Antifungal activity against *Cryptococcus neoformans* strains and genotoxicity assessment in human leukocyte cells of *Euphorbia tirucalli* L.

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Abstract

In the last times, focus on plant research has increased all over the world. *Euphorbia tirucalli* L., a plant known popularly as Aveloz, and originally used in Africa, has been drawing attention for its use in the United States and Latin America, both for use as an ornamental plant and as a medicinal plant. *E. tirucalli* L. is a member of the family Euphorbiaceae and contains many diterpenoids and triterpenoids, in particular phorbol esters, apparently the main constituent of this plant, which are assumed to be responsible for their activities in vivo and in vitro. The in vitro antifungal activities of *Euphorbia tirucalli* (L.) against opportunistic yeasts were studied using microbroth dilution assay. The results showed that aqueous extract and latex preparation were effective against ten clinical strains of *Cryptococcus neoformans* in vitro (Latex and extract MIC range of 3.2 - 411 µg/mL). Aiming the safe use in humans, the genotoxic effects of *E. tirucalli* were evaluated in human leukocytes cells. Our data show that both aqueous extract and latex preparation have no genotoxic effect in human leukocytes cells in vitro. Although the results cannot be extrapolated by itself for use in vivo, they suggest a good perspective for a therapeutic application in future. In conclusion, our results show that the aqueous extract and latex preparation from *E. tirucalli* L. are antifungal agents effective against several strains of *C. neoformans* and do not provoke DNA damage in human leukocyte cells, considering the concentrations tested.

Key words: *Euphorbia tirucalli* L., aveloz, antifungal, *Cryptococcus neoformans*.

Introduction

The *Euphorbia tirucalli* (L.), a native plant from Africa, but well adapted in Brazil has been used to treat various ills by popular medicine (Valadares et al., 2006), e.g., for treat victims of snake bites, relieve asthma symptoms and spasms. Moreover, antiviral and antimicrobial properties have also been reported, as well as the molluscicidal and larvicidal activities, beyond cytotoxicity against tumoral cells (Jueberg et al., 1985; Yadav et al., 2002; Madureira et al., 2004). This plant presents a diverse range of bioactive constituents including the isoeuphoroltriterpenoic, quercetin, rutin, gallic acid, caffeic acid, taraxasterol, tirucallol, 12-O-tetradecanoylphorbol-13-acetate (TPA, a phorbol ester), ingenane, togliane, and diterpenic acid derivatives (Furstenberger, 1986).

Infections caused by opportunistic pathogenic yeasts, particularly non-*Candida albicans*, *Cryptococcus* spp., *Trichosporon* spp., *Rhodotorula* spp. and others became a serious medical problem in immunocompromised patients, which are highly susceptible to such infections. *Cryptococcus neoformans* is an encapsulated opportunistic yeast that causes criptococosis, an disease which affects mainly immunocompromised individuals leading them to lung infection, which may spread toward the brain, causing meningoencephalitis (Mitchell and Perfect, 1995; Garcia-
The treatment choice for cryptococcosis depends on the patient’s overall condition (host’s immune status, brain injuries or only pulmonary lesions) and the extent of the cryptococcal infection (Perea et al., 2002). This yeast often shows resistance to limited option of antifungal therapy used nowadays, i.e., amphotericin B associated or not to 5-flucytosine and fluconazole. Faced with this reasoning new researches are necessary to discover new antifungal drugs (Silva et al., 2008; Perea et al., 2002).

The aim of this study was to evaluate the antifungal activity of the aqueous extract and latex preparation from *E. tirucalli* (L.) against *C. neoformans* strains and also assess the genotoxicity in human leucocyte cells.

**Material and Methods**

**Chemicals**

All the chemicals were of analytical grade. Solvents were purchased from Merck (Darmstadt, Germany). All the others reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

**Plant material**

Aerial parts of *E. tirucalli* L. were harvested in Bagé (31°19'51" S / 54°6'25" W) (State of Rio Grande do Sul, Brazil) on March of 2008. Samples of the collected material were identified by Botanist Dr. Thais Scott do Canto Dorow and archived as voucher specimens in the herbarium of Department of Biology in Federal University of Santa Maria by register number SMD 10127. The plant was cut into small pieces and dried at 37 °C for two days. Both the aqueous extract and latex preparation from *E. tirucalli* were made according to popular medicine. To prepare the aqueous extract was used 140 g of chopped aerial part of the plant in 200 mL previously boiled water per 15 min (wield = 2.35 mg/g fresh plant). The latex preparation was made mixing one fresh latex drop into 200 mL of warm water (wield = 4.02 mg/g fresh plant). After that, 5 mL of each these preparations were transferred to porcelain crucibles previously weighed and subjected to 37 °C for two days to obtain their respective dry residues, which will be added to the culture media.

**Determination of total polyphenolic contents**

The crude extract was prepared following an standardized procedure: 0.5 g of each dried sample, *i.e.*, extract and latex, was dissolved in 10 mL of ethanol and the volume adjusted to 100 mL with water. An aliquot of 3 mL of each solution was dissolved in 100 mL of water. Final concentration was 0.15 mg/mL. The total polyphenol concentration in aqueous extract from the aerial parts was measured spectrophotometrically in triplicate as described by modified Folin-Ciocalteau method (Chandra and Mejia, 2004) and read at 730 nm in a Shimadzu-UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan). The total polyphenol content was expressed as milligram equivalents of pyrogallol per milliliter of the extract or as milligram equivalents of pyrogallol per gram of fresh fraction (FF). The equation for standard curve of pyrogallol was made in the range of 0.005-0.030 mg/mL.

**Determination of condensed tannins**

The aqueous extract was prepared following a standardized procedure: 0.25 g of each dried sample was dissolved in 10 mL of methanol. Final concentration of each fraction was 25 mg/mL. The total condensed tannins concentrations in aqueous extract was measured spectrophotometrically in triplicate as described by modified Vanilin method (Morrison et al., 1995). The solution was heated for 10 min at 60 °C before reading at 730 nm in a Shimadzu-UV-1201 spectrophotometer. The contents were expressed as milligram equivalents of pyrogallol per milliliter of the extracts or as milligram equivalents of pyrogallol per gram of fresh fraction (FF). The equation for standard curve of pyrogallol was made in the range of 2.5-20 mg/mL.

**Determination of flavonoids**

Methanolic solutions of quercetin in the range of 4.0-12.0 μg/mL were used as references. To 2 mL of each reference solution, 20 mL of methanol and 1 mL of 5% AlCl₃ (w/v) were added and the volume made up to 50 mL with methanol at 20 °C. After 30 min, the absorbances were measured at 425 nm in a Shimadzu-UV-1201 spectrophotometer. The same procedure was made to analyse of the aqueous extract and latex (in triplicate) (Woisky and Slatino, 1998). The contents were expressed as milligram equivalents of quercetin per milliliter of the extracts or as milligram equivalents of quercetin per gram of fresh fraction (FF). The equation for standard curve of quercetin was made in the range of 4.0-12.0 μg/mL.

**High performance liquid chromatographic**

High performance liquid chromatography (HPLC) of the samples was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence auto sampler (SIL-20A), equipped with Shimadzu LC-20 AT recirculating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1.

**Analyze of quercetin and rutin**

Chromatographic analyses were carried out in isocratic conditions using RP-C18 column (4.6 mm x 250 mm) packed with 5 μm diameter particles. The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. The mobile phase was filtered through a 0.45 μm membrane filter and degassed in ultrasonic bath previous to use. Flow rate and injection volume were...
1.0 mL/min and 10 μL, respectively. Quercetin reference standards and samples were quantified at 368 nm. Rutin reference Standards and samples were quantified at 257 nm (Zu et al., 2006). The equation for standard curve of quercetin was made in the range of 18-280 μg/mL.

**Analyze of gallic acid and caffeic acid**

Chromatographic analyses were carried out in isocratic conditions using RP-C18 column (4.6 mm x 250 mm) packed with 5 μm diameter particles. Running conditions included injection volume: 5 μL, mobile phase composed by methanol-0.4% acetic acid (80:20, v/v), flow rate: 1 mL/min, detection at 290 nm. Gallic acid and caffeic acid reference standards, crude extract and latex were quantified at 290 nm (Singh et al., 2008). The equation for standard curve of gallic acid was made in the range of 25-1000 μg/mL, while to caffeic acid was made in the range of 25-1000 μg/mL.

**Analyze of 12-O-tetradecanoylphorbol-13-acetate (TPA)**

The TPA was identified by a gradient elution high performance liquid chromatography (HPLC) method described by Makkar et al. (1997). The equation for standard curve of TPA in the range of 10-100 μg/mL was made at 280 nm.

**Susceptibility testing**

A total of 19 opportunistic yeasts isolates were tested for the antifungal susceptibility test in triplicate. The yeasts tested were Candida albicans (ATCC90028), C. krusei (CKR01), C. parapsilosis (CPA05), C. glabrata (CG04), C. tropicalis (ATCC750), C. guillermondii (CG40039), Trichosporon asahii (TBE01), Geotrichum candidum (GEO01), Rhodotorula mucilaginosa (RHO07) and Cryptococcus neoformans (HCCR01, CRY 14, CRY 16, CR 18, CRY 15, CRY 19, CRY 20, CR 22, CRY 25, CRY 26). Inoculums of all opportunistic yeasts were prepared according the Clinical Laboratory and Standards Institute (CLSI, 2008). Minimal inhibitory concentration (MIC) of active components both aequous extract and latex preparation from E. tirucalli (L.) was carry out by the broth microdilution method following M27-A3 CLSI guidelines, with RPMI-MOPS medium (RPMI 1640 medium containing L-glutamine, without sodium bicarbonate - Sigma-Aldrich Co., St Louis, USA - buffered to pH 7.0 with 0.165 mol/L MOPS buffer -Sigma). Microtitre plates were covered with 100 μL of different concentrations of the antifungal agents and added with 100 μL of the yeast suspension. A final inoculum of 0.5 x 10⁵ to 2.5 x 10⁶ cfu/mL and the final concentrations of the antifungal agents ranged from 0.8 to 822.5 μg/mL for E. tirucalli latex and extract, 0.03 to 64 μg/mL for fluconazole and 0.007 to 16 μg/mL and amphotericin B. MIC values were determined after incubation at 72 h at 35 °C for Cryptococcus isolates and 48 h at 35 °C for Candida, Rhodotorula, Geotrichum and Trichosporon isolates. Visual determination of MIC end points was based on the lowest concentration that produced a 100% inhibition for E. tirucalli latex and extract. The MIC end points were defined for Amphotericin B as the lowest concentration of drug which resulted in a complete inhibition of visible growth, while for fluconazole was defined as the lowest concentration of drug that produced a 50% reduction in fungal growth compared to that one of drug-free growth control. Candida parapsilosis (ATCC 22019) and C. krusei (ATCC 6258) were included with each testing for quality control. All susceptibility tests were performed twice by each antifungal agent.

The minimal fungicidal concentration (MFC) was determined by sub-culturing volumes of 10 μL from wells without visible grown in Sabouraud dextrose agar (SDA) with Cloranfenicol (Difco, Detroit, USA) and incubated at 35 °C for 48 h. Minimum fungicidal concentration (MFC) was defined as the lowest concentration yielding negative subcultures.

Determination of antimicrobial percent activity (A%), total antimicrobial activity (TAA) and fungal susceptible index (FSI)

These parameters were determinate according the equations (Ellof, 2000; Rangasamy et al., 2007) listed below:

\[
A\% = \frac{100 \times (\text{Susceptible strains} - \text{Total nº of tested strains})}{\text{Total nº of tested strains}}
\]

\[
TAA = \frac{\text{Quantity of material extract from 1 g of plant material} \times \text{MIC}}{\text{FSI}}
\]

\[
FSI = \frac{100 \times \text{nº of extract effective against each fungal strain}}{\text{nº of total samples}}
\]

**Genotoxicity evaluation on human leukocytes**

A sample of whole blood was collected by venipuncture from the forearm vein and immediately placed in tubes containing heparin. This protocol was approved by the Ethics Committee of the Federal University of Santa Maria (23081.012330/2006-94). Aliquots of whole blood (4 mL) were placed in contact with 1 mL of aqueous extract and latex preparation samples (diluted in 2% DMSO in PBS Buffer pH 7.4) over a period of 2 h at 37 °C. Apart from these two groups, we performed other two group, negative and positive controls, which was incubated with a dilution vehicle (2% DMSO in PBS buffer pH 7.4) and hydrogen peroxide 25 μM, respectively. After this period, counts were made of total leukocytes and testing of cell viability and DNA damage.

In order to perform the genotoxicity tests, previously the amount of total leukocytes is achieved through counting in a Neubauer chamber (Montagner et al., 2010). Viability is assessed by the loss of membrane integrity, using the trypan blue (Burow et al., 1998). Three hundred cells are counted in this technique. The genotoxicity test was evalu-
ated by Comet Assay (Singh et al., 1995). The comet assay is not the only way to measure oxidative DNA damage, but it is one of the most sensitive and accurate, being relatively free of artefacts (Collins, 2009). One hundred cells in slides were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). Additionally, we shows the selectivity Index (SI), who is calculated as the higher tested concentration divided by the MIC50, accord-
ing. The equation of standard curve from different pharmacognostic groups show a \( r^2 \) range between 0.9937 to 0.9999, while the \( r^2 \) from compounds the range was 0.9703 to 0.9998.

Table 2 shows the fungistatic and fungicidal activities of the extract and latex preparations of Euphorbia tirucalli (L.) against Cryptococcus neoformans clinical strains, with a MIC range values /MIC50 of 3.2 - > 411/205.5 \( \mu \)g/mL and 3.2 - > 411/ > 411 \( \mu \)g/mL, respectively. The E. tirucalli L. preparations did not show activity against Candida albicans, C. krusei, C. tropicalis, C. glabrata, C. parapsilosis, C. guilliermondii, Geotrichum candidum, and Rhodotorula mucilaginosa (data not show).

There was not difference between extract and latex preparation antimicrobial percent activity (A%) and fungal susceptible index (FSI), since the strains of C. neoformans was vulnerable with both A% and FSI equal to 100%. Total antimicrobial activity (TTA) to extract and latex preparation was 185.59 mg/mL and 628.13 mg/mL, respectively.

Table 3 shows the cell viability and DNA damage index. In the present study, we did not observe differences in cell viability between the negative control (98 ± 1%) and E. tirucalli L. aqueous extract and latex preparations (97 ± 2% and 99 ± 2%, respectively) (\( p = 0.439 \)). However there was differences between the positive control vs. negative control, \( F(47.1) = 98 \pm 27 t = 9.68; p < 0.001 \); as well as between positive control vs. aqueous extract and latex preparation, \( t = 8.13 \) and \( t = 8.04 \), respectively, \( p < 0.001 \).

Table 4 shows the fungistatic and fungicidal activities of the extract and latex preparations of Euphorbia tirucalli (L.) against C. neoformans has few alternatives, which are basically represented by amphotericin B and 5-fluorocytosine in association with azole drugs. However, whether the strains have resistance against these drugs the patients show an increase in risk of death. Moreover, the amphotericin B may cause important unwanted effect, such as impairment of glomerular filtration and hepatic function, hypokalaemia and hypomagnesaemia, anaemia, thrombocytopenia, anaphylactic reactions, and neurotoxicity. In attempt to decrease these unwanted effects, liposome-encapsulated and lipid-complexed preparations have been used, however they are significantly more expensive and less efficient than native drug (Gruszczekiet al., 2003; Blau and Fauser, 2000). Consequently, there is an increasing need for new compounds with antifungal activity. Natural products, including plants, may be a source of compounds with

| Table 1 - Concentrations of some biologically important groups and compounds presents in the samples used of Euphorbia tirucalli L. |
|-----------------|---------------|-----------------|-----------------|
| Group/compound  | Extract (\( \mu \)g/mL) | Latex (\( \mu \)g/mL) | Equation of standard curve | \( r^2 \) |
| Polyphenol compounds | 2072 ± 4.07 | 592.00 ± 8.02 | \( y = 34.443x - 0.0942 \) | 0.9937 |
| Condensed tannins | 689.5 ± 24.64 | 197.00 ± 7.04 | \( y = 0.0423x - 0.1362 \) | 0.9849 |
| Total flavonoids | 882 ± 41.61 | 252.00 ± 11.89 | \( y = 0.0202x - 0.0031 \) | 0.9999 |
| Quercetin | 1.47 ± 0.11 | 0.42 ± 0.03 | \( y = 32214x - 259717 \) | 0.9968 |
| Rutin | 0.49 ± 0.07 | 0.14 ± 0.02 | \( y = 19271x - 16913 \) | 0.9998 |
| Gallic acid | 30.52 ± 1.19 | 8.72 ± 0.34 | \( y = 7606.8x - 132936 \) | 0.9703 |
| Caffeic acid | 15.61 ± 3.12 | 4.46 ± 0.89 | \( y = 20367x - 1162400 \) | 0.9890 |
| 12-O-tetradecanoylphorbol-13-acetate (TPA) | 3.12 ± 2.87 | 0.89 ± 0.82 | \( y = 27228x - 31278 \) | 0.9971 |

Data from extract and latex preparations are expressed as means ± S.D. Results were confirmed by an experiment that was repeated three times in triplicate.
Antifungal effects and therefore possible candidates for the development of new antifungal agents.

Phenolic compounds are broadly distributed in the plant kingdom and are the most abundant secondary metabolites found in plants (Macheix et al., 1990). The key role of phenolic compounds as antibacterial is emphasized in several reports (Komali et al., 1999; Moller et al., 1999). Flavonoids occur naturally in plant foods and are a common component of our diet. Flavonoids demonstrated a wide range of biochemical and pharmacological effects, including antioxidant, anti-inflammatory and antifungals (Macheix et al., 1990). Tannins have been reported to exert other physiological effects; e.g., they can reduce blood pressure, accelerate blood clotting, decrease the serum lipid level, modulate immuneresponses and produce liver necrosis (Muchuweti et al., 2006).

Natural products have been widely studied as an alternative for treating yeasts such as C. neoformans. Cáceres et al. (2012) tested the ethanol extract of Smilax domingensis against C. neoformans, showing a MIC of 500 g/mL. In the same year, Manoj and Muragan (2012) tested the methanol extract of Plagiochila beddomei against C. neoformans (MTCC 6333) also showing a MIC of 500 g/mL. In another recent article tested the fungicides activities of seven species of Lippia, the results showed an anti-C. neoformans only for the species L. sidoides, with the MIC of 625 g/mL (Fabri et al., 2011).

The data from the scientific literature are widely varied, ranging from highly active to non-active. Some works such as that published by Cos and et al. (2006) has attempted to establish standards for interpretation and evaluation of these results. According to this article, relevant and selective activity relates to IC_{50} values below 100 g/mL for extracts and below 25 M for pure compounds. If we use this article as base of results obtained in this study, we observed a significant activity of the aqueous extract of E. tirucalli against clinical isolates HCCRY01, CRY 15, CRY 22, and considerable effects (MIC 102.8 µg/mL) for CRY 19 and CRY 20. In the same parameter, the latex preparation shows a high activity on CRY 15, CRY 22 and CRY 26. In addition, the A%, TTA, and FSI are useful tools, which help to choose the better plant parts and respective preparation that should be study in deep. The values found to these parameters to the extract and latex preparation corroborate to a good perspective to E. tirucalli L. as antifungal drug.

Although the E. tirucalli L. belongs to toxic plant family, there are not reports that describe toxic effects or tissue injury at low doses as the used by non-traditional medicine. Furthermore, various factors corroborate with the use of this plant, such as its easy cultivation, the low cost of its preparations, and the low concentrations need to have antifungal activity.
Table 3 - Genotoxicity evaluation on human leukocytes.

| Sample / Test | Negative Control (vehicle) | Positive Control (H2O2 25 μM) | E. tirucalli - Aqueous extract | E. tirucalli - Latex preparation |
|---------------|----------------------------|-------------------------------|-------------------------------|--------------------------------|
| Total leukocytes/mm3 | 6500 ± 100 | 4900 ± 250* | 6400 ± 200 | 6350 ± 100 |
| Cell viability (%) | 98 ± 1 | 76 ± 5* | 97 ± 2 | 99 ± 1 |
| Index of DNA damage | 7 ± 1 | 98 ± 27* | 8 ± 1 | 9 ± 2 |
| Selective Index (SI)** | - | - | > 724.71 | > 362.53 |

Tests were carried out in triplicates, and the data are as mean ± standard error.

*Indicate statistically significant different values when compared to the negative control (p < 0.001).

** S.I., selectivity index calculated as the higher tested concentration divided by the MIC50.

In summary, our data showed that aqueous extract and latex preparation from *E. tirucalli* L. confirmed their potential as source of new alternative against *C. neoformans* infections. Is worth noting that latex antifungal activity showed a three times higher than latex. Furthermore, at the concentrations and preparations tested, the preliminary results indicate there is no genotoxicity, although, of course, results from in vitro tests cannot be extrapolated without due caution. The antifungal activity of the isolated compounds should be further investigated to confirm the impressions of this study as well as to determine their mechanism of action.

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