Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Cytotoxicity, Phagocytic Activity, and Leishmanicidal Potential of Extract Standardized in Geranylgeraniol Obtained from the Fruit of *Pterodon emarginatus* Vogel

Suzana Ferreira Alves[1,2], Clayson Moura Gomes[2,3], Matheus Gabriel de Oliveira[1], Wanessa Machado de Andrade[2,4], Larissa Cleres Moreira[4], Leonardo Luiz Borges[2,5], Cláudio Carlos da Silva[6], Graciella Ottoni de Souza[6], Vinicius Barreto da Silva[2], Marize Campos Valadares[4], Maria Teresa Freitas Bara[1]

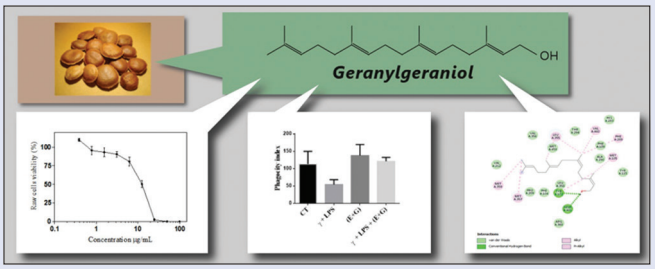
[1]Faculty of Pharmacy, Natural Products Research Laboratory, Federal University of Goiás, [2]Pharmacy Course, School of Medical Sciences, Pharmaceuticals and Biomedical Sciences, Pontifical Catholic University of Goiás, [3]Institute of Tropical Pathology and Public Health, [4]Faculty of Pharmacy Laboratory of Pharmacology and Cell Toxicology – Farmatec, Federal University of Goiás, [5]Pharmacy Course, University Unit of Exact and Technological Sciences, State University of Goiás, Anápolis, Go, [6]Replicon Research Center, Department of Biology, Pontifical Catholic University of Goiás, Goiânia, Brazil

Submitted: 29-05-2019 Revised: 27-06-2019 Published: 11-02-2020

ABSTRACT

Aim: The propouse of this study was to available the cytotoxicity, phagocytic activity, and leishmanicidal potential of extract standardized from the fruits of *Pterodon emarginatus* Vogel. **Background:** *P. emarginatus* is a Brazilian medicinal plant with several constituents shown to have anti-inflammatory, antinociceptive, antimicrobial, and antiproliferative activities. **Materials and Methods:** The standardized extract in geranylgeraniol obtained from the fruit of *P. emarginatus* was investigated for clastogenic and cytotoxic effects, as well as phagocytic activity against *Leishmania guyanensis*. **Results:** A 24-h exposure of *Allium cepa* roots to standardized geranylgeraniol extract (9.77 mg/mL) promoted clastogenic effects, with a mitotic index (MI) of 10.33 ± 0.61. Extending the exposure time to 48 or 72 h significantly reduced the MI ($P < 0.05$). The IC_{50} for geranylgeraniol in 3T3 and RAW 264.7 cells was found to be 15.5 µg/mL and 12.2 µg/mL, respectively. The standardized extract in geranylgeraniol exhibited high phagocytic and microbicidal activities in *Leishmania,* promoting a >60% increase in macrophage infection, with greater internalization and destruction of the parasite. *In silico* study results suggest that geranylgeraniol acts in *Leishmania* by interfering with the biosynthesis of sterols through steric hindrance of the active site of the enzyme CYP51. **Conclusion:** The standardized extract in geranylgeraniol may be useful as an antiproliferative agent, macrophage immunomodulator, and as a cytotoxic treatment against infectious agents.

Key words: Biological safety, clastogenic effects, docking, geranylgeraniol, phagocytic activity, potential leishmanicide



Correspondence:
Dr. Suzana Ferreira Alves,
Pharmacy Course, School of Medical Sciences, Pharmaceuticals and Biomedical Sciences, Pontifical Catholic University of Goiás, Brazil.
E-mail: suzanafal@gmail.com
DOI: 10.4103/pm.pm_231_19

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Pterodon emarginatus Vogel (family Fabaceae), popularly known as "sucupira-branca" or "faveiro," is found in the Cerrado region of Brazil. Having proven anti-inflammatory and analgesic properties,[1-6] its oil resin and fruit extract are used in folk medicine for the treatment of rheumatism, sore throats, and respiratory disorders (bronchitis and tonsillitis). Studies have ascribed cercaricide, antimicrobial and anti-inflammatory,[7-9] analgesic,[10] antinociceptive,[8,11-13] and anticancer properties[14-16] to the oil and fruit extracts of *P. emarginatus*.

The compounds involved in the antinociceptive and anticancer activities were isolated from *Pterodon* genus and identified as geranylgeraniol, 6α-acetoxy, 7α-hydroxylvouacapan, 6α,7α-di-hydroxy-vouacapan-1 7α-oate methyl ester, and its isomers 6α-hydroxy-7α-acetoxy-vouac apan-17α-oate methyl ester, and 6α-acetoxy-7α-hydroxy-vouacapan -17α-oate methyl ester.[11,17] Geranylgeraniol (3,7,11,15-tetramethyl-2,6,10,14-hexadecatraen-ol) [Figure 1] is an acyclic diterpene that has shown antinociceptive activity in capsaicin and glutamate animal models, and the likely pharmacological mechanisms involved have been described.[18,19] In addition, geranylgeraniol inhibits the proliferation of the amastigote form of the parasite *Trypanosoma cruzi* at concentrations that did not affect cell viability in the mammalian host.[20]

In vitro studies of the essential oil extracted from the fruit of *P. emarginatus* have investigated its cytotoxic and antiproliferative activities, as well as its activity against the protozoan *Leishmania*.[21,22] However, these studies were not performed using standardized extracts of geranylgeraniol.

Cite this article as: Alves SF, Gomes CM, de Oliveira MG, de Andrade WM, Moreira LC, Borges LL, *et al*. Cytotoxicity, phagocytic activity, and leishmanicidal potential of extract standardized in geranylgeraniol obtained from the fruit of *Pterodon emarginatus* Vogel. Phcog Mag 2020;16:140-7.

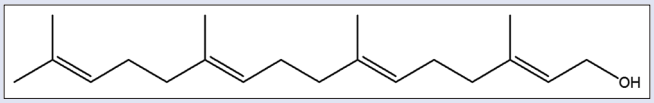


Figure 1: Chemical structure of geranylgeraniol (acyclic diterpene)

Proper evaluation of the toxic properties of plant extracts is important for safety considerations since exposure to complex chemicals can have adverse effects in humans.[23] Thus, toxicity studies of the diverse bioactivity of compounds found in the *Pterodon* genus are needed.

In a study of the potential clastogenic effects of oil extracted from the seeds of *Pterodon pubescens*, Dias *et al.*[24] reported no chromosomal abnormalities. Sabino *et al.*[25] also observed no mutagenic or toxic effects of this seed oil in the cells of humans or other animals. Similar toxicity studies of the standardized geranylgeraniol extracts of *P. emarginatus* fruit are needed. Accordingly, this study aims to assess the cytotoxic potential of standardized extracts of geranylgeraniol in 3T3-A31 fibroblasts and RAW macrophage cells. In addition, the mutagenic and clastogenic effects of geranylgeraniol are investigated in meristematic cells of *Allium cepa* and its possible biological sites of action are predicted *in silico.*

MATERIALS AND METHODS

Cell culture

BALB/c 3T3-A31 fibroblasts were kindly donated by Dr. Mari Cleide Sogayar from the Chemistry Institute, São Paulo University, Brazil. These cells were cultured in Dulbecco's Modified Eagle Medium® medium containing inactivated fetal bovine serum (FBS) (10%), glutamine (1%), erythromycin, and streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 in the air. RAW 264.7 cells were donated by Dr. Milton Adriano Pelli Oliveira from the Institute of Tropical Pathology and Public Health, Federal University of Goiás (UFG), Brazil. These cells were grown in RPMI-1640 (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% FBS (Cripion, São Paulo, Brazil). Cells were inactivated by incubation at 56ºC for 30 min in RPMI containing 2 mM L-glutamine (Sigma Chemical Co.), 50 µM 2-mercaptoethanol (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), and 2 mM Hepes (Sigma-Aldrich). Cells (2×10^5) were grown in 2 mL of RPMI in a well of 6-well culture plates (Costar, Nova York, USA) in a 37°C incubator at 5% CO_2.

Plant material and standardized geranylgeraniol extract

The fruit of *P. emarginatus* was collected in the city of Campestre in the state of Goiás, Brazil (S 16º 46' 01"; W 49º 42' 06"; 612 m). A voucher specimen (UFG-41714) was deposited at the UFG herbarium. The crushed fruits were subjected to filtration using 95% alcohol as the solvent. After obtaining the crude alcoholic extract, it was concentrated in a rotary evaporator (Buchi R-220 SE), under reduced pressure (30 rpm, 40ºC, –600 bar). The concentrated extract was standardized with respect to geranylgeraniol using the standard method for quantification by high-performance liquid chromatography (HPLC) using a Waters model HPLC Alliance with separation module e2695, photodiode array detector (PDA) 2998, and data-processing system Enpower 2.0. Chromatographic separations were conducted using the Zorbax Eclipse column XDB C_{18} (5 µm, 250 × 4.6 mm, Agilent) at a wavelength of 210 nm, elution mode gradient, with the mobile phase consisting of acetonitrile acidified with phosphoric acid 0.2% (v/v) (A), methanol (B), and phosphoric acid 0.5% (v/v) in water (C) [Table 1]. The mobile phase flow rate was 1 mL/min, injection volume was 10 µL, and the column heater was maintained at a temperature of 30ºC. The external standard of geranylgeraniol (purity ≥98.5%, Sigma-Aldrich) and the concentrated extract were diluted with methanol and filtered through a Millex filter (Millipore, Billerica, MA, USA).

Table 1: Elution model gradient system

Chromatographic conditions: C_{18} column; Flow rate, 1 mL/min; Tc, 30°C; λ, 210 nm			
Time (min)	Solvent (%)		
	A	B	C
0-10	65	15	20
10-15	65→90	15→0	20→10
15-32	90	0	10
32-36	90→65	0→15	10→20
36-40	65	15	20

C_{18}: Column chromatography; Tc: Column temperature; λ: Wavelength; A: ACN acidified with phosphoric acid 0.2% (v/v); B: Methanol; C: Phosphoric acid 0.5% (v/v) in water; ACN: Acetonitrile

Mutagenicity and clastogenicity test

A. cepa bulbs were exposed to geranylgeraniol at concentrations of 3.24, 6.48, 9.77, 12.95, and 16.19 mg/mL for 24, 48, or 72 h. Control tests were carried out using Milli-Q water. After reaching 1.5 cm in length, the geranylgeraniol-exposed *A. cepa* roots were collected and fixed in 3:1 (v/v) ethanol/glacial acetic acid (Carnoy's solution). Microscope slides were prepared by carefully squashing the material, as described by Matsumoto *et al.*[26] Meristems previously fixed in Carnoy's solution were washed with distilled water in three baths of 5 min each. The material was then hydrolyzed in HCl 1 M at 60°C for 11 min, followed by three baths of 5 min in distilled water. The slides were stained with acetic carmine (2%) and later covered with coverslips. One thousand cells were observed by optical microscopy, noting those that had undergone any type of change. The mitotic index (MI) was calculated according to:

$$MI(\%) = \frac{\text{number of scored cells } (1000 \text{ per root})}{\text{number of dividing cells}} \times 100$$

We also calculated the average number of changes at each concentration for exposures of 24, 48, and 72 h. Disorders of mitosis, including c-metaphases, chromosome bridges, sticky and lagging chromosomes, "budding" nuclei, nucleoli partly outside nuclei, micronuclei, and binucleated cells, were also investigated using previously described methods.[27,28] Statistical analysis was performed using ANOVA followed by posttests (for group comparisons, Tukey's test) to a significance level of 5% ($P < 0.05$) using the Statistical Analysis System software version 7.0. (SAS Institute Inc., North Carolina, USA).

Neutral red uptake assay

The viability of 3T3 cells (3×10^4 cells/mL) after treatment with nine concentrations of standardized extract in geranylgeraniol (0.39–100 µg/mL) for 48 h was evaluated by measuring the uptake of neutral red by lysosomes in viable cells. Cell suspensions (3×10^4 cells/mL) were distributed in 96-well plates (TPP, Trasadingen, Switzerland) and incubated at 37°C at 5% CO_2 for 24 h. Next, the cells were exposed to 50 µL of the standardized extract at a concentration of 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, or 100 µg/mL and incubated for 48 h under the same conditions. The adhered cells were exposed to a 250 µL of a solution containing 5% FBS (Cultilab), 1% neutral red dye at 0.3% (Sigma-Aldrich), and culture medium DMEM-1640 (Sigma, St. Louis, MO, USA) for 3 h at 37°C in an oven with CO_2 at 5%. The solution containing neutral red was removed, and the cells were washed with 250 µL of PBS at 37°C, resuspended in 100 µL of a solution of 1%

glacial acetic acid (Vetec, Rio de Janeiro, RJ, Brazil), 50% ethanol (Vetec, Rio de Janeiro, RJ, Brazil), and 49% water, and incubated for 20 min. The absorbance of each sample at 540 nm was determined using an ELISA plate reader (Start fax 2100, Awareness Technology, Dusseldorf, Germany).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

This assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction test as previously described by Mosmann[29] and modified by Mota *et al.*[30] Briefly, the RAW 264.7 cells (1×10^6 cells/mL) were plated in triplicate in 96-well plates in RPMI-1640 medium supplemented with 10% FBS and treated with one of 9 concentrations of standardized geranylgeraniol extract (0.39–100 μg/mL). After 48 h of incubation, 10 μL of MTT (5 mg/mL) was added to each well and the plates were incubated for an additional 3 h. The blue MTT formed a precipitate, which was then dissolved in Dimethyl sulfoxide solution (DMSO). The absorbance was measured using a spectrophotometer (Start fax 2100, Awareness Technology, Dusseldorf, Germany) at 550 nm. Cell viability is expressed as a percentage of the control, and IC_{50} values (concentration that inhibited cell growth in 50% relative to untreated group) were obtained.

Phagocytic activity

The RAW 264.7 macrophages cells were counted in a Neubauer chamber, plated at 1×10^6 cells per well on glass coverslips in 24-well culture plates, and infected with 5×10^5 metacyclic forms of *Leishmania guyanensis* (M4147) and also counted in a Neubauer chamber. The negative control contained cells, the parasite and RPMI; the positive control contained cells, parasite, 1 ng/mL interferon-γ (IFN-γ) and 20 ng/mL lipopolysaccharide (LPS). Test 1 (T1) contained cells, the parasite and 10 μL of the standardized extract (6.25 μg/mL) in RPMI and test 2 (T2) contained cells, the parasite, 1 ng/mL IFN-γ, 20 ng/mL LPS and 10 μL of the standardized extract (6.25 μg/mL) in RPMI. After exposure, the plates were incubated at 37°C at 5% CO_2 for 1 h or 48 h. After incubation, the coverslips were removed from the wells and immediately stained using Kit Instant Prov (Newprov, Pinhais, PR, Brazil) and then fixed on microscope slides. Under optical microscopy (magnification, ×1000), 300 cells were observed and the number of parasites in the infected cells was counted. The phagocytic activity was determined as the percentage of infected cells versus the average number of parasites per infected cell.

Prediction of biological activity spectra

Bioactivity prediction based on the two-dimensional (2D) structure of geranylgeraniol was performed using PASS[31] and SwissTargetPrediction[32] tools, which operate based on the assumption that bioactive compounds with similar structures may similar biological activity. The top-ranked activities indicated by each tool were selected for analysis.

Molecular docking

Docking simulations using geranylgeraniol and *Leishmania infantum* sterol 14-alpha demethylase (CYP51), originally complexed with fluconazole (PDB code: 3L4D), were performed using the DockThor server.[33] The calculations were performed inside a sphere with a 5 Å radius centered at the N_2 atom of the fluconazole-complexed ligand. The advanced options were maintained as default. The docked orientations were clustered according to the root-mean-square deviation for the analysis.

RESULTS AND DISCUSSION

Standardized extract in geranylgeraniol

The alcoholic extract of the fruit of *P. emarginatus* yielded a total solid content of 32.39 g/100 g of extract. Thus, after quantification of geranylgeraniol by HPLC [Figure 2], the average content of geranylgeraniol in the extract was determined to be 5.55% (w/w). The content of geranylgeraniol in the standardized extract is 1.80 g/100 g of extract. Menna-Barreto *et al.*[20] determined that geranylgeraniol is the major component of the oil of *P. pubescens*. Isolation and characterization studies of the oil and extracts from fruits of species of the genus *Pterodon* have identified geranylgeraniol as a component of relevance for genus identity and monitoring.[11,17,19] The question of dosage represents a major obstacle to ensure the safety and efficacy of medicinal herbs, even in the case of plants with confirmed biological activities. A small dose may not have any effect, whereas a higher dose may be dangerous to the organism.[34] Thus, a standardized extract allows for greater biological safety and obtaining an analytical quality profile of the pharmacological activities reported for the vegetal extract.

Allium cepa test

Data on the frequency of metaphase and anaphase abnormalities in 1000 interphase cells and the MI for each concentration of geranylgeraniol

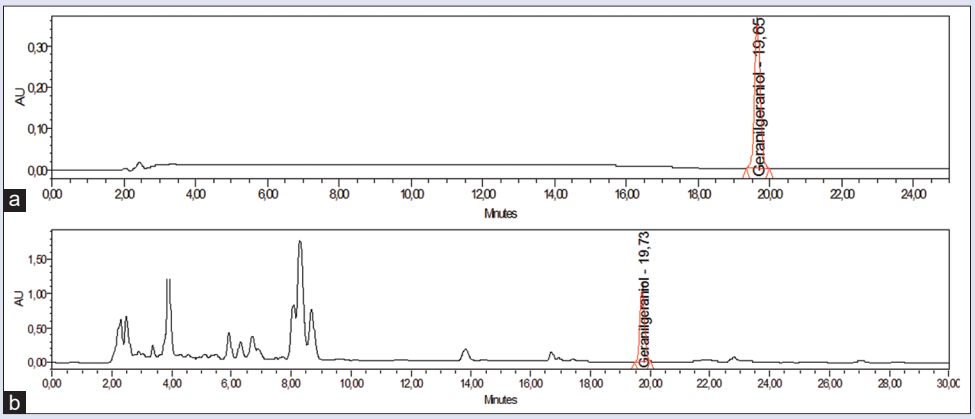


Figure 2: Two-dimensional chromatographic profile at 210 nm. (a) Geranylgeraniol standard (Sigma-Aldrich), with integration, at 19.65 min. (b) Alcoholic extract marker geranylgeraniol identified and standardized, with integration, at 19.73 min. High-performance liquid chromatography-photodiode array (μ, 210 nm); column, C_{18} Zorbax (250 mm × 4.6 mm × 5 μm). Mobile phase in a gradient system; flow rate, 1.0 mL/min; and column temperature, 30°C. Empower 2.0 software

extract tested are shown in Figure 3. In the five concentrations tested, no micronuclei were observed, indicating the absence of mutagenicity.

The interaction of concentration and time exerts a significant influence on the number of abnormalities observed during metaphase and anaphase ($P < 0.05$), as observed at concentrations of 9.77 mg/mL and 16.19 mg/mL, for which there were significant differences in MI between exposure times at the same concentration [Figure 3].

After exposure to standardized extract in geranylgeraniol, meristematic cells of the roots were observed for chromosomal abnormalities such as chromosomal fragments, chromosomal bridges, delayed chromosomes, and polyploidy [Figure 4].

The cytogenetic changes observed suggest that bioactive components of the extract interact with proteins that control the mitotic spindle, resulting in delays in chromosomal migration. The appearance of micronuclei is the consequence of chromosomal breakdown, demonstrating disturbances of the mitotic process[35,36] that did not occur after treatment with standardized extracts. The MI and replication index are indicators of cell proliferation.[37] The MI differenced significantly between exposure times for cells treated with 9.77 mg/mL and 16.19 mg/mL standardized geranylgeraniol extracts ($P < 0.05$) [Figure 3]. The MI for both concentrations was higher after treatment for 24 h than that after 48 or 72 h; at 16.19 mg/mL, the MI after treatment for 72 h was lower than that after 24 or 48 h.

The MI increased in a concentration-dependent manner [Table 2], showing that the standardized geranylgeraniol extract induces cell proliferation with 24 h of exposure. However, after 48 and 72 h of exposure, the MI decreased significantly ($P < 0.05$), indicating that the time of exposure may influence the antiproliferative activity of *A. cepa,* as observed by Peron *et al.*[38] using extracts of *Hypericum perforatum*.

Table 2: Frequency of clastogenic events expressed as the mitotic index in metaphase and anaphase in 1000 cells at interphase at 24, 48, and 72 h

Treatments	MI (mean±SD)		
	24 h	48 h	72 h
Control	2.19±0.81	2.19±0.81	2.19±0.81
$[\ ]_1$: 3.24 (mg/mL)	7.70±1.39	6.60±0.60	7.07±1.10
$[\ ]_2$: 6.48 (mg/mL)	4.13±1.94	3.07±1.70	7.20±2.62
$[\ ]_3$: 9.77 (mg/mL)	10.33±0.61	4.47±1.94	4.87±2.20
$[\ ]_4$: 12.95 (mg/mL)	8.00±4.0	5.00±1.74	3.87±0.42
$[\ ]_5$: 16.19 (mg/mL)	9.33±3.29	8.67±0.61	3.80±1.06

All data are presented as the mean±SD of triplicate assays. Control: Milli-Q water; [ ]: Concentration test; SD: Standard deviation; MI: Mitotic index

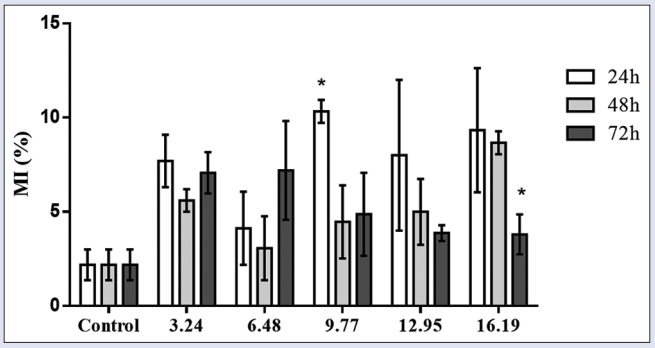


Figure 3: The mean percentage and standard deviation of the mitotic index for the control group (Milli-Q water) and the groups treated with standardized geranylgeraniol extract at five concentrations (mg/mL) for different times (24, 48, and 72 h). ANOVA factorial followed by posttest of grouping (Tukey's test) to a significance level of 5% ($P < 0.05$). *Compared to control ($P < 0.05$)

According to Lloret *et al.*,[17] geranylgeraniol is a precursor of vouacapanic derivatives, among them 6α-hydroxy-7α-acetoxyvouacapan, which plays an important role in inhibiting cell growth in cell lines such as cancer PC-03. In this same study, the concentration of geranylgeraniol was inversely proportional to the content of vouacapans, which may explain the lag in the antiproliferative effect observed in our study after 48 and 72 h, as indicated by the significant decrease in MI.

Neutral red uptake assay

A decline was observed in the viability of 3T3 basal cells after treatment with all nine concentrations of standardized geranylgeraniol extract (0.39–100 μg/mL) for 48 h in a concentration-dependent manner. The IC_{50} value was 15.5 μg/mL [Figure 5].

The neutral red assay is a biological safety assay that evaluates cell viability after exposure to chemicals capable of causing cytotoxicity. The geranylgeraniol compound treats an acyclic diterpene precursor of other vouacapan compounds that were evaluated for cytotoxicity in 3T3 cells, giving the following results: 6α-acetoxy-7β-hydroxyvouacapan, IC_{50}, 34.33 μg/mL; 6α,7β-dihydroxyvouacapan-17β-oate methyl ester, IC_{50}, 22.83 μg/mL; and 6α,7β-dihydroxyvouacapan-17β-methylene -ol, IC_{50}, 23.55 μg/mL; and exhibited pronounced cytotoxic activity (antitumor/antiproliferative activity) in the following cancer cells *in vitro*: UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), K562 (leukemia), and NCI-ADR/RES (ovarian expressing multidrug-resistant phenotype).[15] Although the geranylgeraniol compound has a low IC_{50}, suggesting cytotoxicity, its antinociceptive, anti-inflammatory, and antitumor properties already observed in oils and extracts of various species of the genus *Pterodon*[9,12,14,16,18,19] also make it promising for treating numerous pathological conditions. The oil extracted from the fruit of *P. emarginatus*, which is known to have diterpene compounds such as geranylgeraniol, demonstrated

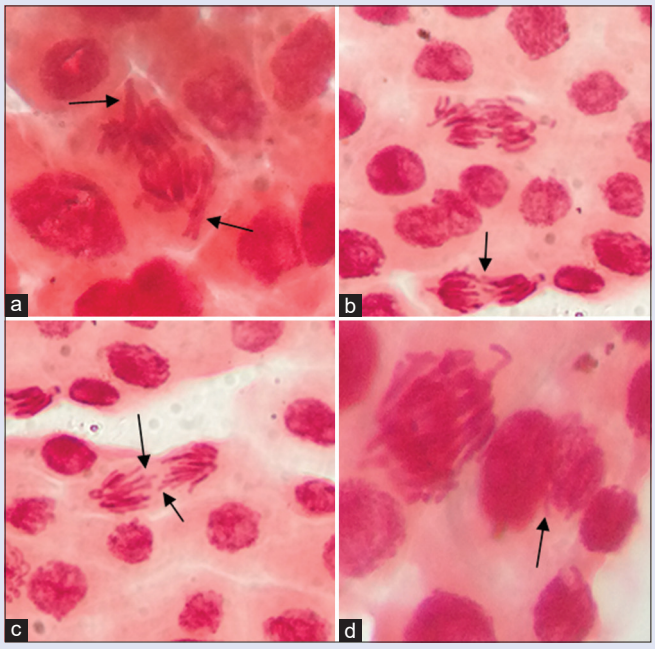


Figure 4: Photomicrograph of chromosomal abnormalities observed after analysis of *Allium cepa* meristematic cells exposed to standardized extracts of geranylgeraniol at 24, 48, and 72 h. Chromosome fragments (a), chromosomal bridges (b), straggler chromosomes (c), and polyploid cells (d). Images obtained in optical photomicroscope (objective, 100×)

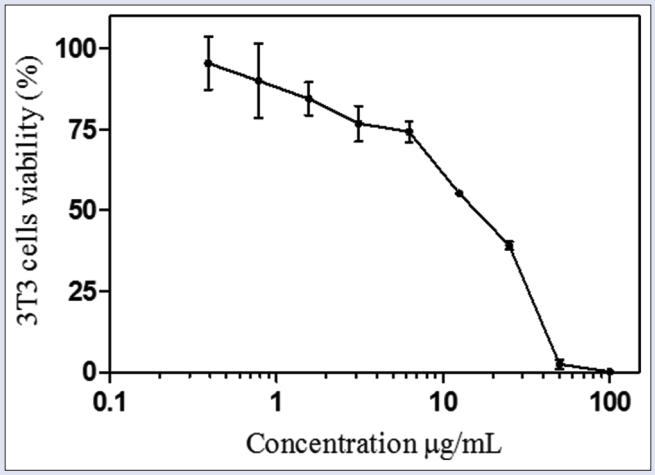


Figure 5: Cytotoxic effects of standardized geranylgeraniol extract in 3T3 basal cells. The cells (3 × 10^4 cells/mL) were treated with nine concentrations of geranylgeraniol (0.39–100 µg/mL) for 48 h, and cell viability was evaluated by neutral red uptake assay. Results represent the mean ± standard deviation of three independent experiments using six replicates

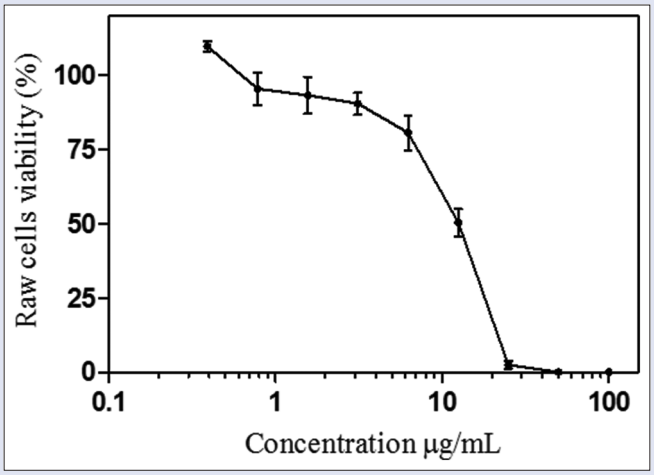


Figure 6: Cytotoxic effects of standardized geranylgeraniol extract in RAW 264.7 cells (macrophages). The cells (3 × 10^4 cells/mL) were treated with nine concentrations of extract (0.39–100 µg/mL) for 48 h and the cell viability evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Results are presented as the mean ± standard deviation of three independent experiments using six replicates

cytotoxic activity against cancer cell lines (line C6) (IC_{50}, 24.9 µg/mL) but had no effect on peripheral blood mononuclear cells (PBMCs), which demonstrated a viability of 94 ± 1.5%, 93 ± 3.5%, and 95 ± 3.3%, after 12, 24, and 48 h of treatment at 100 µg/mL, respectively.[22]

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

A concentration-dependent decline in the viability of RAW 264.7 cells was observed after treatment with nine concentrations of standardized geranylgeraniol extract (0.39–100 µg/mL) for 48 h. The IC_{50} value was 12.2 µg/mL [Figure 6].

The viability of RAW murine cells (macrophages) as assessed by MTT assay was dose-dependent, with an IC_{50} of 12.2 µg/mL. This IC_{50} was higher than that observed by Sabino *et al.*[25] for human PBMC treated with oil extracted from *P. pubescens* after 24 (2 µg/mL) and 48 (1 µg/mL) h. These authors did not consider the material evaluated as cytotoxic for PBMC. This result is consistent with that present study, as no dead cells were observed after treatment with standardized extract (6.25 µg/mL). Determination of the geranylgeraniol IC_{50} in RAW 264.7 cells was essential for evaluating phagocytic activity, clarifying the optimal concentration for verifying the microbicidal potential of the standardized extract geranylgeraniol. We determined this concentration to be 6.25 µg/mL against *L. guyanensis*, an important parasite of murine macrophages. Our study showed that geranylgeraniol has a high phagocytic capacity and microbicidal potential against the experimental model of *L. guyanensis*, corroborating the findings of Menna-Barreto *et al.*[20] using a similar infection model in which geranylgeraniol inhibited the proliferation of intracellular amastigote forms of *T. cruzi*. The treatment of macrophages infected with the amastigote forms of *T. cruzi* with 2 and 6 µg/mL of geranylgeraniol showed a decrease in the dose and time dependence of infection and a decrease in the number of internalized parasites. The mechanism underlying the geranylgeraniol-induced induction of phagocytosis involves the rupture of the mitochondrial membrane in trypomastigotes and epimastigotes.

Phagocytic activity

Figure 7 shows the number of parasites in all cells counted for each treatment (a), the percentage of infected macrophages (b), the phagocytic activity of the macrophages (c), and the relationship of parasites by infected macrophages (d). During the 24 or 48 h of the experiment, endocytosis of parasites should be observed. However, this did not occur in this study because RAW 264.7 cells are known to release reactive oxygen and nitrogen during this time period, killing the parasite. In light of this observation, we evaluated the phagocytic and microbicidal activity during the 1st hour after treatment and compared the results to those of positive controls treated with INF-γ and LPS. We observed that treatment with the standardized geranylgeraniol extract inhibited the action of INF-γ and LPS, as shown in Figure 7.

Because of the similarity between the model used in the present study and that of Menna-Barreto *et al.*[20] including the geranylgeraniol concentration and the use of parasites of the same family, it is likely that the pronounced phagocytic activity observed in RAW 264.7 cells is related to the treatment with geranylgeraniol. The 1-h period for evaluation of phagocytic activity was considered adequate since it corresponds to the macrophage activation period observed by Dias *et al.*[39] who showed that the maximum activation of macrophages in the peritoneal cavity of *Brycon amazonicus* inoculated with *Saccharomyces cerevisiae* yeast occurred during a 2-h study period, allowing determination of the phagocytic index.

Cardoso *et al.*[40] observed that the ethanolic extract from the seeds of *P. pubescens* inhibits nitric oxide (NO) production *in vivo*, suggesting that it affects macrophage function. Because NO is one of the main microbicidal agents produced by macrophages, this observation suggests that the extract would promote a decrease in phagocytic activity, a prediction not supported by the findings of our present study. However, a study reporting that NO synthase inhibition in macrophages contributes to anti-inflammatory and immunosuppressive actions[41] is consistent with findings of studies of the anti-inflammatory activity of *P. emarginatus* extracts and oils.[4,8,9] Several compounds of vegetal origin exhibit immunomodulatory activities,[42,43] including the phagocytosis of bacteria by mononuclear phagocytes, a microbicidal activity stimulated by immunomodulatory agents.[44] Immunomodulatory compounds

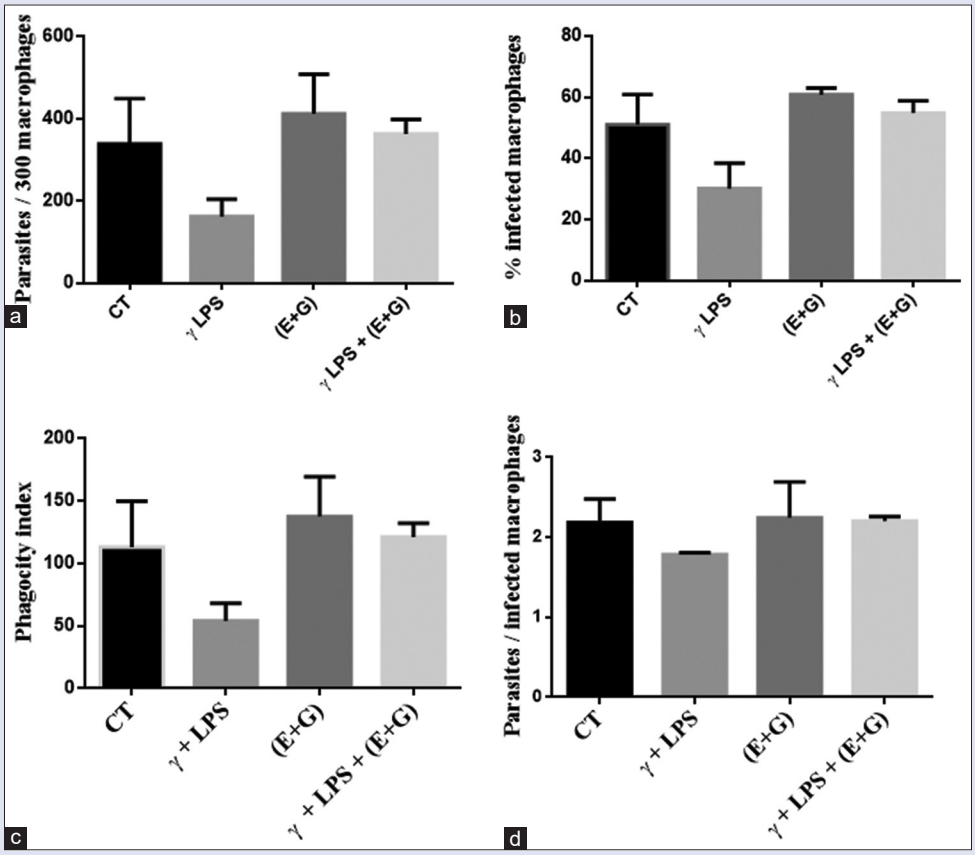


Figure 7: Index of phagocytosis and microbicidal activity (%) of mononuclear phagocytes (RAW 264.7 cells) against *Leishmania guyanensis* exposed to the standardized geranylgeraniol extract for 1 h. CT, negative control; γ + lipopolysaccharide, positive control containing cells, parasite, 1 ng/mL interferon-γ, and 20 ng/mL lipopolysaccharide; E + G, test 1 (T1) containing cells, parasite, 10 mL standardized extract (6.25 mg/mL); γ + lipopolysaccharide + (E + G), test 2 (T2) containing cells, parasite, 1 ng/mL interferon-γ, 20 ng/mL lipopolysaccharide, and 10 mL standardized extract (6.25 mg/mL). (a) Phagocity activity; (b) % infection macrophages; (c) Phagocity Index; (d) Number de parasites infective por macrophages. The data are presented as mean standard deviation of three experiments performed in triplicate were compared for significance by Student's test or ANOVA followed by Bonferroni test using the GraphPad Prism software 5.0 (Inc. San Diego, CA, USA). $P < 0.05$ was considered significant

capable of modifying immune system responses are considered alternative treatments for infections.

Biological activity prediction *in silico* and docking of geranylgeraniol

The PASS and SwissTargetPrediction tools were used to predict the bioactivity of geranylgeraniol. This analysis revealed that it may have anti-inflammatory and antineoplastic activities, regulate lipid metabolism, and interfere with enzymes involved in sterol biosynthesis. The inhibition of farnesyltranstransferase and 2,3-oxidosqualene-lanosterol cyclase was suggested by the PASS tool, and the inhibition of squalene monooxygenase was suggested by the SwissTargetPrediction tool.

Docking calculations for geranylgeraniol within the *Leishmania* CYP51 active site revealed that 20% of the orientations obtained were capable of interacting directly with the heme group through hydrogen bonds between the hydroxyl group of geranylgeraniol and the heme propionate moiety. Another hydrogen bond was observed with TYR102. In addition, van der Waals interactions were observed between geranylgeraniol and Val^{212}, Met^{459}, Phe^{104}, Phe^{109}, Tyr^{115}, and Thr^{294} [Figure 8].

The hexane and butanol fractions obtained from oil extracted from *P. emarginatus* fruit exhibited activity against promastigote forms of *Leishmania amazonensis* (hexane fraction IC_{50}, 50.06 μg/mL; butanol fraction IC_{50}, 46.65 μg/mL). These fractions had no activity against *Leishmania chagasi*.[21] This observation suggests that further studies to evaluate the leishmanicidal potential of standardized derivatives and substances isolated *P. emarginatus* extracts would be worthwhile.

Because the sterol biosynthesis pathway is vital to *Leishmania*, it is an important source of antimicrobial targets.[45] Inhibition of farnesyltranstransferase, a target of bisphosphonates, may interfere with the production of substrates for enzymes catalyzing the first committed step in sterol biosynthesis. Another target in this pathway is squalene monooxygenase, a known target of the allylamines. Squalene monooxygenase participates in the conversion of the 30-carbon chain squalene to a tetracyclic sterol skeleton.[45,46]

Geranylgeraniol may also be an inhibitor of 2,3-oxidosqualene-lanosterol cyclase, which catalyzes the cyclization of 2,3-oxidosqualene to lanosterol. This enzyme has been confirmed as a therapeutic target against Trypanosomatidae.[46] Thus, the findings obtained in our bioactivity prediction analysis indicate that geranylgeraniol might interfere with enzymes involved in the *Leishmania* sterol biosynthesis pathway.

Sterol 14-alpha demethylase (CYP51) is a key enzyme of the sterol biosynthesis pathway. This enzyme catalyzes the removal of the C-14 alpha-methyl group from sterol precursors, a reaction essential for membrane biogenesis.[47] This enzyme was selected for molecular

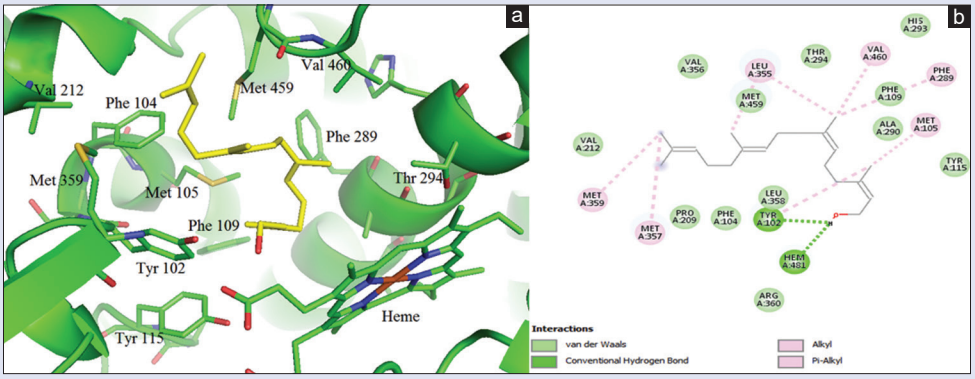


Figure 8: (a) Docking 3D model of geranylgeraniol (carbon atoms in yellow) within the *Leishmania* CYP51 active site, highlighting the main intermolecular interactions with the heme group and amino acid residues. (b) Two-dimensional diagram of interaction between geranylgeraniol and the *Leishmania* CYP51 heme group and amino acid residues

docking simulations to determine whether geranylgeraniol also can interact with this *Leishmania* target.

The 3D structure of *L. infantum* CYP51 is 95% similar to four *Leishmania* species, qualifying it as a suitable molecule for predictive docking in other *Leishmania* species. Docking simulations revealed that geranylgeraniol is accommodated by the CYP51 active site and interacts with it both electrostatically and hydrophobically. The hydrogen bonding between geranylgeraniol and the enzyme active site is of interest because the formation of the fluconazole/CYP51 complex is reported to involve hydrogen bonding.[47]

In addition, the hydrophobic pocket in the active site offers a favorable binding region for the geranylgeraniol alkyl chain. These hydrophobic interactions may be important for the binding affinity since the natural substrate of this enzyme is also a lipophilic molecule.[47,48]

CONCLUSION

That standardized geranylgeraniol extract increases cell division in *A. cepa* radicles after 24 h of exposure; further exposure results in antiproliferative effects. The preliminary cytotoxicity study revealed a low IC_{50} for geranylgeraniol in 3T3 and RAW 264.7 cells and cytotoxic effects in the parasite *L. guyanensis*. This result explains the pronounced phagocytic and microbicidal potential in treated cells, suggesting that geranylgeraniol has an important immunomodulatory activity in macrophages. *In silico* findings support the hypothesis that the effects of geranylgeraniol on *Leishmania* involve interference with enzymes of the sterol biosynthesis pathway. The present study indicates that the standardized geranylgeraniol extract from *P. emarginatus* might be useful as an immunomodulator and microbicide against *Leishmania* species.

Acknowledgements

We are thankful to all the sponsors of this research: Goiás State Research Support Foundation; Institute of Tropical Pathology and Public Health at the Federal University of Goiás; Replicon Research Center of the Pontifical Catholic University of Goiás; and Cellular Pharmacology and Toxicology Laboratory - Farmatec, Federal University of Goiás.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Agra MD, Freitas PF, Barbosa-Filho JM. Synopsis of the plants known as medicinal and poisonous in Northeast of Brazil. Braz J Pharmacogn 2007;17:114-40.
2. Arriaga A, Castro MA, Silveira ER, Braz-Filho R. Further diterpenoids isolated from *Pterodon polygalaeflorus*. J Braz Chem Soc 2000;11:187-90.
3. Evangelista GL, Coelho-de-Souza AN, Santos CF, *et al.* Essential oil of *pterodon polygalaeflorus* inhibits electromechanical coupling on rat isolated trachea. J Ethnopharmacol 2007;109:515-22.
4. Dutra RC, Pittella F, Ferreira AS, Larcher P, Farias RE, Barbosa NR. Efeito Cicatrizante das Sementes de *Pterodon emarginatus* Vogel em modelos de úlceras dérmicas experimentais em coelhos. Latin Am J Pharm 2009;28:375-82.
5. Hansen D, Haraguchi M, Alonso A. Pharmaceutical properties of "sucupira" (*Pterodon* spp). Braz J Pharm Sci 2010;46:607-16.
6. Hoscheid J, Cardoso ML. Sucupira as a potential plant for arthritis treatment and other diseases. Arthritis 2015:379-459.
7. Ojo OA, Bakare SB, Babatunde AO, Expressway B. Research paper. World Health 2001;1:30-5.
8. de Moraes WF, de Matos LG, Mariano Nascimento MV, *et al.* Anti-inflammatory and anti-nociceptive effects of *Pterodon emarginatus* stem bark alcohol extract. Pharm Biol 2009;47:146-50.
9. de Moraes WF, Galdino PM, Nascimento MV, *et al.* Triterpenes involved in the anti-inflammatory effect of ethanolic extract of *Pterodon emarginatus* Vogel stem bark. J Nat Med 2012;66:202-7.
10. Silva MC, Gayer CR, Lopes CS, *et al.* Acute and topic anti-edematogenic fractions isolated from the seeds of *Pterodon pubescens*. J Pharm Pharmacol 2004;56:135-41.
11. Spindola HM, Servat L, Denny C, Rodrigues RA, Eberlin MN, Cabral E, *et al.* Antinociceptive effect of geranylgeraniol and 6alpha, 7beta-dihydroxyvouacapan-17beta-oate methyl ester isolated from *Pterodon pubescens* benth. BMC Pharmacol 2010;10:1.
12. Oliveira PC, Conceição EC, Oliveira PA, *et al.* Obtaining a dry extract of *Pterodon emarginatus* (Fabaceae) fruits by spray-drying. J Pharm Res 2012;5:641-5.
13. Negri G, Mattei R, Mendes FR. Antinociceptive activity of the HPLC-and MS-standardized hydroethanolic extract of *Pterodon emarginatus* Vogel leaves. Phytomedicine 2014;21:1062-9.
14. Euzébio FP, dos Santos FJ, Piló-Veloso D, *et al.* Effect of 6α,7β-dihydroxyvouacapan-17β -oic acid and its lactone derivatives on the growth of human cancer cells. Bioorg Chem 2009;37:96-100.
15. Spindola HM, Rodrigues RA, Denny C, Sousa IM, Tamashiro JY, Foglio MA. Furanoditerpenes from *Pterodon pubescens* benth with selective *in vitro* anticancer activity for prostate cell line. J Braz Chem Soc 2009;20:569-75.
16. Euzébio FP, Santos FJ, Piló-Veloso D, *et al.* Synthesis, antiproliferative activity in cancer cells and theoretical studies of novel 6a, 7b-dihydroxyvouacapan-17b-oic acid mannich base derivatives. Bioorg Med Chem 2010;18:8172-7.
17. FC, CC. 6α-Acetoxy-7α-hydroxy-vouacapan isolated from *Pterodon pubescens* benth. fruit's with selective activity against prostate cancer cell line: Artifact or natural product? Med Aromat Plants J 2016;5:2-7.
18. Spindola HM, Servat L, Denny C, *et al.* Antinociceptive effect of geranylgeraniol and 6alpha,

7beta-dihydroxyvouacapan-17beta-oate methyl ester isolated from *Pterodon pubescens* Benth. BMC Pharmacol 2010;10:1.

19. Spindola HM, Servat L, Rodrigues RA, Sousa IM, Carvalho JE, Foglio MA. Geranylgeraniol and 6α,7β-dihydroxyvouacapan-17β-oate methyl ester isolated from *Pterodon pubescens* Benth: Further investigation on the antinociceptive mechanisms of action. Eur J Pharmacol 2011;656:1-3.
20. Menna-Barreto RF, Laranja GA, Silva MC, *et al*. Anti-trypanosoma cruzi activity of *Pterodon pubescens* seed oil: Geranylgeraniol as the major bioactive component. Parasitol Res 2008;103:111-7.
21. Dutra RC, Braga FG, Coimbra ES, Silva AD, Barbosa NR. Atividades antimicrobiana e leishmanicida das sementes de *Pterodon emarginatus* Vogel. Braz J Pharmacogn 2009;19:429-35.
22. Dutra RC, Pittella F, Dittz D, *et al*. Chemical composition and cytotoxicity activity of the essential oil of *Pterodon emarginatus*. Braz J Pharmacogn 2012;22:971-8.
23. Jothy SL, Chen Y, Kanwar JR, Sasidharan S. Evaluation of the genotoxic potential against h 2 o 2 -radical-mediated DNA damage and acute oral toxicity of standardized extract of *Polyalthia longifolia* leaf. Evid Based Complement Alternat Med 2013;2013:1-13.
24. Dias FD, Takahashi CS, Sakamoto-Hojo ET, Vichnewski W, Sarti SJ. Genotoxicity of the natural cercaricides 'sucupira' oil and eremanthine in mammalian cells *in vitro* and *in vivo*. Environ Mol Mutagen 1995;26:338-44.
25. Sabino KC, Gayer CR, Vaz LC, Santos LR, Felzenszwalb I, Coelho MG. *In vitro* and *in vivo* toxicological study of the *Pterodon pubescens* seed oil. Toxicol Lett 1999;108:27-35.
26. Matsumoto ST, Mantovani MS, Malaguttii MI, Dias AL, Fonseca IC, Marin-Morales MA. Genotoxicity and mutagenicity of water contaminated with tannery effluents as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. Genet Mol Biol 2006;29:148-58.
27. Ateeq B, Abul Farah M, Niamat Ali M, Ahmad W. Clastogenicity of pentachlorophenol, 2,4-D and butachlor evaluated by *Allium* root tip test. Mutat Res Genet Toxicol Environ Mutagen 2002;514:105-13.
28. Glińska S, Bartczak M, Oleksiak S, *et al*. Effects of anthocyanin-rich extract from red cabbage leaves on meristematic cells of *Allium cepa* L. roots treated with heavy metals. Ecotoxicol Environ Saf 2007;68:343-50.
29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
30. Da Mota MF, Benfica PL, Batista AC, Martins FS, De Paula JR, Valadares MC. Investigation of Ehrlich ascites tumor cell death mechanisms induced by *Synadenium umbellatum* Pax. J Ethnopharmacol 2012;139:319-29.
31. Lagunin A, Stepanchikova A, Filimonov D, Poroikov V. PASS: prediction of activity spectra for biologically active substances. Bioinformatics 2000;16:747-8.
32. Gfeller D, Michielin O, Zoete V. Shaping the interaction landscape of bioactive molecules. Bioinformatics 2013;29:3073-9.
33. De Magalhães CS, Almeida DM, Barbosa HJ, Dardenne LE. A dynamic niching genetic algorithm strategy for docking highly flexible ligands. Inf Sci (Ny) 2014;289:206-24.
34. Li SP, Zhao J, Yang B. Strategies for quality control of Chinese medicines. J Pharm Biomed Anal 2011;55:802-9.
35. Grover IS, Kaur S. Genotoxicity of wastewater samples from sewage and industrial effluent detected by the *Allium* root anaphase aberration and micronucleus assays. Mutat Res Fundam Mol Mech Mutagen 1999;426:183-8.
36. Bagatini MD, Ferreira Da Silva AC, Tedesco SB. Uso do sistema teste de *Allium cepa* como bioindicador de genotoxicidade de infusões de plantas medicinais. Braz J Pharmacogn 2007;17:444-7.
37. Gadano A, Gurni A, López P, Ferraro G, Carballo M. *In vitro* genotoxic evaluation of the medicinal plant *Chenopodium ambrosioides* L. J Ethnopharmacol 2002;81:11-6.
38. Peron AP, Mariucci RG, de Almeida IV, Düsman E, Mantovani MS, Vicentini VE. Evaluation of the cytotoxicity, mutagenicity and antimutagenicity of a natural antidepressant, *Hypericum perforatum* L. (St. John's wort), on vegetal and animal test systems. BMC Complement Alternat Med 2013;13:97.
39. Dias DD, Tachibana L, Seriani R, Santos AA, Ranzani-Paiva MJ, Romagosa E. Tempo de migração dos macrófagos em matrinxã, Brycon amazonicus, por meio da técnica de inoculação de leveduras *Saccharomyces cerevisiae*. Acta Amazon 2011;41:421-4.
40. Cardoso CC, Pinto AC, Marques PR, *et al*. Suppression of T and B cell responses by *Pterodon pubescens* seeds ethanolic extract. Pak J Biol Sci 2008;11:2308-13.
41. Di Rosa M, Radomski M, Carnuccio R, Moncada S. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. Biochem Biophys Res Commun 1990;172:1246-52.
42. Corrêa VS, Maynié JC, França EL, Honório-França AC. Atividade funcional de fagócitos na presença do fitoterápico 'Mais Vida'. Rev Bras Plantas Med 2006;8:26-32.
43. Côrtes MA, França EL, Reinaque AP, Scherer EF, Honorio-França AC. Imunomodulação de fagócitos do sangue humano pelo extrato de *Strychnos Pseudoquina* ST. HILL adsorvido em microesferas de polietilenoglicol. Polimeros 2013;23:1-8.
44. França EL, Morceli G, Fagundes DL, Rudge MV, Calderon ID, Honorio-França AC. Secretory IgA-Fcα receptor interaction modulating phagocytosis and microbicidal activity by phagocytes in human colostrum of diabetics. Acta Pathol Microbiol Immunol Scand (Apmis) 2011;119:710-9.
45. de Souza W, Rodrigues JC. Sterol biosynthesis pathway as target for anti-trypanosomatid drugs. Interdiscip Perspectives Infectious Dis 2009;2009:502-642.
46. Oliveira IA, Freire-de-lima L, Penha LL, Dias WB, Todeschini AR. Proteins and proteomics of *Leishmania* and *Trypanosoma*. Subcell Biochem 2014:181-201.
47. Hargrove TY, Wawrzak Z, Liu J, Nes WD, Waterman MR, Lepesheva GI. Substrate preferences and catalytic parameters determined by structural characteristics of sterol 14a-demethylase (CYP51) from *Leishmania infantum*. J Biol Chem 2011;286:26838-48.
48. Lepesheva GI, Waterman MR. Sterol 14a-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim Biophys Acta Gen Subj 2007;1770:467-77.