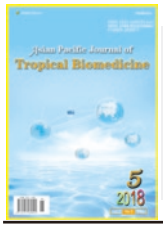

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Phytochemical bioprospecting, antioxidant, antimicrobial and cytotoxicity activities of saline extract from *Tithonia diversifolia* (Hemsl) A. Gray leaves

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ABSTRACT

Objective: To evaluate antimicrobial and antioxidant properties of saline extract from *Tithonia diversifolia* leaves by phytochemical bioprospecting, and investigate its safety against animal cells. **Methods:** The saline extract was prepared, with NaCl (0.15 M), by constant stirring of the dried and pulverized leaves, followed by volume reduction by lyophilization. The extract was phytochemical characterized using ultra-performance liquid chromatography, and total phenol and flavonoid analysis also was performed. The antioxidant capacity was determined through DPPH$^{\bullet}$ radical, the antimicrobial property was evaluated against standard bacteria and fungi, and the viability assays were performed against mice splenocytes. **Results:** Fifteen compounds were identified belonging to two main classes terpenoids and phenolics. The extract showed 22.185 mg GAE/g of total phenolic compounds and 3.220 mg QE/g of flavonoid. Moreover, extract showed higher antioxidant ability similar to butylated hydroxytoluene a standard molecule [(3.042±0.019) mg AAE/g and (4.12±0.10) mg AAE/g to saline extract and butylated hydroxytoluene, respectively]. The antimicrobial assays demonstrated that the extract had a significant antifungal potential against *Candida* species and could be used with safety against mice splenocytes, in concentrations lower than 50 μg/mL, promoting higher proliferation in these cells. **Conclusions:** Saline extract from *Tithonia diversifolia* leaves presents potential antioxidant, antifungal properties and induces immunostimulation in mice splenocytes.

1. Introduction

Many therapeutic agents are used in medicine against diseases and different plants comprise of the sources of these compounds[1,2]. In fact, according to Pan *et al.*[2], more than 35 000 species of plants possess curative potentials because of their phytochemical constituents. These chemical constituents are functionally classified

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as primary and secondary metabolites. Primary metabolites, essential for development, growth and reproduction of plant, are proteins, lipids, nucleic acids and carbohydrates. Secondary metabolites, produced in response to the involvement of plant with the environment or as possible defensive mechanisms against aggressive agents, can be represented by many compounds as alkaloids, flavonoids and terpenoids[3].

Most of the commercially available drugs are derived from plants or generated from natural products. Some compounds of plant and/or derived from plant were discovered throughout history. Among these compounds, we can find capsaicin (*Capsicum* spp.), aspirin (*Salix alba* L.), atropin (*Atropa belladonna*), morphine (*Papaver somniferum* L.), quinine (*Cinchona* sp.), pilocarpine (*Pilocarpus jaborandi* and *Pilocarpus microphyllus*), colchinine (*Colchicum autumnale*), etoposide (*Podophyllum peltatum* L.), masoprocol (*Larrea divaricata* Cav.), metformin (*Galega officinalis*), levodopa (*Mucuna pruriens*), dicumarol (*Melilotus officinalis*), vinblastine, vincristine, vinrosidine, vinorelbine and vinflunine (*Catharanthus roseus*), solamargine (*Solanumdu lcamara*), taxol (*Taxus brevifolia*), artemisinin (*Artemisia annua*), maytansine (*Maytenus ovatus*), irinotecan (*Camptotheca acuminata*), and arglabin (*Artemisia glabella* Kar.)[4-30]

Currently, about 80% of the immunosuppressive, cardiovascular, antimicrobial and antitumor drugs are originated from plants. The identification and isolation of compounds and/or phytochemical groups are crucial for the discovery of new therapies[1,2,31-33].

Tithonia diversifolia (*T. diversifolia*) (Hemsl) A. Gray, is a perennial and shrub species of the family Asteraceae, tribe Heliantheae, which is native in Central America and Mexico and popularly known as Mexican sunflower[34-36]. Many representatives of this species are found throughout the world, especially in Asia, Africa and America[35].

In the literature, *T. diversifolia* is described as an important source of biologically active molecules[35], which possesses many compounds in leaves and inflorescences belonging to sesquiterpene lactones and flavonoids classes, and derivatives of trans-cinnamic acid[37,38]. Extracts and secondary metabolites isolated from inflorescences and leaves showed significant biological properties, such as antitumor[39], anti-inflammatory[37], antihyperglycemic[40] and antimicrobial[41].

In this study we explore the phytochemical characterization as well as investigate antioxidant profile, antimicrobial properties against pathogenic species of bacteria and *Candida* sp. and cytotoxicity against mice splenocytes promoted by saline extract of *T. diversifolia* leaves.

2. Materials and methods

2.1. Processing of plant leaves and extract preparation

The plant was collected in city of Paudalho, interior of the State of Pernambuco, Brazil. The botanical identification was carried by Herbarium Geraldo Mariz of the Center of Biosciences of the Federal University of Pernambuco. Plant was deposited, under identification number 82.703. For two days, the humidity was removed for obtainment of dried leaves. These leaves were crushed in reverse black oster and the pulverized material (20 g) was diluted in 200 mL of NaCl (0.15 M/10% (w/v). Therefore, using an orbital and alternative incubator shaker (Lab Companion™ IS-971) the material was kept under agitation (400 rpm) in constant temperature (28 ℃) for 16 hours. After the stirring time, the material was filtered using paper filter and centrifuged (15 min, 10 000 × *g*). The obtained supernatant was collected and called saline extract from *T. diversifolia* leaves. After this, the extract was lyophilized, and 9.2 g of crude material was obtained.

2.2. Phytochemical bioprospecting

2.2.1. Determination of total phenolic compounds

The contents of total phenols were determined through method described by Li *et al.*[42] with few modifications. One mg/mL of diluted extract was dissolved in distilled water. A total of 100 μL of Folin solution (1:10 v/v) was added to 200 μL of this diluted extract. After incubation, the extracts were protected from light for 3 min, and 0.08 mL of Na_2CO_3 (7.5%) was added. Extracts were then incubated again and protected from light, at 21 ℃, for 120 min. After incubation time, the extracts were read in the spectrophotometer (765 nm). Distilled water was the blank on the calibration curve. A calibration curve of the graphical representation of the absorbance as a function of the concentration of gallic acid (0-500 μg/mL) was prepared and the linear equation ($Y = 0.004\ 8x + 0.001\ 6$, $R^2 = 0.999\ 9$). Phenols were indicated in gallic acid equivalent (mg GAE/g of extract).

2.2.2. Investigation of total flavonoids

The measurement of total flavonoid amount was performed similarly to the method used by Woisky and Salatino[43], with some modifications. Using test tubes, 1 mL of the extract, previously diluted (1 mg/mL) was added. After this, 1 mL of the 3% solution of aluminum chloride ($AlCl_3$) prepared with methanol was added in tubes. After incubation (30 min), in dark (21 ℃), the absorbance was obtained (425 nm). A standard curve with quercetin (0-500 μg/mL) was performed to obtain the equation ($Y = 0.023x + 0.150\ 9$, $R^2 = 0.995\ 6$). The assays were obtained in quintuplicate. Flavonoids were indicated in quercetin equivalent (mg QE/g of extract).

2.2.3. Ultra-High Performance Liquid Chromatography coupled to Mass Spectrometry

Ultra-High Performance Liquid Chromatography was performed with an Acquity H-Class (Waters) employing a 2.1 mm × 100 mm BEH column with a particle size of 1.7 μm. The column was maintained

under a constant temperature of 40 ℃ and the auto injector at 10 ℃. We used an aqueous solution (eluent A) containing 2% methanol (MeOH), 5 mM ammonium formate and 0.1% formic acid and a methanol solution (eluent B) containing 0.1% formic acid, which were pumped at a flow rate of 0.3 mL/min. Ten microliters of the *T. diversifolia* extract was injected. Elution was performed in gradient mode and the initial condition (98% A / 2% B) was maintained for 0.25 min. The B ratio increased linearly to 99% in 8.5 min, remaining at 99% B for 1 min, followed by an immediate decrease to 2% B, where it was maintained for up to 11 min. The ultra-performance liquid chromatography system was coupled to a single quadrupole mass spectrometer SQ Detector 2 (Waters). The data were obtained in fullscan mode, analyzing masses between 100 and 1 000 Da, in negative ionization. The acquisition of the chromatograms and mass spectra was obtained through MassLynx™ software (Waters).

2.3. Antioxidant activities in vitro

2.3.1. Free radical sequestration by 2,2-diphenyl-1-picrylhydrazyl (DPPH•)

The antioxidant ability of saline extract was investigated through the stable radical DPPH•, as described by Blois[44]. We performed a serial dilution with 400 μL of the saline extract (3.9; 7.8; 15.6; 31.3; 62.5; 125; 250 and 500 μg/mL). In each concentration was added 0.25 mL of the DPPH• solution (1 mM and OD_{517} = 0.650 ± 0.50). After incubation time (25 min/21 ℃/in the dark), the absorbance was obtained in 517 nm. The DPPH• solution added to water was used as control. The sequestration of DPPH• radicals was measured by using the equation:

DPPH• (%) = [(As - Ac)/Ac] × 100

Where As is the sample absorbance and Ac is the control absorbance.

2.3.2. Total antioxidant activity

The total antioxidant activity of saline extract was measured as a function of ascorbic acid, according to Pietro *et al.*[45]. Saline extract (100 μL in 500 μg/mL) was mixed with ascorbic acid (1 mg/mL) and with 1 mL of phosphomolybdenum solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was incubated in water bath (95 ℃ / 90 min). After cooling, the absorbance was obtained at 695 nm against a blank (1 mL solution with 0.1 mL of water). A standard curve with ascorbic acid (0-500 μg/mL) was performed to obtain the equation (Y = 0.019x + 0.072 3, R^2 = 0.993 7). The total antioxidant activity measured by the equation:

ATT (%) = [(As - Ac)/(Aaa - Ac)] × 100.

Where Ac is the control absorbance, As is the sample absorbance and Aaa is the Ascorbic acid absorbance.

2.3.3. FRAP assay

The stock solution of the FRAP assay was prepared using acetate buffer (300 mM / 16 mL of CH_3COOH and 3.1 g of CH_3COONa) at 3.6 pH, 10 mM TPTZ (2,4,6-tripyridyl-triazine) solubilized in $FeCl_3$ solution (20 mM) and HCl (40 mM) as described by Benzie *et al.*[46]. The assay solution was prepared by combination of the acetate buffer, TPTZ and $FeCl_3$ in a ratio of 10:1:1 (v/v/v) and incubated for 5 min at 37 ℃. Saline extract (25 μL in 1 mg/mL) was added in 0.180 mL of FRAP solution. After stirring, the extract was rested for 30 min at 37 ℃, protected from light. Subsequently the absorbance was obtained at 593 nm. A standard curve with $FeSO_4$ (0–1 000 μg/mL) was performed to obtain the equation (Y = 0.002 4x + 0.001 9, R^2 = 0.995 3). The results were obtained as mg $EFeSO_4$ (Ⅱ)/g of extract.

2.4. Antimicrobial activity test

2.4.1. Bacterial and fungal strains, culture conditions and preparation of samples

Fungal strains were obtained from the culture collections at Mycology Department from Federal University of Pernambuco and bacteria isolates were provided by the culture collection of the Departamento de Antibióticos da Universidade Federal de Pernambuco. Stock cultures were kept under refrigeration (-20 ℃) in sterilized skim milk containing 10% (v/v) glycerol. Microorganisms pathogens used in this investigation were *Candida albicans* (*C. albicans*), *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis* (*C. parapsilosis*), *Candida glabrata*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Staphylococcus saprophyticus*. For antimicrobial activity assay, bacterial and yeast species were cultured in Mueller Hinton and Sabourand Dextrose agar medium, respectively, overnight at 36 ℃, and subsequently the colonies were resuspended in sterile saline solution (0.15M NaCl) and turbidimetrically adjusted at a wavelength of 600 nm (OD_{600}) to obtain the suspension equivalent to 10^6 CFU/mL. For the assay, the samples were filtered on the sterile polyvinylidene difluoride syringe filter (pore size: 0.22 μm; diameter: 13 mm).

2.4.2. Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The MIC of the leaf extract of *T. diversifolia* was determined by the microtiter test based on CLSI[47] criteria. In 96-well microtiter plates, the extract (12 mg/mL) was added (80 μL) into the fourth well from which a serial dilution in sterile Milli-Q water was performed to the twelfth well of the same row. Subsequently, 40 μL of Mueller Hinton (bacteria) or Sabourand Dextrose (yeast) broths were added to all wells, but to the first one was filled with 200 μL of the culture medium, corresponding thus to the sterility medium

control. Antibiotics ampicillin and tetracycline (8 μg/mL) and antifungal fluconazole (64 μg/mL), obtained from Sigma-Aldrich (USA), were used as positive controls in the second well. Finally, the bacterial or yeast suspensions (80 μL, 10^6 CFU/mL) were added in the second well to the last well in the row. The third well (containing microorganisms in the absence of the sample) corresponded to the 100% growth control or negative control. The plates were incubated at 36 ℃ and the optical density was measured at time zero and after 24 h of incubation. The MIC_{90} and MIC_{50} corresponded to the lowest concentration of the sample capable of promoting a reduction of ≥90% or ≥50%, respectively, in optical density, as compared to the 100% growth control. For determination of the MBC and the MFC, aliquots of the wells containing concentrations of ≥MIC_{50} samples were inoculated into petri dishes containing Mueller Hinton or Sabourand Dextrose agar medium, which were subsequently incubated at 36 ℃ for 24 h. The MBC and MFC corresponded to the lower concentration of the sample which was able to reduce the number of CFU in 99.9% in relation to the initial inoculum. For this study, three independent experiments, performed in triplicate, were used.

2.5. Obtainment of murine splenocytes for cytotoxicity assay

2.5.1. Animals

Female BALB/c mice (6–8 weeks old) were obtained from the animal facilities of the Keizo Asami Immunopathology Laboratory–LIKA located in Federal University of Pernambuco, Brazil. Mice were kept under standard laboratory conditions (20–22 ℃/ 12 hours of day and night cycle / standard diet and water). All experimental procedures were performed in accordance with Ethics Committee of Animal Use of Federal University of Pernambuco, Brazil (protocol number: 0048 approved in December of 2016).

2.5.2. Preparation of splenocytes

This procedure was performed in accordance to Melo *et al.*[48]. Cells of the spleens of mice were processed in polystyrene conic tubes (15 mL) with Ficoll-Paque™ PLUS solution with the density adjusted to 1.076 g/mL. After three centrifugations, cells were counted in a Neubauer chamber, using the trypan blue solution. Cells were only used when viability was >95%.

2.5.3. Investigation of cell death

Mice splenocytes (10^6 cells) were treated with *T. diversifolia* saline extract in 50, 25, 12.5, 6 and 3 μg/mL and were maintained in 48-well plates for 24 h to evaluate the cytotoxicity power of the extract. Untreated cells, only in RPMI 1640 medium, were used as control. After this, lymphocytes were centrifuged twice at 26 ℃, 450 × *g* for 10 min. The pellet was resuspended in a cytometer tube containing 300 μL of binding buffer of cell viability kit (Becton Dickinson Biosciences). Annexin Ⅴ-FITC (1:500) and propidium iodide-PE (20 μg/mL) were added to tube. Samples was obtained in FACSCalibur flow cytometer (BD®) and analyzed using Cell Quest Pro software (BD®).

2.5.4. Cell proliferation analysis

Same protocol to obtained splenocytes was used for proliferation assay. After splenocytes obtainment, cell solution was centrifuged at 300 × *g* (21 ℃/5 min) with sterile PBS 1X added with SFB 5% (pH 7.2). After this, the cell solution was adjusted to1 10^6 cells/mL and was incubated (for 10 min) with 5mM of 5(6)-carboxyfluorescein diacetate N-succinimidyl ester. Cells were centrifuged at 300 × *g*/5 min with sterile PBS 1 ×. Cells stained were cultured for 24 and 48 h with 12.5 μg/mL of saline extract from *T. diversifolia* leaves and only culture complete RPMI 1640 medium (negative control). After culture time cells were centrifuged (300 × *g*/5 min), they were carried on FACSCalibur flow cytometer (BD®) and results were analyzed using Cell Quest Pro software (BD®).

2.6. Statistical analysis

Shapiro–Wilke test was applied to evaluate normal hypothesis. The statistical difference between two groups was analyzed by Wilcoxon test and among more than three groups by ANOVA. Significance level considered was 5%. GraphPad Prim 5.01 software was used in our statistical tests.

3. Results

3.1. Phytochemical analysis

The chromatogram and mass spectrum of this extract (Figures 1 A and B, respectively) indicated the presence of important classes of biologically active compounds. Identified fifteen compounds were between terpenes and phenolics, based on retention time, peak area and mass characteristics (Table 1). Total phenol compounds and total flavonoids of saline extract from *T. diversifolia* leaves showed (22.185 ± 0.201) mg GAE/g and (3.220 ± 1.085) mg QE/g, respectively.

3.2. Antioxidant activities in vitro

Results of antioxidant capacity promoted by saline extract of *T. diversifolia* leaves showed that the extract had an antioxidant capacity (3.042±0.019 mg AAE/g) similar to butylated hydroxytoluene (BHT) standard [(4.12±0.10) mg AAE/g]. The extract also promoted lower

Table 1

Compounds identified of extract saline from *T. diversifolia* leaves using ultra-performance liquid chromatography.

Compounds	Retention time	Area	Molecular formula	Molecular weight (g/mol)	[M-H]$^-$
Acetoxyanthecotulide	0.59	40 788.816	$C_{17}H_{22}O_5$	306.147 0	305.138 9
Matricin	0.59	40 788.816	$C_{17}H_{22}O_5$	306.358 0	305.138 9
Pauciflorol A	0.59	40 788.816	$C_{20}H_{34}O_2$	608.205 0	305.248 1
Rutin	0.59	40 788.816	$C_{27}H_{30}O_{16}$	610.153 4	609.145 6
Rhinocerotinoic acid	0.63	5 468.996	$C_{20}H_{30}O_3$	318.457 0	317.211 7
Dihydroferulic acid	0.69	32 424.615	$C_{10}H_{12}O_4$	196.074 0	195.065 7
5-Isopropyl-2-methylphenol	0.90	367 689.219	$C_{10}H_{14}O$	150.221 0	149.096 6
Quercetin-3-*O*-galactoside	3.35	26 832.139	$C_{21}H_{19}N_2O_8$	463.087 7	462.079 8
Gallocatechin	4.21	19 936.754	$C_{15}H_{14}O_7$	306.270 0	305.066 1
Kaempferol 3-*O*-D-glucoside	5.82	7 941.079	$C_{21}H_{19}O_{10}$	447.093 0	430.090 0
Paeoniflorin	6.06	5 254.705	$C_{23}H_{28}O_{11}$	480.466 0	479.155 3
Latrunculin B	6.98	19 184.275	$C_{20}H_{29}NO_5S$	395.177 0	394.168 8
Isotriptophenolide	9.86	46 427.395	$C_{20}H_{24}O_3$	312.409 0	311.164 7
Quercetin	9.86	46 427.395	$C_{15}H_{10}O_7$	302.042 7	301.034 8
p-coumaric acid	10.15	49 493.141	$C_9H_8O_3$	164.047 3	163.039 5

free radical sequestration [(10.11±1.15)% mg/mL; (93.13±0.58)% mg/mL; (90.04±0.16)% mg/mL, to saline extract, BHT and ascorbic acid, respectively] and was able to induce a reduction of ferric ion [(236.53±26.97) mg $EFeSO_4$ (Ⅱ)/g; (679.17±25.98) mg $EFeSO_4$ (Ⅱ)/g; (1 215.00±48.55) mg $EFeSO_4$ (Ⅱ)/g to saline extract, BHT and ascorbic acid, respectively].

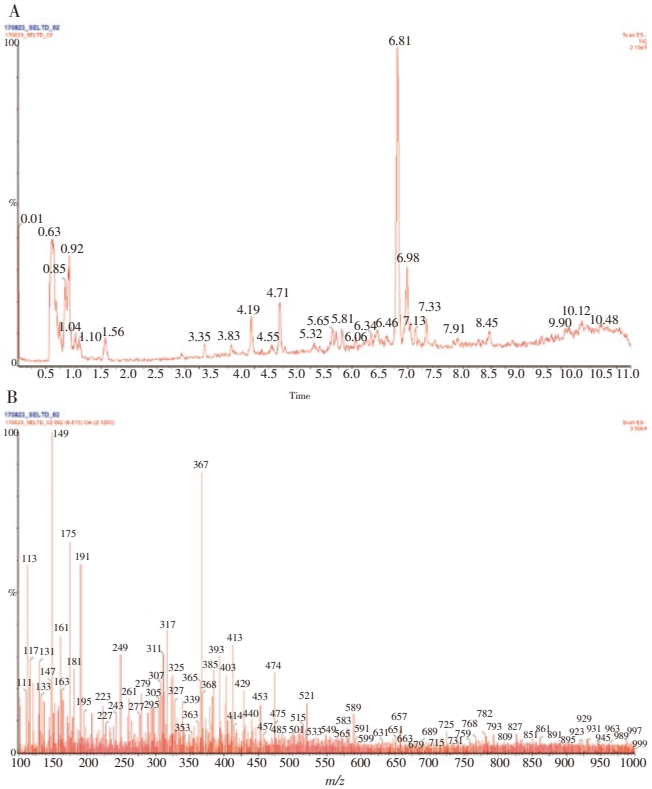

Figure 1. (A) Chromatogram and mass spectrum (B) of saline extract from *T. diversifolia* leaves.

3.3. Antimicrobial assay

The saline extract of *T. diversifolia* did not show antibacterial activity against bacterial isolates tested (data not shown). However, this compound demonstrated potential fungistatic and fungicidal properties *in vitro* against pathogenic species of *Candida*. Results showed that saline extract induced MIC_{50} in *C. albicans* (0.293 μg/mL), *Candida krusei* (0.002 4 μg/mL) and *C. parapsilosis* (0.293 μg/mL). It also induced MIC_{90} in the above same fungal strains (0.293 μg/mL; 0.004 8 μg/mL and 0.293 μg/mL, respectively) and MFC in *C. albicans* (150 μg/mL) and C. parapsilosis (0.375 μg/mL). Fluconazole, used as positive control, showed potential effects to all strains tested (except *Candida tropicalis*) and MIC_{50} values obtained were 0.25 μg/mL, 32 μg/mL, 4 μg/mL and 64 μg/mL to *C. albicans*, *Candida krusei*, *C. parapsilosis* and *Candida glabrata*, respectively.

3.4. Cell viability promoted by saline extract from T. diversifolia leaves

After the antimicrobial properties were evaluated, we performed a cytotoxicity test to investigate if saline extract from *T. diversifolia* leaves was able to kill mice splenocytes. For this test, cell cultures were treated with different concentrations of saline extract and results showed that this extract might be used with safety in concentrations equal to and lower than 25 μg/mL (Figure 2). Moreover, we evaluated if saline extract also was able to promote cell activation and proliferation in these cells. Our results showed that saline extract promoted splenocytes proliferation in higher values especially at 24 hours of assay (Figure 3).

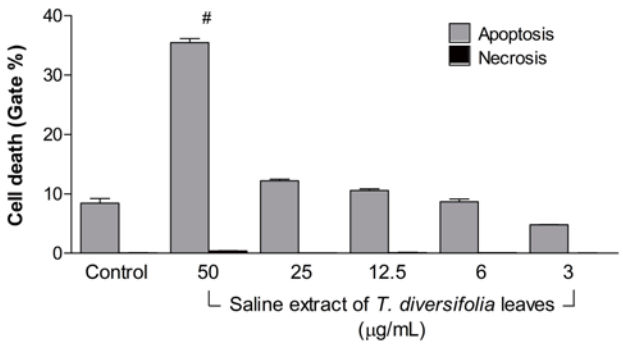

Figure 2. Mice splenocytes viability evaluated using annexin Ⅴ and propidium iodide staining.

Saline extract of *T. diversifolia* leaves promoted significant cell death, by apoptosis, only at 50 μg/mL concentration. Horizontal bars represent the average of three independent experiments performed in duplicate. $^{\#}P$<0.000 1.

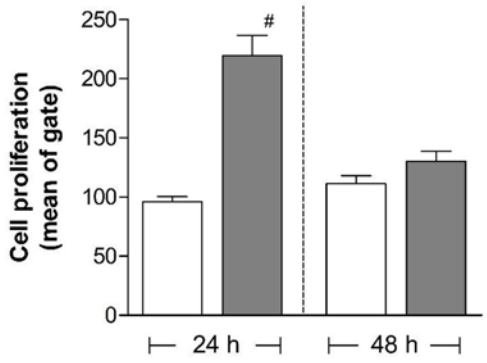

Figure 3. Mice splenocytes proliferation index evaluated using 5(6)-carboxyfluorescein diacetate N-succinimidyl ester staining.

Saline extract from *T. diversifolia* leaves promoted proliferation in mice splenocytes in both times investigated, but only at 24 hours of assay this value was significant. Vertical white bars represented negative control cells (cells+culture medium) and vertical gray bars represented saline extract from *T. diversifolia* leaves at 12.5 μg/mL concentration. This assay was performed using three independent experiments performed in duplicate. $^{\#}P$<0.000 5.

4. Discussion

T. diversifolia is commonly used in folk medicine in several continents[9] due to its phytomedicine and ethnopharmacological applicability in the treatment of various organic disturbances[49]. The extracts of *T. diversifolia* are used orally because of their analgesic capacity[50], antimalarial[51], antipyretic[52] and antidiabetic[53], and are topical applied by their anti-inflammatory potential[52] and in the treatment of microbial infections in the genitals organs[49].

Studies affirm that the species belonging to the Asteraceae family presents high concentrations of sesquiterpenes lactones[35,54] and also shows that compounds such as sesquiterpenes lactones, caffeine-derived acids, diterpenes and flavonoids are commonly found in the aerial parts of *T. diversifolia*[38]. Our results demonstrated that, similar to other extracts from leaves of *T. diversifolia* (using different types of solvents), saline extract used in this study is rich in terpenoids and phenolics compounds[36,37,50,55,56]. In fact, Gama *et al.*[57] and Pantoja *et al.*[58] demonstrated higher antioxidant capacity promoted by ethanol extract of the inflorescences of *T. diversifolia* which can be attributed to the phenols of this species. The phenolic compounds are agents that induce effectively to the antioxidant action due mainly to their reducing properties, which give them the ability to act as reducing agents, hydrogen and/or oxygen donors[59-61]. Our results also showed a similar profile of these studies, especially when compared to the commercial antioxidant BHT, when no significant difference between extract and standard was observed.

T. diversifolia species, as well as the entire Asteraceae family present high amounts of terpenoids, a natural compound with antimicrobial effects[37,38,54,62]. Several tests performed with extracts and compounds isolated from various tissues of *T. diversifolia* proved the potential antimicrobial capacity against many pathogenic species of bacteria and fungi[38,41].

However, we did not find results of antibacterial activity to our saline extract, but a significant antifungal action was observed. These results were reinforced by Ait-Ouazzou and collaborators[63] and Zengin and Baysal[64] that demonstrated that terpenoids compounds present in their preparations did not show *in vitro* inhibitory capacity against growth of the bacterial species. Moreover, Duraipandiyan *et al.*[62] demonstrated that two sesquisterpenic lactones (ostunolide and eremanthin) isolated from *Costus speciosus* (Asteraceae) were not be able to inhibit bacterial growth, but both compounds showed fungistatic properties. Assays with *T. diversifolia*, performed by Obafemi *et al.*[65] and Olayinka *et al.*[66], also demonstrated that ethanolic extracts, aqueous extracts, fractions and sesquiterpenic compound isolated from leaves, roots and stem, respectively, presented a potential antifungal activity against *C. albicans*.

Fluconazole is a potent antifungal agent in microbiology field for the treatment of candidiasis[67,68]. When we compare the fungistatic results observed from saline extract with those of fluconazole, it is indicated that the extract from *T. diversifolia* leaves also possesses antifungal potentials against *Candida* species.

Different plant extracts are indicated by traditional medicine for the treatment of viral[69-71], bacterial and fungal infections[72-75]. However, the misconception that natural products, besides not being toxic, also not possess side effects, induces the population to indiscriminate and inappropriate use, which in turn results in serious health problems, such as poisoning[76]. Many treatments, promoted by natural compounds, may be immunosuppressive or cause serious side effects[77,78]. Here we also investigated if saline extract, prepared using an usual physiological concentration (NaCl 0.15M), is dangerous to normal cells because many communities used *T. diversifolia* tea for some diseases[49,53]. Our results showed that in appropriate dose, saline extract from *T.*

diversifolia leaves is able to activate mice splenocytes and induce higher cell proliferation in these cells at 24 hours of assay, indicating a possible immunostimulant effect. Similar to our findings, other studies show that extracts from *T. diversifolia* leaves were able to promote the differentiation of mesenchymal stem cells[79] and did not promote cytotoxicity against human peripheral blood mononuclear cells and mouse macrophages[80].

In conclusion, saline extract from *T. diversifolia* leaves presented higher amounts of terpenoids and phenolics compounds, being suggestive of the presence of sesquisterpenes lactones. This extract showed antioxidant capacity, reduction of the ferric ion and antifungal properties against *Candida* species. Moreover, the extract promoted cell proliferation in mice splenocytes. These results indicate that, in future, saline extract from *Tithonia diversifolia* leaves can be investigated as a prototype candidate for antifungal agent and/or adjuvant of the antimicrobial conventional therapies.

Conflict of interest statement

The authors declare no conflict of interest.

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