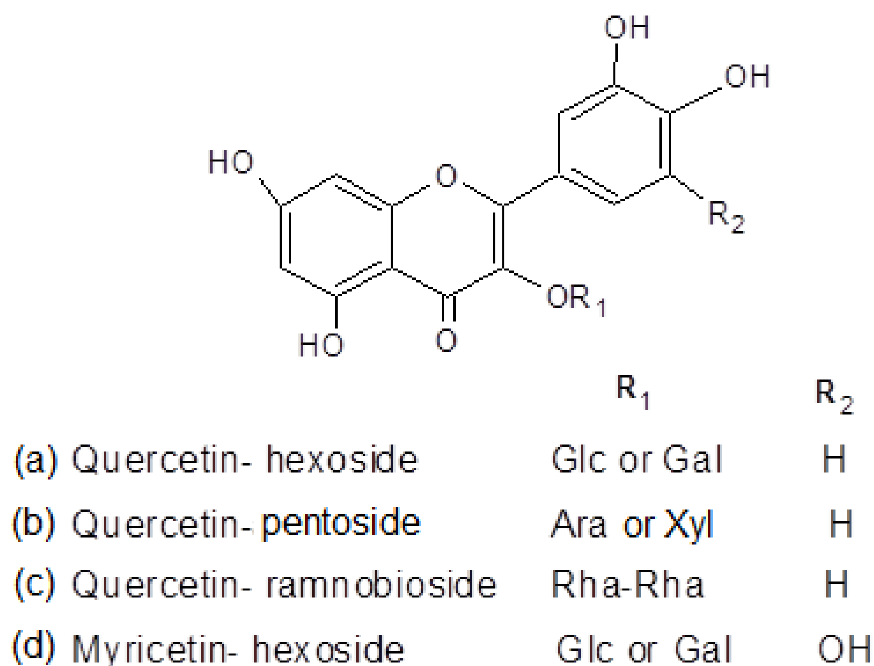

Figure 2. Glycosylated flavonols identified in the crude ethanol extract and fractions of *T. gardneriana*.

exhibited by the crude ethanol extract and fractions is shown in Table 3. All samples exhibited a higher SPF of 6.0 in a concentration in 100 mg L^{-1}, except for the hexane fraction. The SPF values at this concentration were 7.410 ± 0.132, 12.794 ± 0.163, 11.280 ± 0.127 and 11.891 ± 0.291 for crude ethanol extract, the chloroform fraction, ethyl acetate fraction and methanol fraction, respectively. The results of *in vitro* SPF of crude ethanol extract and fractions using the methodology proposed by Mansur (1986) are shown in Table 4.

DISCUSSION

LC-MS analysis

Structural analysis of the individual ions in the mass spectra was performed by comparison with the literature data (Souza et al., 2008). In compound 1, the removal of ring B resulted in the fragmented peak m/z 181 allowing profiling of the sugar substituent in ring C. Generally, sugar substituents linked to a hydroxyl group of the aglycone are located at position 3 or 7; however, substituent located at the 3-position is more easily lost than in position 7 (Cuyckens and Claeys, 2005; Rijke et al., 2006). The hexose identified in compound 1 and 4 possibly refers to glycosides as galactose or glucose, as it has been common in flavonol glycosides in the family Polygonaceae. With this, the compound 1 was identified as quercetin-hexoside (Figure 2a), consistent with the structure of isoquercitrin or hyperoside, both already reported in the family; the compound 4 refers to myricetin-hexoside (Figure 2d). In compound 2 (Figure 2b), the sugar residue may consist of an arabinose or xylose, both already reported in the family Polygonaceae. However, the pentose arabinose appears in most cases in the structures of flavonols in the family, what leads to the supposition that it is the sugar linked in the aglycone. The peaks m/z 169 and 147 that may correspond to a rhamnose unit in compound 3 (Figure 2c) may also be a coumaroyl unit, but there is no report of this acyl group in flavonols isolated in the family. It therefore suggests that it is a quercetin-ramnobioside (Oliveira, 2008).

Determination of total flavonoid content

As expected, the compounds of phenolic nature were mainly present in the samples that have moderate to high polarity as the crude ethanol extract and the ethyl acetate and methanol fractions (Table 2).

Determination of antioxidant activity

Values of antioxidant activity of the crude ethanol extract,

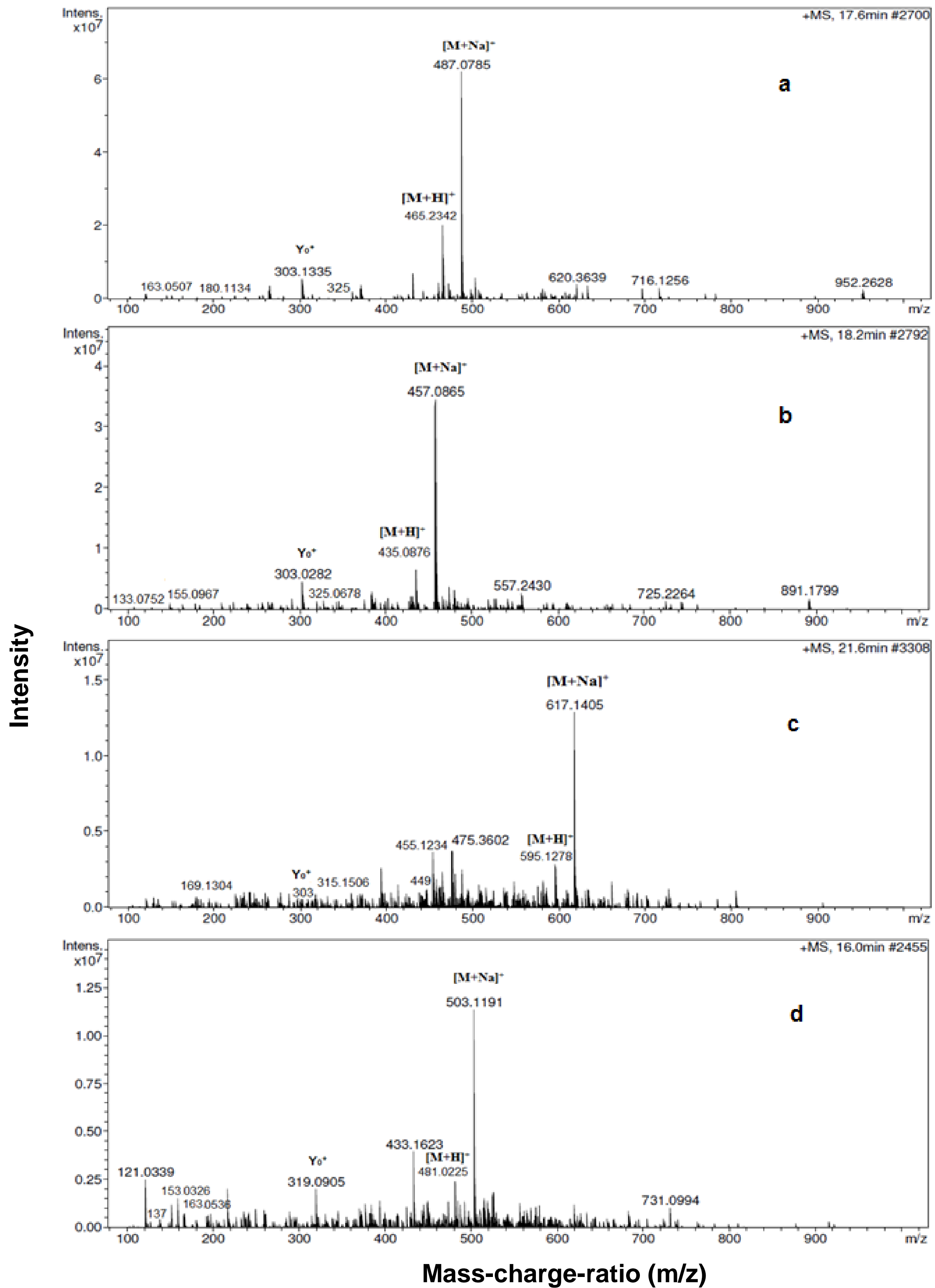

Figure 3. ESI-MS mass spectrum registered in positive mode: (a) quercetin-hexoside, (b) quercetin-pentoside, (c) quercetin-ramnobioside and (d) myricetin-hexoside.

Table 1. Retention time and fragmentation in positive mode of the identified compounds of T. gardneriana.

Compound	T_R EEC	T_R $CHCl_3$	T_R AcOEt	T_R MeOH	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	Fragmentation in positive mode (MS+)
1	17.6	17.6	17.6	17.5	465	487	303, 181, 185, 163
2	18.4	-	18.2	18.3	435	457	303, 325, 133, 155
3	21.6	21.5	-	-	595	617	303, 471, 455, 449, 315, 147, 169
4	16.0	-	-	15.9	481	503	319, 163,153

*EEC= Crude ethanol extract. $CHCl_3$=Chloroform fraction. AcOEt= Ethyl acetate fraction. MeOH= Methanol fraction. T_R= Retention time.

Table 2. Total flavonoids and antioxidant activity of *T. gardneriana*.

Extract/Fractions/Standards	Flavonoids (mg de ECAT/g)	DPPH (EC_{50}, μg ml^{-1})	β-Carotene (%AA)
Crude ethanol extract	281.35± 6.23	2.27± 0.19	67.62± 2.48
Hexane fraction	n.d	32.91± 0.76	48.90± 1.12
Chloroform fraction	61.88± 3.50	50.35± 10.27	54.74± 1.28
Ethyl acetate fraction	287.14± 2.23	5.42± 0.84	45.04± 1.12
Methanol fraction	271.35± 1.32	3.35± 0.15	47.64± 2.59
Ascorbic acid	-	3.21± 0.30	-7.83± 2.58
Butylhydroxyanisole	-	3.05± 0.40	70.68± 0.55
Butylhydroxytoluene	-	5.38± 0.11	69.96± 3.07

*Values are presented as mean ± SD (n = 3). ECAT= Catechin equivalents. EC50 is defined as sufficient for 50% maximal effect concentration. (%AA)= percentage of antioxidant activity. n.d.= not determined.

Table 3. Maximum wavelength and absorption type of crude ethanol extract and fractions of T. gardneriana.

Extract / Fractions	λ maximum (nm)	Absorption region
Crude ethanol extract	280	UVC/UVB
Hexane fraction	-	-
Chloroform fraction	280/330/395	UVA/UVB/UVC
Ethyl acetate fraction	280/370/400	UVA/UVB/UVC
Methanolic fraction	280	UVC/UVB

*UVA (320 to 400 nm), UVB (280 to 320 nm) and UVC (100 to 280 nm). Concentrations of the analysis were 5, 25, 50 and 100 mg L^{-1}.

ethyl acetate and methanol fractions are not statistically different ($P < 0.05$) from that found for standards. The crude ethanol extract showed better antioxidant activity than did the standards used. In the β- carotene method, the values of the hexane, ethyl acetate and methanol fractions were not significantly different (Table 2). According to the results shown in Table 2, all samples exhibited antioxidant activity. These results can be justified by the presence of higher levels of flavonoids in the crude ethanol extract, methanol and ethyl acetate fractions. Phenolic compounds, particularly flavonoids, possess ideal structure for the scavenging of radicals, since they are very reactive as a hydrogen and electron donor (Barreiros et al., 2006). The antioxidant activity presented by the leaf extract and fractions of *T. gardneriana* corroborates studies with seed extract (Farias et al., 2013).

Photoprotective activity

According to RDC Resolution No. 30 (National Health

Table 4. Sun Protection Factor (SPF) of crude ethanol extract and fractions of *T. gardneriana.*

Concentration (mg L^{-1})	Crude ethanol extract	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Methanol fraction
5	0.700±0.127	0.343±0.031	0.669±0.022	0.775±0.052	0.685±0.069
25	2.042±0.050	0.731±0.020	3.169±0.032	2.937±0.123	2.769±0.049
50	3.734±0.051	1.267±0.053	6.324±0.069	5.608±0.084	5.379±0.145
100	7.410±0.132	2.393±0.084	12.794±0.163	11.280±0.127	11.891±0.291

* Values are presented as mean ± SD (n = 3).

Surveillance Agency of Brazil (ANVISA), 2012), a product suitable for use in cosmetics such as sunscreen products should have SPF values of at least 6.0. Therefore, all samples, except for the hexane fraction, showed photoprotective effect. Such a high level of photoprotection can be explained by the content of flavonoids present in the samples (Table 2), since, as already mentioned, the presence of these metabolites indicates a potential in the absorption of UV radiation. Therefore, assays like this are important, since they guide the selection of plant species with potential sun protection factor in a simple and inexpensive manner. According to the literature, the genus has been little explored from the phytochemical point of view, previously reporting only the isolation of five flavonol glycosides and gallic acid from the ethyl acetate fraction of the leaves of *T. cumingiana* (Hussein et al., 2005) and phenylpropanoid, one flavonol glycoside and gallic acid on leaves, stems and fruit of *T. americana* (Oliveira et al., 2008). The presence of glycosylated flavonols and gallic acid in both plant species may serve as useful chemo-taxonomic markers for the genus. Thus, glycosylated flavonols identified in this study are in agreement with the data described in studies of genus and confirm further these chemical markers.

Conclusion

In this study, the coupled LC-MS technique has proved to be a powerful tool for the identification of flavonols, which allowed for the identification of four flavonol glycosides which are described for the first time in the species. The results obtained from this study show that the excellent antioxidant and photoprotective activities can be attributed to its chemical composition rich in flavonols. It also suggests that this species, which is already widely used in folk medicine, may become a great alternative for use in pharmaceuticals.

Conflict of interests

The author(s) have not declared any conflict of interests.

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REFERENCES

Agência de Vigilância Sanitária- ANVISA (2012). RDC Resolution No. 30, approved the Technical Regulation on sunscreens in cosmetics. ANVISA: Brasília. Available at: http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2012/rdc0030_01_06_2012.html Accessed April 2014.

Alves CQ, David JM, David JP, Bahia MV, Aguiar RM (2010). Methods for determination of in vitro antioxidant activity in organic substrates. Quim. Nova 33(10):2202-2210.

Barreiros ALBS, David JM, David JP (2006). Oxidative stress: relationship between generation of reactive species and organism defense. Quim. Nova 29(1):113-123.

Boulekbache-Makhlouf L, Meudec E, Mazauric JP, Madani K, Cheynier V (2012). Qualitative and semi-quantitative analysis of phenolics in *Eucalyptus globulus* leaves by High- performance Liquid Chromatography coupled with Diode Array Detection and Electrospray Ionisation Mass Spectrometry. Phytochem. Anal. 24:162-170.

Cartaxo SL Souza MMA, Albuquerque UP (2010). Medicinal plants with bioprospecting potential used in semi-arid Northeastern Brazil. J. Ethnopharmacol. 131:326-342.

Cuyckens F, Claeys M (2005). Determination of the glycosylation site in flavonoid mono-O- glycosides by collision-induced dissociation of electrospray-generated deprotonated and sodiated molecules. J. Mass Spectrometry 40:364-372.

Dewanto V, Wu X, Adom KK, Liu RH (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food Chem. 50(10):3010-3014.

Farias DF, Souza TM, Viana MP, Soares BM, Cunha AP, Vasconcelos IM, Ricardo NMPS, Ferreira PMP, Melo VMM, Carvalho AFU (2013). Antibacterial, antioxidant, and anticholinesterase activities of plant seed extracts from Brazilian semiarid region. Biomed. Res. Int. 510736:9.

Hussein AA, Barberena I, Correa M, Coley PD, Solis PN, Gupta MP (2005). Cytotoxic flavonol glycosides from *Triplaris cumingiana*. J. Nat. Prod. 68:231-233.

Mansur JS, Breder MNR, Mansur MCA, Azulary RD (1986). Determination of the sun protection factor by spectrophotometry. Anal. Bras. Dermatol. 61:121-124.

Mensor LL, Menezes F, Leitão GG, Reis AS, Santos TC, Coube TS, Leitão SG (2001). Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15:127-130.

Oliveira PES, Conserva LM, Lemos RPL (2008). Chemical constituents from *Triplaris americana* L. (Polygonaceae). Biochem. Syst. Ecol. 36:134-137.

Rijke E, Out P, Niessen WMA, Ariese F, Gooijer C, Brinkman UAT (2006). Analytical separation and detection methods for flavonoids. J. Chromatogr. A. 1112:31-63.

Souza FP, Campos GR, Packer FJ (2013). Determination of the photoprotective and antioxidant activity in emulsions containing extract *Malpighia glabra* L. – Acerola. Rev. Cienc. Farm. Basica Appl. 34(1):69-77.

Souza LM, Cipriani TR, Serrato RV, Costa DE, Iacomini M, Gorin FAJ, Sassaki GL (2008). Analysis of flavonol glycoside isomers from leaves of *Maytenus ilicifolia* by offline and online high performance liquid chromatography–electrospray mass spectrometry. J. Chromatogr. A. 1207:101-109.

Violante IMP, Souza IM, Venturini CL, Ramalho AFS, Santos RAN, Ferrari M (2009). In vitro evaluation of the photoprotective activity of plant extracts from the Cerrado of Mato Grosso. Rev. Bras. Farmacogn. 19(2A):452-57.

Wannes WA, Mhamdi B, Sriti J, Jemia MB, Ouchikh O, Hamdaoui G, Kchouk ME, Marzouk B (2010). Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. Food Chem. Toxicol. 48(5):1362-1370.

Zuanazzi JAS, Montanha JA (2012). Flavonoids. In: Simões CMO, Schenkel EP, Gosmann G, Mello JCP, Mentz LA, Petrovick PR (eds.), Pharmacognosy of the plant to the medicine. Federal University of Rio Grande do Sul: Porto Alegre. pp. 577-614.

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Pharmaco-toxic characterization of the aqueous extract from *Pereskia grandifolia* leaves

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Medicinal plants are rich sources of bioactive compounds with potential for therapeutic, cosmetic and food use, and the species *Pereskia grandifolia* is widely used by low-income populations for nutritional and medicinal purposes. The objective of this study was to evaluate the pharmaco-toxic potential of the aqueous extract from *P. grandifolia* leaves, in order to obtain information on the safety of its use. *P. grandifolia* leaves were dried, ground and the obtained flour was subjected to decoction at a 1:25 (wv^{-1}) ratio. The extract was then centrifuged and the supernatant was collected and lyophilized. The genotoxic potential on human leukocytes (comet assay) was evaluated, as well as phospholipase, hemolytic, coagulant and fibrinogenolytic activities, besides potential interactions with isolated hyaluronidases (evaluated in polyacrylamide gel). The evaluated doses of the aqueous extract from *P. grandifolia* leaves did not induce damage to the genetic material of human leukocytes, phospholipase, coagulant and fibrinogenolytic activities, nor altered the electrophoretic migration profile of hyaluronidases in polyacrylamide gel. However, the extract induced *in vitro* hemolysis, in doses higher than 10 µg. Therefore, it is premature to recommend the use of this plant for therapeutic purposes regarding possible risks and benefits to human health and additional studies of toxicity, efficacy and safety are necessary, especially using *in vivo* assays.

Key words: *Pereskia grandifolia*, genotoxicity, hemolysis, coagulation, molecular interactions.

INTRODUCTION

Ora-pro-nobis corresponds to different species of the genus *Pereskia*, cacti known as unconventional vegetables, consumed by rural and urban populations and which contribute to suplement food and family economy (Souza, 2009). *Pereskia aculeata* Miller is the most widely investigated species, and it is consumed in various regions of Brazil (Takeiti et al., 2009). On the other hand, *Pereskia grandifolia* is very little studied regarding its chemical constituents and toxicity.

P. grandifolia is widely distributed throughout Brazil

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(Turra et al., 2007). However, the lack of information on their nutritional value and toxicity results in a reduction in their consumption in Brazil (Rocha et al., 2008).

In Malaysia, the leaves of this plant are traditionally used in the treatment of cancer, hypertension, diabetes mellitus and diseases associated with rheumatism, inflammation, headache, atopic dermatitis and hemorrhoids (Sim et al., 2010). They can also be used for gastric pain and ulcer (Goh, 2000); however, for most of these effects, there are no studies which confirm these therapeutic actions, highlighting the importance of research with the objective to increase the pharmacological and toxicological characterization of this plant.

Cacti of the genus *Pereskia* have a high content of proteins with high digestibility, mucilage fiber and minerals such as iron and calcium, thus arousing the interest of pharmaceutical and food industries. Furthermore, different *Pereskia* species have large amounts of bioactive substances, which allow to classify them as functional food (Takeiti et al., 2009). Various secondary metabolites found in cacti, such as alkaloids, terpenoids, anthocyanins, steroids, flavonoids, quinones and lignans are commercially used as pharmacons, natural dyes, flavoring substances and insecticides (Simões et al., 2004).

Despite their beneficial effects, bioactive compounds present in medicinal plants, in certain situations, may not be innocuous and present toxic, genotoxic and carcinogenic effects (Boeira et al., 2010; Osowski et al., 2010), highlighting the need for wide *in vitro* and *in vivo* pharmaco-toxic characterizations of the extracts from these plants in order to evidence their therapeutic effects, as well as the determination of effective and safe conditions for their use. Therefore, the objective of this study was to evaluate the damage-inducing potential of the aqueous extract from *P. grandifolia* leaves to the genetic material of human leukocytes, degradation of phospholipids, hemolytic, coagulant, fibrinogenolytic and interaction with hyaluronidases, in order to investigate the safety of its use by the population and in formulations of new products.

MATERIALS AND METHODS

Preparation of the extract

P. grandifolia leaves were harvested from a garden, namely Horto de plantas Medicinais, at Universidade Federal de Lavras in January, 2014. The leaves were washed in tap water, kept in a 0.1% sodium hypochlorite solution for 1 h, washed in distilled water and dried in an oven for 48 h, at a temperature of 35°C. The dried leaves were grind in a Willey mill and the obtained flour was subjected to decoction in boiling water at a 1:25 (wv^{-1}) ratio. The extract was then centrifuged at 10,000 × g for 10 min (206 BL Fanem Baby®I), the supernatant was collected and the precipitate was subjected twice to the extraction process. The extracts were then lyophilized (FreeZone LABCONCO 4.5 L benchtop lyophilizer), weighed and dissolved in water for the assays. In this study, all reagents were of analytical grade.

Comet assay

Obtention of human blood and preparation of treatments

The experiment was conducted in accordance with the standards of the Ethics Committee on Human Research (COEP) from Universidade Federal de Lavras, and has been approved by this committee (Protocol 545/281). The peripheral blood of five volunteers aged between 21 and 40 years old was used, without disease symptoms, and they declared not to have used prescription drugs for a minimum of 30 days prior to collection, after free informed consent. The blood was collected in vacuum tubes containing the anticoagulant heparin and was immediately protected from light. The aqueous extract from *P. grandifolia* leaves was then added to 300 µl aliquots of blood diluted in 300 µl phosphate buffered saline (PBS) at doses of 10, 30, 60 and 100 µg. Negative controls received water in replacement of the treatments. The concentration 60 µg chosen for the genotoxicity evaluation of the aqueous extract from *P. grandifolia* leaves correspond to the equivalent dose recommended for human consumption of teas, infusions and decoctions, seeking benefits to health, considering the equivalency between the blood volumes of an adult individual and that used in the assay. The blood samples were incubated in an oven at 37°C, in the presence of the treatments for 4 h. The comet assay was then performed.

Obtention of nucleoids and electrophoretic run

The comet assay was performed according to the methodology described by Singh et al. (1988), with modifications. Therefore, an aliquot (15 µl) of each cell suspension containing the treatments was mixed with 100 µl of low melting point agarose (0.5% wv^{-1} in PBS), applied to a microscope slide previously coated with standard agarose solution (1% wv^{-1} in PBS), immediately overlaid with a coverslip and kept at ±4°C for 5 min. For each treatment/volunteer, 3 slides were prepared. The coverslips were then removed and the slides were immersed in lysis solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmol L^{-1} Tris, 1% Triton X-100, 10% dimethyl sulphoxide (DMSO); pH 10), where they remained for 24 h at 4°C, allowing the removal of anucleate cells and plasma molecules, as well as the lysis of leukocytes, in order to isolate only their nucleoids for analysis. After lysis, the slides were kept at ± 10°C for 25 min in a freshly prepared electrophoresis solution (1 mmol L^{-1} EDTA, 300 mmol L^{-1} NaOH; pH 13), in order to expose alkali-labile sites, and they were then subjected to electrophoresis, conducted at 25 V for 35 min. After electrophoresis, the slides were kept in a neutralization solution (0.4 mol L^{-1} Tris; pH 7.5) for 30 min, dried and fixed with 100% ethanol. All procedures were performed in the dark.

Staining and analysis

The slides were stained with 45 µl of propidium iodide solution (1 mg ml^{-1}), overlaid with a coverslip and analyzed in an epifluorescence microscope (Nikon ECLIPSE E400) at 200× and 400× magnifications. In order to measure damage levels in DNA

molecules of leukocytes, 100 nucleoids of each slide, 3 slides per treatment/individual (totaling 300 nucleoids per treatment/individual) were counted and classified by the same evaluator, using visual score patterns described by Singh et al. (1988). The cells were classified according to the size of the “tail” and the diameter of the “head” in Class 0: no damage (damage < 5%); Class 1: low damage level (5 to 20%); Class 2: intermediate damage level (20 to 40%); Class 3: high damage (40 to 85%); Class 4: totally damaged (damage > 85%). The average damage frequency was calculated from the sum of the percentages of damage 1, 2, 3 and 4.

Phospholipase activity

Phospholipase activity was evaluated using solid media as described by Gutiérrez et al. (1988), replacing agarose with agar. The gel was prepared with 0.01 mol L^{-1} $CaCl_2$; 1:3 vv^{-1} egg yolk in PBS; 0.005% sodium azide; and 1% bacteriological agar, at pH 7.2. After the gel solidified in petri dishes, the aqueous extract from *P. grandifolia* leaves was applied to ~ 0.5 cm gel holes at doses of 1, 5, 10, 25, 50, 100 and 400 µg, and phospholipase activity was evaluated after the plates were kept in a cell culture chamber at 37°C for 24 h. The formation of a clear halo around the orifice in the gel characterized phospholipase activity, which was measured according to the halo diameter.

Hemolysis of human erythrocytes

For the evaluation of hemolytic activity in solid medium, a gel similar to that cited in the methodology for phospholipase activity was prepared, with the replacement of egg yolk by erythrocytes in PBS solution. The blood was collected in vacuum tubes containing the anticoagulant sodium citrate, immediately centrifuged at 400 × g (206 BL Fanem Baby®I) for 10 min and subjected to 3 washes at the same PBS volume; the erythrocytes were then obtained at hematocrit of 100%. After solidification of the gel in a Petri dish, the aqueous extract from *P. grandifolia* leaves was applied to ~ 0.5 cm gel holes at doses of 1, 5, 10, 25, 50, 100 and 400 µg, and hemolytic activity was evaluated after the plates were kept in a cell culture chamber at 37°C for 24 h. The formation of a translucent halo around the gel hole characterized hemolytic activity, which was measured by the diameter of the halo.

Coagulant activity

The coagulant activity was evaluated according to the methodology described by Rodrigues et al. (2000) using citrated human plasma (200 µl), stabilized at 37°C. The aqueous extract from *P. grandifolia* leaves was added to plasma at doses of 1, 5, 10, 25, 50, 100 and 400 µg, with subsequent counting of clotting time, gentle agitation and constant observation until the formation of a solid clot. Incubated plasma and the extracts were observed for 120 min, allowing to confirm the absence of coagulant action.

Proteolytic activity on fibrinogen

In order to evaluate fibrinogenolytic activity, polyacrylamide gel electrophoresis was performed under reducing conditions, as described by Laemmli (1970). Different doses of the aqueous extract from *P. grandifolia* leaves (60, 100, 200 and 400 µg) were incubated with bovine fibrinogen (80 µg) for 60 min at 37°C. The samples were analyzed in a polyacrylamide gel prepared at 12% (wv^{-1}) under denaturing conditions, allowing the observation of α, β, and γ chains of the fibrinogen control, as well as the presence of fibrinopeptides in the samples in which venom-induced proteolysis occurred.

Interaction of the aqueous extract from *Pereskia grandifolia* leaves with hyaluronidases

For the evaluation of possible interactions between constituents of the extract from *P. grandifolia* and hyaluronidases, considering enzyme fragmentation or structure binding, the polyacrylamide gel electrophoresis method described by Leber and Balkwill (1997) was used. The hyaluronidases and the extract was incubated at the ratios 1:0.5, 1:1, 1:5, 1:10, 1:50 and 1:100 (hyaluronidase/extract; ww^{-1}) for 30 min at 37°C. Samples were analyzed in a 10% polyacrylamide gel in the absence of denaturing agents and without boiling to preserve the native structure of the enzymes. After the electrophoretic run, staining in Coomassie Blue was carried out for 30 min and destaining in a 10% acetic acid solution. The analysis of the results was performed visually, and migration profiles were reported.

Statistical analysis

The results of the comet assay were statistically evaluated by analysis of variance and the means were compared using the Scott Knott test (P < 0.05) with the aid of the R software (R Development Core Team, 2011).

RESULTS AND DISCUSSION

In the comet assay, in which fragmentation levels of DNA molecules obtained from human leukocytes were evaluated, there was no statistical difference between the nucleoids treated with extracts from *P. grandifolia* leaves and those untreated (negative control), for all damage classes (Table 1). It was possible to observe the predominance of undamaged nucleoids (class 0), with variation in average percentages from 50.97 to 58.63% for different doses of the extract and with a low damage level (class 1), the averages observed ranged between 37.60 and 41.10%. Nucleoids with intermediate damage levels (class 2) represented low average percentages, ranging between 1.84 and 3.70%, and for all doses evaluated, nucleoids with high fragmentation levels (class 3) or totally fragmented DNA (class 4) were absent (Table 1). The average damage frequency (Figure 1) demonstrates that, in general, there was no significant increase in the amount of damaged nucleoids with increasing doses of the extract, showing the lack of genotoxicity, considering the experimental conditions used, thus suggesting an absence, in the composition of the aqueous extract from *P. grandifolia* leaves, of molecules capable of inducing genotoxicity and possibly mutagenicity to leukocyte DNA.

Table 1. Average converted into percentage of nucleoid number per comet class after treatment with the aqueous extract from *Pereskia grandifolia* leaves.

Treatment	Comet class (%)[1]				
	0	1	2	3	4
C (-)[2]	61.00 ± 10.33[a]	37.67 ± 9.68[a]	1.94 ± 0.68[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
P. grandifolia					
10 µg	56.57 ± 10.14[a]	39.60 ± 9.24[a]	3.60 ± 1.19[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
30 µg	55.17 ± 10.47[a]	40.20 ± 10.96[a]	3.70 ± 1.52[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
60 µg	50.97 ± 10.88[a]	41.10 ± 7.95[a]	3.41 ± 1.51[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
100 µg	58.63 ± 14.23[a]	37.60 ± 14.56[a]	1.84 ± 0.63[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]

Data represent the average values obtained in 300 nucleoids/treatment/volunteer. 5 volunteers (1 volunteer/experiment) were used. Same letters in the columns do not differ by the Scott-Knott test (P <0.05). Classes[1]: (0 = damage<5%); (1 = 5 to 20%); (2 = 20 to 40%); (3 = 40 to 85%) and (4 = damage>85%). C (-)[2] = negative control prepared with the addition of water.

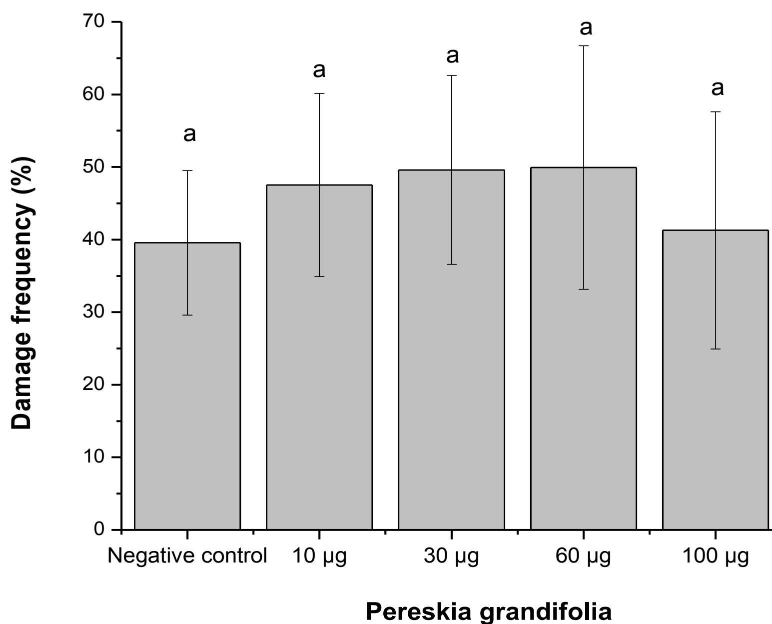

Figure 1. Damage frequency (DF) in the DNA of human leukocytes treated with different doses of the aqueous extract from *Pereskia grandifolia* leaves. DF: sum of damage from class 1 to 4. Same letters in the columns do not differ by the Scott-Knott test (P < 0.05).

Some researchers have used the comet assay to complement the toxicological characterization of plant extracts, thus investigating the genotoxic effects of secondary metabolites (for example, phenolic compounds, alkaloids and terpenes). Pereira et al. (2012) investigated the genotoxic potential of white bean flour, containing tannins and other phenolic compounds, on the genetic material of human leukocytes, and described the predominance of low fragmentation levels of DNA molecules in their results, under the evaluated conditions. Wan Ibrahim et al. (2010) evaluated 20 aqueous plant extracts and found that only two of them, *Vitex Pinnata* L. and *Quercus infectoria* Oliver, caused damage higher than 50% to the DNA of human leukocytes, and they are attributed to phenolic content, more specifically to tannic acid. Strange et al. (2009) analyzed natural products such as guaco (*Mikania glomerata* Spreng), espinheira-santa (*Maytenus ilicifolia* Mart. ex Reiss) and salvia (*Lippia alba* Mill. NE), and observed cytotoxic and genotoxic activity induced by metabolites such as coumarins, tannins and terpenes, suggesting the need for a toxicological characterization of plant metabolites, since

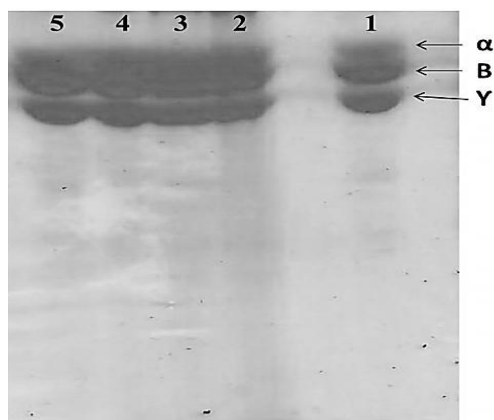

Figure 2. PAGE for visualization of fibrinogenolytic activity. Evaluation of the interaction between fibrinogen and the aqueous extract from *Pereskia grandifolia* leaves (AEPGL). Samples: 1- Control, Fibrinogen (80 µg); 2- Fibrinogen (80 µg) + AEPGL (60 µg); 3- Fibrinogen (80 µg) + AEPGL (100 µg ml^{-1}); 4- Fibrinogen (80 µg) + AEPGL (200 µg); 5- Fibrinogen (80 µg) + AEPGL (400 µg ml^{-1}).

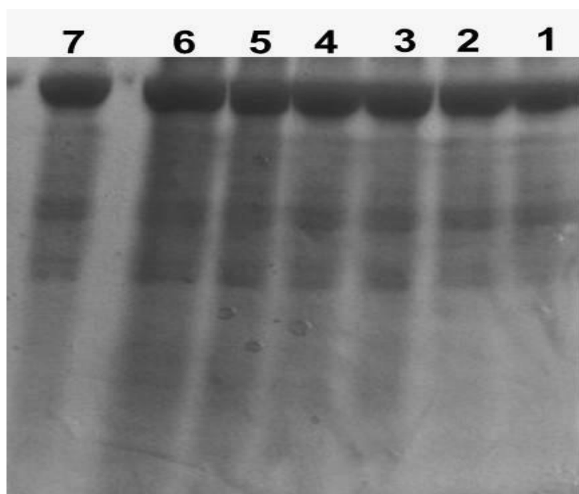

Figure 3. Effect of the aqueous extract from *Pereskia grandifolia* leaves on the structure of isolated hyaluronidases. Samples: 1- pure hyaluronidase (10 µg); 2- hyaluronidase + aqueous extract from *Pereskia grandifolia* leaves (AEPGL) (1:0.5, w/w), 5 µg extract; 3 hyaluronidase + AEPGL (1:1, w/w), 10 µg extract; 4- hyaluronidase + AEPGL (1:5, w/w), 50 µg extract; 5- hyaluronidase + AEPGL (1:10, w/w), 100 µg extract; 6- hyaluronidase + AEPGL (1:50, w/w), 500 µg extract; 7- hyaluronidase + AEPGL (1:100, w/w), 1000 µg extract.

they can cause adverse and/or therapeutic effects depending on factors such as dose and duration of use. Studies conducted with *P. grandifolia* leaves showed the presence of some metabolites with pharmacological properties such as saponins (Almeida et al., 2014), tannins, flavonoids (Turra et al., 2007) and alkaloids (Doetsch et al., 1980). However, some scientific reports have demonstrated that these compounds can be toxic to mammalian cells, even at low concentrations (Boeira et al., 2010; Osowski et al., 2010). In the present study, the conditions for the preparation of the extract, the evaluated doses and the parameters used in the assays resulted in the lack of genotoxicity, even considering the presence of compounds from secondary metabolism in the extract from *P. grandifolia* leaves. At the tested doses, the aqueous extract from *P. grandifolia* leaves did not induce lysis of phospholipids (results not shown); however, it was hemolytic at doses higher than 10 µg (results not shown), possibly due to the presence of interactions of extract constituents with different structures present in erythrocyte membranes, namely, proteins or steroids.

From the metabolites reported in the literature to be present in *P. grandifolia* leaves, saponins have hemolytic potential. The ability of saponins to interact with sterols, present in the plasma membrane of erythrocytes, increases the permeability of this membrane, allowing ions and water into the cells, resulting in their rupture and release of hemoglobin (Karabaliev and Kochev, 2003). Although surfactant and hemolytic properties are striking features of saponins, they are not common to all saponins and, therefore, it is not possible to conclude that they are responsible for the hemolysis observed in present study. Thus, additional studies should be conducted to verify the hemolytic potential of the aqueous extract from *P. grandifolia* leaves.

Regarding coagulant activity, none of the tested doses was able to induce coagulation, thus suggesting the absence of molecules capable of cleaving or interacting with components of the coagulation cascade (results not shown) in the composition of the evaluated aqueous extract. The aqueous extract from *P. grandifolia* leaves was not proteolytic on the fibrinogen molecule or with binding properties of this protein in the evaluated concentrations (60, 100, 200 and 400 µg), since the electrophoretic profile of α, β and γ chains resulting from reduced fibrinogen molecules remained unchanged after incubation in the presence of the extract for a period of 60 min (Figure 2). This result corroborates the lack of the coagulation effect of the extract on citrated human plasma, since one of the coagulation-inducing mechanisms is related to the cleavage of fibrinogen molecules for the formation of fibrin networks.

At the evaluated ratios and incubation conditions used, no change was observed in the migration profile of the enzyme hyaluronidase, commercially obtained, which characterizes binding of enzymes to constituents of the extract or hyaluronidase fragmentation (Figure 3). In the

samples containing higher doses of the extract, it is possible to observe darkened bands in the migration profile, which correspond to a higher concentration of non-protein compounds, probably secondary metabolites and/or carbohydrates present in the extract, but without any changes in the electrophoretic profile of hyaluronidase molecules.

Hyaluronidases are enzymes present in our organism responsible for the degradation of hyaluronic acid, which is part of the composition of interstitial spaces (Stern and Jedrzejas, 2006). These enzymes have been widely used by cosmetic (mainly to increase skin elasticity, allowing a better hydration) and pharmaceutical industries (to loosen the tissues, facilitating drug administration), besides being a valuable tool for research, since its inhibition results in changes in inflammatory response (Dunn et al., 2010; Menzel and Farr, 1998; Pirrelo et al., 2007).

Studies conducted with medicinal plants and compounds isolated from these plants show that they have potential inhibition of hyaluronidases. Flavonol kaempferol competitively inhibits this enzyme (Middleton et al., 2000). Polyphenols found in wine also inhibit hyaluronidase (Weisse, 1995). Machiah et al. (2006) observed a hyaluronidase inhibitor, purified from medicinal plants (*Withania somnifera*). Choi et al. (2006) also studied the inhibition of hyaluronidase using plants (*Schisandra*), and found that the extract of this plant was effective in the inhibition of hyaluronidase. These studies show that medicinal plants have compounds with properties to interact or inhibit hyaluronidases in their composition, highlighting the need for an investigation of the interaction of plant extracts and their metabolites with hyaluronidase. Therefore, the results obtained in the present study suggest that the constituents of aqueous extract from *P. grandifolia* leaves have neither bound, nor induced cleavage in hyaluronidase molecules, under the evaluated conditions, since have not influence the electrophoretic migration profile of hyaluronidase.

Conclusion

The aqueous extract from *P. grandifolia* leaves has no genotoxic, phospholipase, coagulant, fibrinogenolytic potential and interaction and/or cleavage on isolated hyaluronidases at the evaluated concentrations. However, it is premature to recommend the use of this plant regarding possible risks and benefits to human health, mainly due to the hemolytic activity observed, and additional studies of toxicity, efficacy and safety are necessary for defining doses, formulations and an appropriate usage for human consumption.

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Conflict of interest

The authors declare that they have no conflict of interest to disclose.

REFERENCES

Almeida MFE, Junqueira AMB, Simão AA, Corrêa AD (2014). Chemical characterization of the non-conventional vegetable known as ora-pro-nobis. Biosci. J. 30(1):431-439.

Boeira JM, Fenner R, Betti AH, Provensi G, Lacerda LA, Barbosa PR (2010). Toxicity and genotoxicity evaluation of *Passiflora alata* Curtis (Passifloraceae). J. Ethnopharmacol. 128(2):526-532.

Choi SI, Park SR, Heo TR (2006). Matrix Degradation Inhibitory Effect of Schisandra Fructus on Human Articular Cartilage and Chondrocytes. J. Ethnopharmacol. 106(2):279-284.

Doetsch PW, Cassady JM, Mclaughlin JL (1980). Cactus alkaloids. XL. Identification of mescaline and other β- phenethylamines in *Pereskia*, *Pereskiopsis* and *Islaya* by use of fluorescamine conjugates. J. Chromatogr. 189(1):79-85.

Dunn A, Heavner JE, Recz G, Day M (2010). Hyaluronidase: a review of approved formulations, indications and off-label use in chronic pain management. Expert Opin. Biol. Ther. 10(1):127-131.

Goh KL (2000). Malaysian herbaceous plants millennium. Malaysia: Advanco, 2000. 142 p.

Gutiérrez JM, Avila C, Rojas E, Cerdas L (1988). An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. Toxicon. 26(4):411-413.

Karabaliev M, Kochev V (2003). Interaction of solid supported thin lipid films with saponin. Sens. Actuators B, 88:101-105.

Laemmli UK (1970). Cleavage of structural proteins during the assembly to the head of bacteriophage T4. Nature. 227:680-685.

Leber TM, Balkwill FR (1997). Zymography: a single-step staining method for quantification of proteolytic activity on substrate gels. Anal. Biochem. 249:24-28.

Machiah DK, Girish KS, Gowda TV (2006). A glycoprotein from a Folk Medicinal Plant, *Withania somnifera*, Inhibits Hyaluronidase Activity of Snake Venoms. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 143(2):158-161.

Menzel EJ, Farr C (1998). Hyaluronidase and its substrate hyaluronan: biochemistry, biological activities and therapeutic uses. Cancer Lett. 131(1):3-11.

Middleton E, Kandaswami C, Theoharides C (2000). The effects of Plant Flavonoids on Mammalian Cells: Implications for Inflamamation, Heart Disease, and Cancer. Pharmacol. Exp. Ther. 52:673-751.

Osowski A, Pietrzak M, Wieczorek Z, Wieczorek J (2010). Natural compounds in the human diet and their ability to bind mutagens prevents DNA-mutagen intercalation. J.Toxicol. Environ. Health: Part A, 73(17-18):1141-1149.

Pereira LLS, Marcussi S, Sátiro LC, Pereira CA, Andrade LF, Davide LC, Santos CD (2012). Application of Comet assay to assess the effects of white bean meal on DNA of human lymphocytes. Braz. J. Pharm. Sci. 48 (1):103-108.

Pirrelo RD, Ting-Chen C, Thomas SH (2007). Initial experiences with subcutaneous recombinant human hyaluronidase. J. Palliat. Med. 10(4):861-864.

Rocha DRC, Pereira-Júnior GA, Vieira G, Pantoja L, Santos AS, Pinto NAVD (2008). Macarrão adicionado de ora-pro-nobis (*Pereskia aculeata* Miller). Alim. Nutr. 19(4):459-465.

Rodrigues V, Soares AM, Guerra-Sá R, Rodrigues V, Fontes MRM,

Giglio JR (2000). Structural and Functional Characterization of Neuwiedase, a Nonhemorrhagic Fibrin(ogen)olytic Metalloprotease from *Bothrops neuwiedi* Snake Venom. Arch. Biochem. Biophys. 381:213-224.

R Development Core Team. R: A language and environment for statistical computing (2011). Viena: R Foundation for Statistical Computing. http://www.R-project.org/.

Sim KS, Sri-Nurestri AM, Sinniah SK, Kim KH, Norhanom AW (2010). Acute oral toxicity of *Pereskia bleo* and *Pereskia grandifolia* in mice. Pharmacogn. Mag. 6(21):67-70.

Simões CMO, Schenkel EP, Gosman G, Mello JCP, Mentz LA, Petrovick PR (2004). Farmacognosia: da planta ao medicamento. 5. ed. Porto Alegre/Florianópolis: Editora da UFRGS, 2004.

Singh NP, Mccoy MT, Tice RR, Scheider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175(1):184-191.

Souza MRR (2009). O potencial do ora-pro-nobis na diversificação da produção agrícola familiar. Rev. Bras. Agroecol. 4(2):3550-3554.

Stern R, Jedrzejas MJ (2006). The Hyaluronidases: Their Genomics, Structures, and Mechanisms of Action. Chem. Rev. 106(3):818-839.

Strange VS, Gomes TDUH, Andrade MA, Batitucci MCP (2009). Avaliação do efeito mutagênico do extrato hidroalcoólico bruto, por meio de bioensaios *in vivo* e prospecção fitoquímica de *Cecropia glaziovii* Sneth (embaúba), Cecropiaceae. Rev. Bras. Farmacogn. 19(2):637-642.

Takeiti CY, Antonio GC, Motta EMP, Collares-Queiroz FP, Park KJ (2009). Nutritive evaluation of non-conventional leafy vegetable (*Pereskia aculeata* Miller). Int. J. Food Sci. Nutr. 60(1):148-160.

Turra AF, Marçal FJB, Baretta IP, Takemura OS, Laverde JRA (2007). Avaliação das propriedades antioxidantes e susceptibilidade antimicrobiana de *Pereskia grandifolia* Haworth (Cactaceae). Arq. Cienc. Saúde Unipar. 11(1):9-14.

Wan-Ibrahim WI, Sidik K, Kuppusamy UR (2010). A high antioxidant level in edible plants is associated with genotoxic properties. Food Chem. 122(4):1139-1144.

Weisse ME (1995). Wine as a digestive aid: comparative antimicrobial effects of bismuth salicylate and red and white wine. Br. Med. J. 311: 1657-1660.

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Full Length Research Paper

Assessment of the genotoxic and antigenotoxic potential of crude extracts and fractions of *Schwartzia brasiliensis* (Choisy) Bedell ex Giraldo-Cañas

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Schwartzia brasiliensis **(Marcgraviaceae) is a native Brazilian shrub species with neotropical distribution in shoal environments. Recent studies have revealed its medicinal potential for some human diseases; therefore, this study aimed to evaluate the genotoxic and antigenotoxic potential of extracts and fractions obtained from different organs of *S. brasiliensis*. To accomplish this, plant extracts at different concentrations (25, 125 and 250 µg/ml) were incubated with plasmid DNA, either alone, or in the presence of stannous chloride as a positive control. Samples were then examined to detect any plasmid strand breaks or the absence of such breaks, which would indicate protection of these molecules against stannous chloride-induced lesions. Methanol and aqueous extracts of leaves and stems showed the ability to withstand the effects of stannous chloride in that no DNA damage was observed. Moreover, no other extracts or fractions used in the experimental conditions assayed resulted in DNA damage. These findings suggest that *S. brasiliensis* has antigenotoxic properties, indicating, in turn, that its biological activities deserve further study given the medicinal relevance of this plant.**

Key words: Medicinal plant, Marcgraviaceae, stannous chloride, flavonoids.

INTRODUCTION

Medicinal plants and their derivatives have been used as important sources of biologically active substances (Newman and Craag, 2012) that are known to promote health, treat illness, and cure, or prevent many diseases

(Efferth and Grefen, 2014; Gurib-Fakim, 2006; Jachak and Saklani, 2007). Such phytomedicines are usually alternative or complementary options to treatments with synthetic drugs (Dragan et al., 2015; Olasehinde et al., 2014). However, even though considered therapeutic, some bioactive plant derivatives may have toxic properties that will cause damage to the human organism, including the induction of genetic damage (Bednarczuk et al., 2010; Düsman et al., 2012; Efferth and Grefen, 2012).

Schwartzia brasiliensis belongs to the Marcgraviaceae family, and it is found in shoal environments of the Brazilian Atlantic Forest. This is a shrub species whose flowering takes place in summer (Ferreira, 1995; Zamith and Scarano, 2004) and whose conspicuous inflorescences might be used for ornamental purposes. *S. brasiliensis* also presents nectaries to attract bird pollinators (Rocca et al., 2006; Rocca and Sazima, 2008). Initially, the species was included in the *Norantea* genus (Giraldo-Cañas, 2004), but it now belongs to the *Schwartzia* genus. Pharmacological studies with some species of the Marcgraviaceae family indicate antifungal (Jones et al., 2000) and anxiolytic activities (Mullally et al., 2011). Studies investigating the medicinal potential of *S. brasiliensis* also reported antibacterial activities (Mello et al., 2014), and Agripino et al. (2004) showed that ethanol extracts of stems of *S. brasiliensis* could protect against DNA damage. In folk medicine, *S. brasiliensis* tea is used to prevent heart disease (Agra, 2008), but no scientific studies have shown either efficacy or safety. In this context, the present work aims to evaluate the genotoxic and antigenotoxic potential of crude extract and different fractions of *S. brasiliensis* grown in a natural environment.

MATERIALS AND METHODS

Plant

The plants were collected in the morning, around 10:00 a.m., in March, 2012, on a preserved sandbank area, located in Barra da Tijuca, Rio de Janeiro City, 22° 59' 29.7" S to 43° 20' 48.4" W, Rio de Janeiro State, Brazil. The material was collected in vegetative stage under license from SISBIO/IBAMA number 3299651. A voucher (HRJ 11749) has been deposited in the Herbário da Universidade do Estado do Rio de Janeiro (HRJ).

Preparation and fractionation of the extracts

Leaf, stem and root samples of *S. brasiliensis* were fragmented, dried at 40°C for 24 h, and added to methanol (MeOH) for 15 days at environmental conditions. The extracts obtained were filtered using Whatman paper n°.1, evaporated at 40°C and concentrated in vacuum. Then, approximately 3 g of each crude extract were subjected to fractionation with different polarities of chemical solvents (n-hexane, ethyl acetate and distilled water). The fractions were evaporated at 40°C and concentrated in vacuum. Immediately before the use, both the crude methanol extracts and the fractions were solubilized and diluted in ultrapure water until reaching the concentrations required to carry out the experiments.

Evaluation of the genotoxic and antigenotoxic potential through agarose gel electrophoresis assay with plasmid DNA

Genotoxic and antigenotoxic potentials of *S. brasiliensis* were evaluated according to Caldeira-de-Araujo et al. (1996). This assay is based on the ability of the reducing agent stannous chloride ($SnCl_2$) to induce DNA strand breaks. During agarose gel electrophoresis, $SnCl_2$-induced lesions can cause plasmid DNA conformational changes, leading to modification in migration pattern. Gel electrophoresis was performed in order to separate different conformations of plasmid DNA: native conformation (supercoiled or form I); open circle (or form II), resulting from single-strand DNA breaks; and linear (or form III) generated through double-strand breaks.

The plasmid DNA used in this investigation was pUC 9.1, as maintained in DH5αF'IQ *E. coli* cells. The plasmidial molecules were purified from DH5αF'IQ *E. coli* cultures, in stationary growth phase, according to the Invisorb® Spin Plasmid Mini Two (Invitek) protocol. In order to evaluate genotoxic and antigenotoxic potential, three different concentrations (25, 125 and 250 µg/ml) of each extract and fraction were incubated with 200 ng of plasmid DNA in the presence, or not, of $SnCl_2$ (200 µg/ml). All dilutions were done in ultrapure water (Milli-Q system, EMD Millipore, Billerica, MA, USA, and the reaction mixtures were incubated for 40 min at room temperature. Then, aliquots of each sample (10 µl) were mixed with 2 µl of loading buffer (0.25% xylene cyanol; 0.25% bromophenol blue; 30% glycerol in water), applied on agarose gel (0.8%) in TAE 1X buffer and submitted to electrophoresis at 7 V/cm for 30 min. Afterwards, the gel was stained with ethidium bromide (0.5 mg/ml), and the DNA bands were visualized by fluorescence in an ultraviolet transilluminator system (UVP, LLC, Upland, CA, USA). Each experiment was repeated three times, and the best result was selected for presentation. The gel bands were then digitized, and the results obtained provide a qualitative analysis. A quantitative evaluation in Plasmid DNA conformational structure was also performed by using NIH ImageJ software.

Statistical analysis of DNA strand breaks

The data presented in Figure 1 were analyzed through densitometer scanning, and percentage of form I was used to obtain the average number of breaks per pUC 9.1 plasmid DNA molecule. According to Remington and Schor (1985), the Poisson distribution could be used to obtain the average number of breaks from the percentage of DNA supercoiled forms, as $\mu = -\ln p(0; \mu)$, Considering no breaks = $p(0; \mu)$.

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Analysis by high-performance liquid chromatography with diode-array detection (HPLC/DAD)

The analysis by HPLC/DAD was performed under the following conditions: Mode: isocratic ACN/aqueous 0.05% TFA (30:70) for 55 min and flow 0.5 ml/min in Shim-pack XR-ODS column (100 mm × 2.0 mm, 2.2 µm, Shimadzu). All injections were performed with a volume of 20 µl loop. Detection was performed in the ultraviolet wavelengths of 254 and 325 nm.

RESULTS AND DISCUSSION

In the present study, electrophoresis assay with plasmid DNA was used in order to evaluate both genotoxic and antigenotoxic properties of *S. brasiliensis*. Based on its genotoxic oxidative properties, which result in DNA strand breaks, stannous chloride ($SnCl_2$) was used as a positive control in this study. Thus, the incubation of *S. brasiliensis* extracts or fractions with plasmid DNA alone, or in combination with $SnCl_2$, could provide the basis for determining genotoxic *vs*. antigenotoxic activity. In particular, alterations in plasmid DNA pattern migration through agarose gel in samples incubated with plant extracts or fractions could reflect the potential to promote DNA strand breaks. Conversely, the ability of samples to prevent damage to plasmid DNA caused by $SnCl_2$ could also reflect the antigenotoxic potential of *S. brasiliensis*. This gel electrophoresis assay is fully described in De Mattos et al. (2004), and the assay has been applied elsewhere to other medicinal species (Simões et al., 2006; Biso et al., 2010; Hamedt et al., 2014). Moreover, since $SnCl_2$ can induce reactive oxygen species, especially hydroxyl radicals (Caldeira-de-Araujo et al., 1996), the antioxidant properties of *S. brasiliensis* extracts or fractions could prevent DNA damage. However, a full assessment of this property is outside the scope of this paper.

Methanol extract of leaves was able to fully protect DNA against stannous chloride-induced damage, but only when tested at the concentration of 250 µg/ml (Figure 1A), as indicated in the control sample (form 1, lane 1) at 88.6% *vs*. 10.3% in lane 2 (pUC 9.1 plus $SnCl_2$), and 85.4% when methanol extract of leaves was added (Figure 1A, lane 8).

Aqueous fraction of leaves also seems to possess an antigenotoxic effect because it was also able to reduce the level of DNA breaks induced by stannous chloride (Figure 1B; lanes 6, 7 and 8). Lane 7 presents 28.23% of plasmid molecules in form I (control native conformation), whereas lane 2 in the same figure shows only 18.78%, supporting the antigenotoxic action of *S. brasiliensis* leaves in aqueous fraction. Both sets of results are based on assessment of the average number of breaks per genome (Figure 2A and B). The chromatograms obtained by High-performance liquid chromatography with photodiode array detection (HPLC/DAD) revealed that methanol extracts have the majority of signals with retention time values equal to 8.659 and 9.452 min (Figure 3A). Aqueous fraction showed a majority of signals with retention time equal to 8.710 and 9.570 (Figure 3B), being related to the chemical class of flavonoids. Phytochemical analysis of leaves of *S. brasiliensis*, according to Barbosa et al. (2004) showed the presence of phenolic compounds, mainly flavonoids (Mello et al., 2012).

The antigenotoxic effect of *S. brasiliensis* leaves may be related to their chemical constituents. Flavonoids are described as having antioxidant (Brunetti et al., 2013; Procházková et al., 2011; Romano et al., 2013) and antigenotoxic activities (Boubaker et al., 2013; Chaabane et al., 2012). In addition, flavonoids have antineoplastic effects and can also protect against cardiovascular and neurodegenerative diseases (Obrenovich et al., 2010; Obrenovich et al., 2011; Simões et al., 2006).

The methanol extract and aqueous fraction of stems of *S. brasiliensis* also revealed antigenotoxic potential (Figure 1E and F, lane 8), as confirmed by evaluation of the average number of breaks per genome (Figure 2E and F, lane 8). These data corroborate a previous study showing that the ethanol extract of stems is able to reduce DNA damage in *Saccharomyces cerevisiae* (Agripino et al., 2004).

In the present report, it was observed that the methanol extract and aqueous fraction of stems decreased DNA damage caused by $SnCl_2$, suggesting a dose-dependent effect (Figure 1E and F). This protective effect has already been observed elsewhere, using the same experimental approach, with extracts of *Cleome rosea* (Simões et al., 2006), *Daphne gnidium* (Chaabane et al., 2012) and *Nitraria retusa* (Boubaker et al., 2013). The chromatograms obtained from the methanol extract and aqueous fraction of stems, using the method proposed, were insufficient to determine their constituents accurately.

In the evaluation of genotoxic potential, the obtained data from methanol extract and aqueous fraction of stems show no DNA damage (Figure 1; lanes 3, 4 and 5), exhibiting instead, antigenotoxic properties. Although antigenotoxic effect was found in stem extracts, analyses of leaf extracts showed more promising results, justifying the choice of this organ for further phytochemical analysis. On the other hand, no antigenotoxic effect was shown for root extracts. This experimental approach has been used elsewhere in order to evaluate a genotoxic or antigenotoxic profile induced by different plant species (Biso et al., 2010; Ferreira-Machado et al., 2004; Hamedt et al., 2014). Therefore, the use of other study designs, both *in vivo* and *in vitro*, is necessary to ensure the safety and efficacy of *S. brasiliensis* as a medicinal plant.

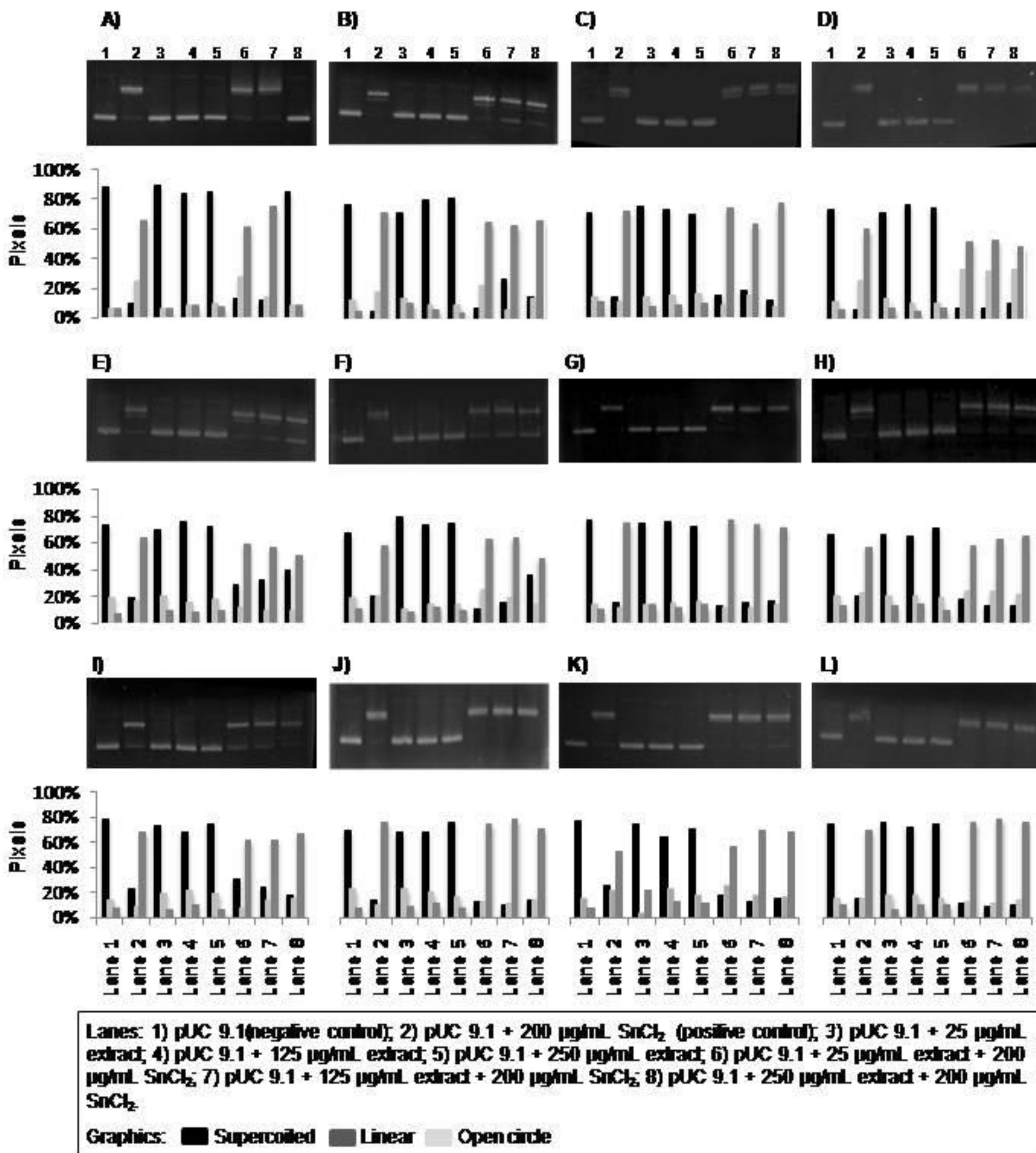

Figure 1. Qualitative and quantitative analysis of the genotoxic and antigenotoxic potential of *S. brasiliensis* extracts and fractions on plasmid pUC 9.1 DNA bands corresponding to aliquots of the plasmid solution (200 ng) treated with extracts and fractions (25, 125 and 250 µg/ml). A) methanol leaf extract; B) aqueous leaf fraction; C) n-hexane leaf fraction; D) ethyl acetate leaf fraction; E) methanol stem extract; F) aqueous stem fraction; G) n-hexane stem fraction; H) ethyl acetate stem fraction; I) methanol stem extract; J) aqueous root fraction; K) n-hexane root fraction; L) ethyl acetate root fraction. Bars represents DNA densitometric measures (%) by image J

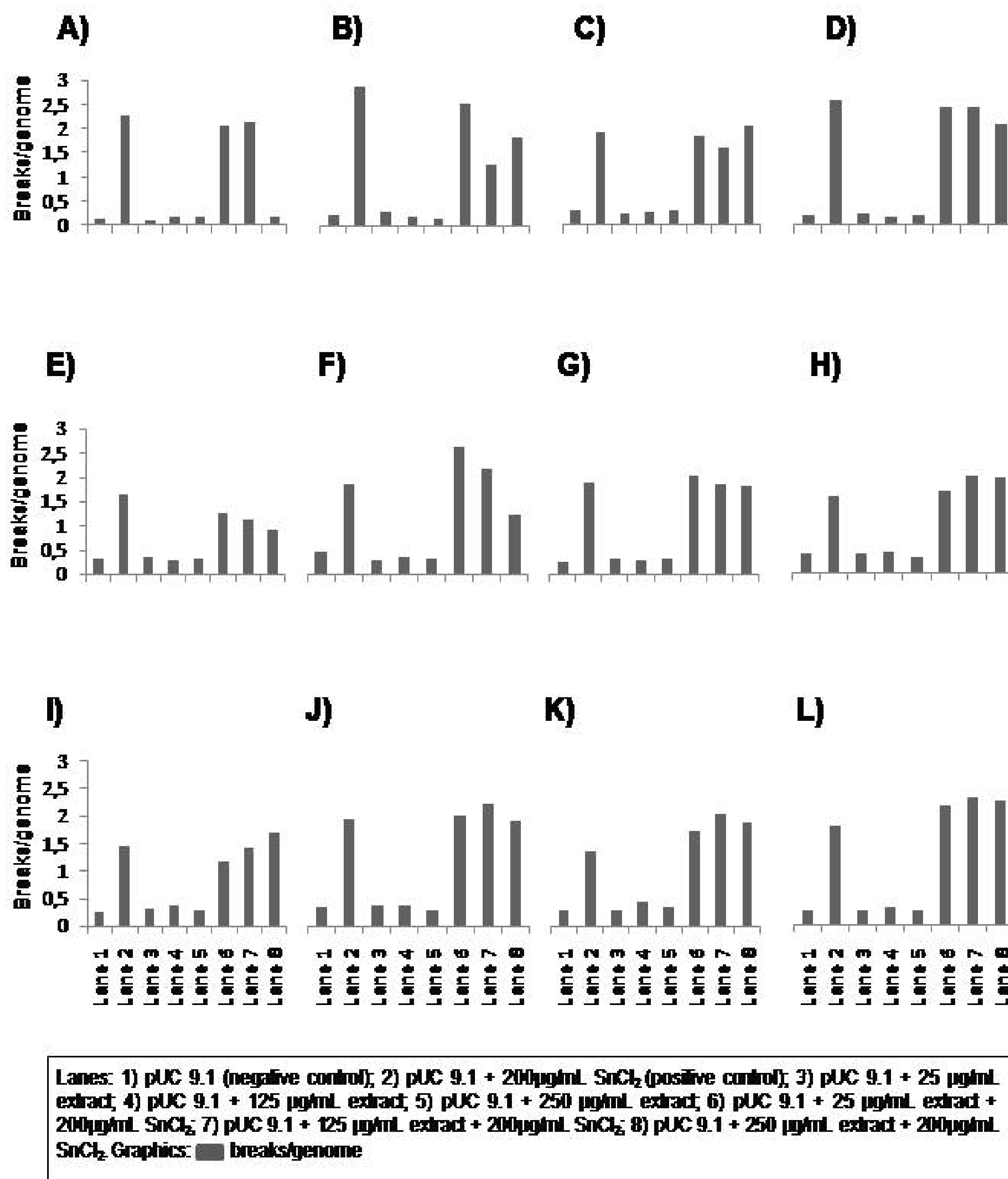

Figure 2. Number of single strand breaks/genome in plasmid pUC 9.1 DNA treated with *S. brasiliensis* extracts and fraction. Lanes corresponding to aliquots of the plasmid solution (200 ng) treated with the extracts and fractions (25, 125 and 250 µg/ml). A) methanol leaf extract; B) aqueous leaf fraction; C) n-hexane leaf fraction; D) ethyl acetate leaf fraction; E) methanol stem extract; F) aqueous stem fraction; G) n-hexane stem fraction; H) ethyl acetate stem fraction; I) methanol stem extract; J) aqueous root fraction; K)n-hexane root fraction; L) ethyl acetate root fraction. Bars represents the number of single strand breaks/genome of three independent experiments.

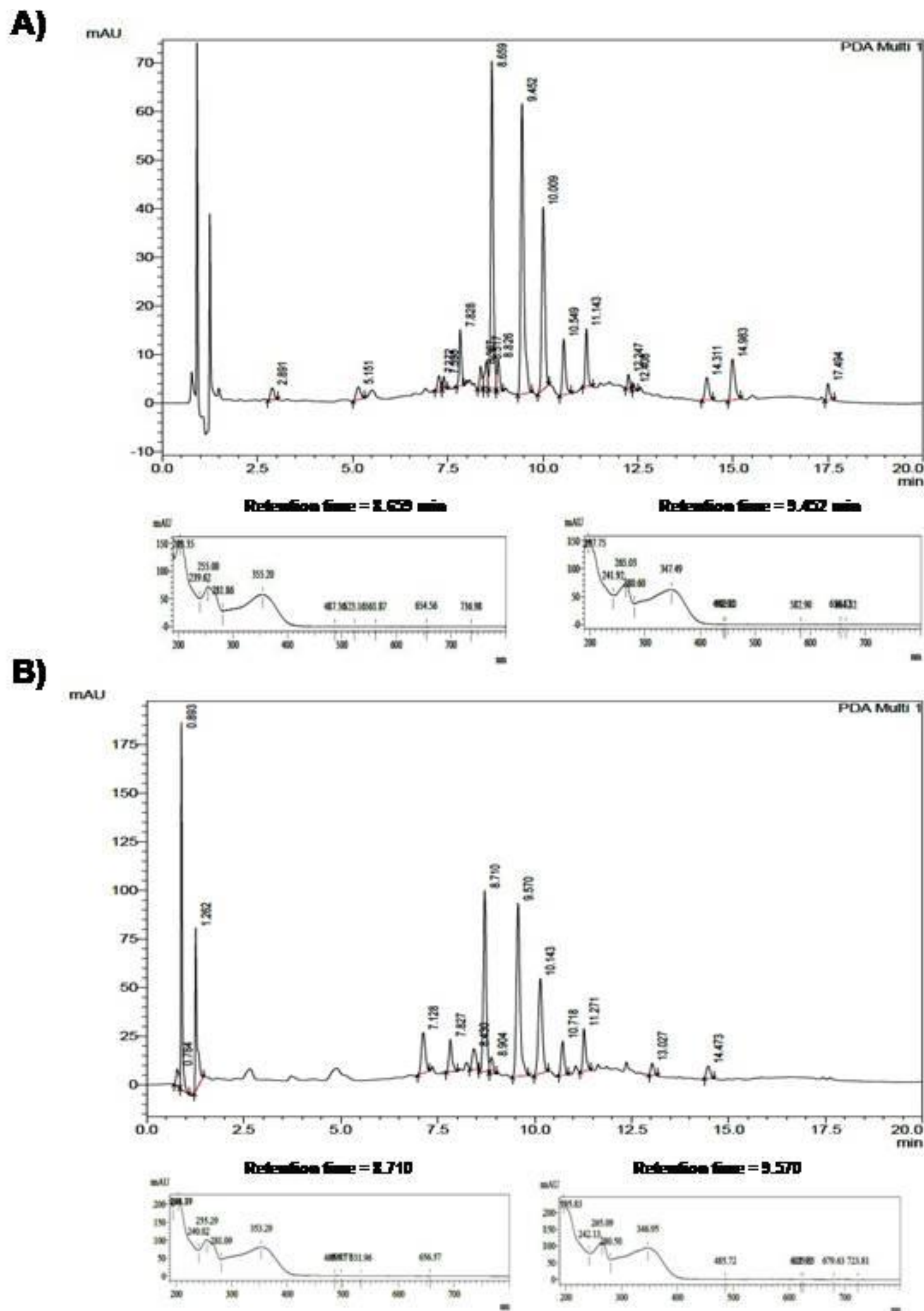

Figure 3. HPLC/DAD analysis of major compounds of methanol extract and aqueous fractions of *S. brasiliensis* in 254 nm and their absorption spectra in the ultraviolet region. A) methanol leaf extract; B) aqueous leaf fraction.

In conclusion, this work constitutes the first report on the genotoxic potential and antigenotoxic properties of *S. brasiliensis*. Results showed no genotoxic effect on DNA plasmid pUC 9.1. Moreover, it was shown that methanol and aqueous fractions of leaves and stems, at the highest concentration tested, have antigenotoxic activity protecting DNA from the breakdown caused by the stannous chloride. Considering the phytochemical profile obtained, we suggest that flavonoid content may be responsible for the activities evaluated.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

REFERENCES

Agra MF (2008). Survey of medicinal plants used in the region Northeast of Brazil. J. Pharmacol. 18:472-508.

Agripino DG, Lima MEL, Silva MR, Meda CI, Bolzani VS, Cordeiro I, Young MCM, Moreno PRH (2004). Screening of Brazilian plants for antimicrobial and DNA-damaging activities. I. Atlantic rain forest. Ecological Station Juréia-Itatins. Biota. Neotrop. 4:1-15.

Barbosa WLR, Quignard E, Tavares ICC, Pinto LN, Oliveira FQ, Oliveira RM. (2004). Manual para Análise Fitoquímica e Cromatográfica de Extratos Vegetais. Rev. Cien. UFPA. 4.

Bednarczuk VO, Verdam MCS, Miguel MD, Miguel OG (2010). Tests *in vitro* and *in vivo* used in the toxicological screening of natural products. Vis. Acad. 11:43-50.

Biso FI, Rodrigues CM, Rinaldo D, Reis MB, Bernardi CC, De Mattos JCP, Caldeira-de-Araujo A (2010). Assessment of DNA damage induced by extracts fractions and isolated compounds of *Davilla nitida* and *Davilla elliptica* (Dilleniaceae). Mutat. Res. 702:92-99.

Boubaker J, Ghedira Z, Ghedira K, Chekir-Ghedira L (2013). Antigenotoxic and antioxidant activity in human chronic myelogenous leukaemia cell line K562 enhanced by *Nitraria retusa* leaf extracts. Cell Biol. Int. Rep. 20:5-12.

Brunetti C, Ferdinando MD, Fini A, Pollastri S, Tattini M (2013). Flavonoids as Antioxidants and Developmental Regulators: Relative Significance in Plants and Humans. Int. J. Mol. Sci. 14:3540-3555.

Caldeira-de-Araujo A, Dantas FJS, Moraes MO, Felzenszwalb I, Bernardo-filho M (1996). Stannous chloride participates in the generation of reactive oxygen species. J. Braz. Assoc. Adv. Sci. 48:109-113.

Chaabane F, Boubaker J, Loussaif A, Neffati A, Kilani-Jaziri S, Ghedira K, Chekir-Ghedira L (2012). Antioxidant, genotoxic and antigenotoxic activities of *Daphne gnidium* leaf extracts. Complement. Altern. Med. 12:1-10.

De Mattos JCP, Dantas FJS, Caldeira-de-Araújo A, Moraes MO (2004). Agarose gel electrophoresis system in the classroom: detection of DNA strand breaks through the alteration of plasmid topology. Biochem. Mol. Biol. Educ. 32:254-257.

Dragan S, Andrica F, Serban, MC, Timar R (2015). Polyphenols-Rich Natural Products for Treatment of Diabetes. Curr. Med. Chem. 22:14-22.

Düsman E, Berti AP, Soares LC, Vicentini VEP (2012). The main mutagens and carcinogens agents of human exposure. Rev. Saúd. Biol. 7:66-81.

Efferth T, Greten HJ (2012). Quality Control for Medicinal Plants. Med. Aromat. Plants. 1:e131.

Efferth T, Greten HJ (2014). Traditional Medicine with Plants – Present and Past. Med. Aromat. Plants. 3:e151.

Ferreira G (1995). Estudo taxonômico das espécies brasileiras do gênero Norantea Aublet (Marcgraviaceae). Arq. Jard. Bot. 33:9-53.

Ferreira-Machado SC, Rodrigues MP, Nunes APM, Dantas FJS, De Mattos JCP, Silva CR (2004). Genotoxic potentiality of aqueous extract prepared from *Chrysobalanus icaco* L. leaves. Toxicol. Lett. 151:481-487.

Giraldo-Cañas D (2004). Las especies del género Schwartzia (complejo *Norantea*, Marcgraviaceae) en Brasil. Darwiniana. 42:169-175.

Gurib-Fakim A (2006). Medicinal plants: Traditions of yesterday and drug of tomorrow. Mol. Aspects Med. 27:1-93.

Hamedt AL, Ortiz IC, Garcia-Huertasa PA, Sáenzc J, Caldeira-De-Araujo A, De Mattos JCP, Rodríguez-Gasqueze MA, Triana-Chávez O (2014). Cytotoxic, mutagenic and genotoxic evaluation of crude extracts andfractions from *Piper jericoense* with trypanocidal. Acta Trop. 131:92-97.

Jachak SM, Saklani A (2007). Challenges and opportunities in drug discovery from plants. Curr. Sci. 92:1251-1257.

Jones NP, Arnason JT, Aboud-zaid M, Akpagana K, Sanchez-Vinda P, Smith ML (2000). Antifungal activity of extracts from medicinal plants used by first nations peoples of Eastern Canada. J. Ethnopharmacol. 73:191-198.

Mello GS, Ignacio ACPR, Albarello N (2014). Potencial antibacteriano de *Schwartzia brasiliensis*. Novas Edições Acadêmicas. OmniScriptum GmbH & Co. Saarbrüken, Deutschland. 52 p.

Mello GS, Moraes BA, Sá AF, Vilardo AF, Rocha AS, Cordeiro LS, Simões-Gurgel C, Albarello N (2012). Estudos fitoquímicos e de toxicidade de *Cleome rosea* e *Norantea brasiliensis* cultivadas *in vivo* e *in vitro*. Anais do II Simpósio Flora das Restingas Fluminenses; fitoquímica e bioatividade.

Mullally M, Kramp K, Cayer C, Saleem A, Ahmed F, Mcrae C, Baker J, Goulah A, Otorola M, Sanchez P, Garcia M, Poveda L, Merali Z, Durst T, Trudeau VL, Arnason JT (2011). Anxiolytic activity of a supercritical carbon dioxide extract of *Souroubea sympetala* (Marcgraviaceae). Phytother. Res. 25:264-270.

Newman DJ, Cragg GM (2012). Natural Products as Sources of new drugs over the 30 Years from 1981 to 2010. J. Nat. Prod. 75:311-335.

Obrenovich ME, Nair NG, Beyaz A, Aliev G, Reddy P (2010). The role of polyphenolic antioxidants in health, disease and aging. Rejuvenation Res. 13:1-13.

Obrenovich ME, Li Y, Parvathaneni K, Yendluri BB, Palacios HH, Leszek J, Aliev G (2011). Antioxidants in health, disease and aging. CNS Neurol. Disord. Drug Targets 10:192-207.

Olasehinde GI, Ojurongbe O, Adeyeba AO, Fagade OE, Valecha N, Ayanda, IO, Ajayi AA, Egwari LO (2014). *In vitro* studies on the sensitivity pattern of *Plasmodium falciparum* to anti-malarial drugs and local herbal extracts. Malaria J. 13:1-7.

Procházková D, Boušová I, Wilhelmová N (2011). Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82:513-523.

Remington RD, Schor MA (1985). Some important distributions. In: Johnson RA, Wichem DW (Ed.), Statistics with Applications to the Biological and Health Sciences. Prentice-Hall, Inc., Englewood Cliffs: New Jersey. 415p.

Rocca MA, Sazima M, Sazima I (2006). Woody woodpecker enjoys soft drinks: the blond-crested woodpecker seeks nectar and pollinates canopy plants in South-Eastern Brazil. Biota Neotrop. 6:1-9.

Rocca MA, Sazima M (2008). *Ornithophilous canopy* species in the Atlantic rain Forest of southeastern Brazil. J. Field Ornithol. 79:130-137.
Romano B, Pagano E, Montanaro V, Fortunato AL, Milic N, Borelli F (2013). Novel Insights into the Pharmacology of Flavonoids. Phytother. Res. 27:1588-1596.
Simões C, De Mattos JCP, Sabino KCC, Caldeira-De-Araújo A, Coelho MGP, Albarello N, Figueiredo SFL (2006). Medicinal potential from *in vivo* and acclimatized plants of *Cleome rosea*. Fitoterapia. 77:94-99.
Zamith LR, Scarano FR (2004). Seedlings of species of Restingas the city of Rio de Janeiro, RJ, Brazil. Acta Bot. Bras. 18:161-176.

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