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Research Paper

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 effectives 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-

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Hermoso *et al.*, 1999; Kobayashi *et al.*, 2005; Paschoal *et al.*, 2004).

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 leukocyte 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 200mL 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 millilitre 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% $AlCl_3$ (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 millilitre 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 reciprocating 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. guilliermondi* (CG40039), *Trichosporon asahii* (TBE01), *Geotrichum candidum* (GEO01), *Rhodotorula mucilaginosa* (RHO07) and *Cryptococcus neoformans* (HCCRY01, CRY 14, CRY 16, CRY 18, CRY 15, CRY 19, CRY 20, CRY 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 aqueous 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 10^3 to 2.5 x 10^3 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% = 100 X of susceptible strains to a specific extract / Total nº of tested strains

TTA = Quantity of material extract from 1 g of plant material / MIC

FSI = 100 X nº of extract effective against each fungal strain / 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 MIC_{50}, according to Cappoen and co-workers (2013). The tests were carried out in triplicates, and the data are presented as mean ± standard error. All results were statistically analyzed by one-way ANOVA followed by Bonferroni's*post hoc* test. The alpha value considered to be statistically significant was $p < 0.05$.

Results and Discussion

Table 1 shows the presence of three pharmacognostic groups and five compounds determined by HPLC analysis both the latex and extract preparations from E. *tirucalli* L. All of these compounds has been reported as bioactive. How we can see, the extract preparation presents compounds concentration about three times than latex preparation. 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 µg/mL and 3.2 - > 411/ > 411 µg/mL, respectively. The *E. tirucalli* L. preparations did not show activity against *Candida albicans, C. krusei, C. tropicallis, C. glabrata, C. parapsilosis, C. guilhermondii, 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, $p < 0.001$.

There was not difference in DNA damage index between negative control *vs.* aqueous extract and latex preparation ($p = 0.187$). On the other hand, the aqueous extract ($t = 8.13$) and latex preparation ($t = 8.04$) show a lower DNA damage index when compared to the positive control, $F (33.07) = 98 \pm 27$, $p < 0.001$.

Nowadays, the scheme therapeutic to treat *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 (Gruszecki*et 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 (µg/mL)	Latex (µg/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 = 19217x - 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.

Table 2 - Fungistatic and fungicidal activities of the extract and latex preparations of *Euphorbia tirucalli* (L.) against *Cryptococcus neoformans* clinical strains.

Clinical isolates	Aqueous Extract MIC / MFC (µg/mL)	Aqueous Extract ange MIC50/MIC90sting: MIC Range* / MIC50** / MIC90*** (µg/mL)	Latex Preparation MIC / MFC (µg/mL)	Latex Preparation MIC Range* / MIC50** / MIC90*** (µg/mL)	Amphotericin MIC Range* / MIC50** / MIC90*** (µg/mL)	Fluconazole MIC Range* / MIC50** / MIC90*** (µg/mL)
HCCRY01	12.8 / 3.2		205.5 / 102.8			
CRY14	> 411 / > 411		> 411 / > 411			
CRY16	> 411 / > 411		> 411 / > 411			
CRY18	205.5 / 102.8	3.2 - > 411*	205.5 / 102.8	3.2 - > 411*	0.06-1.0*	0.125-8*
CRY 15	12.8 / 3.2	102.8**	6.4 / 3.2	205.5**	0.5**	2.0**
CRY 19	102.8 / 25.7	> 411***	> 411 / > 411	> 411***	1.0***	4.0***
CRY 20	102.8 / 25.7		> 411 / > 411			
CRY 22	12.8 / 3.2		12.8 / 3.2			
CRY 25	> 411 / > 411		> 411 / > 411			
CRY 26	205.5 / 51.4		12.8 / 3.2			

MIC50 = Minimum Inhibitory Concentration required to inhibit the growth of 50% of the isolates. MIC90 = Minimum Inhibitory Concentration required to inhibit the growth of 50% of the isolates; MFC = Minimum fungicidal concentration.

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 µg/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 µg/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 µg/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 µg/mL for extracts and below 25 µM 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 (H_2O_2 25 μM)	*E. tirucalli* - Aqueous extract	*E. tirucalli* - Latex preparation
Total leukocytes/mm3	6500 ± 100	4900 ± 250*	6400 ± 200	6350 ± 100
Cellviability (%)	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 statistical significantly different values when compared to the negative control ($p < 0.001$).
** S.I., selectivity index calculated as the higher tested concentration divided by the MIC_{50}.

In summary, our data shown 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.

References

Blau IW, Fauser AA (2000) Review of comparative studies between conventional and liposomal amphotericin B (Ambisome) in neutropenic patients with fever of unknown origin and patients with systemic mycosis. Mycoses 43:325-332.

Burow ME, Weldon CB, Tang Y, Navar GL, Krajewski S, Reed JC, Hammond TG, Clejan S, Backman BS (1998) Differences in Susceptibility to Tumor Necrosis Factor α-induced Apoptosis among MCF-7 Breast Cancer Cell Variants. Cancer Res 58:4940-4946.

Cáceres A, Cruz SM, Martínez V, Gaitán I, Santizo A, Gattuso S, Gattuso M (2012) Ethnobotanical, pharmacognostical, pharmacological and phytochemical studies on *Smilax domingensis* in Guatemala. Braz J Pharmacogn 22:239-248.

Cappoen D, Forge D, Vercammen F, Mathys V, Kiass M, Roupie V, Anthonissen R, Eynde JJV, Huygen K (2013). Biological evaluation of bisbenzaldehydes against four *Mycobacterium* Species. Eur J Med Chem 63:731-738.

Chandra S, Mejia EG (2004) Polyphenolic Compounds, Antioxidant Capacity, and Quinone Reductase Activity of an Aqueous Extract of *Ardisia compressa* in Comparison to Mate (*Ilex paraguariensis*) and Green (*Camellia sinensis*) Teas. J Agr Food Chem 52:3583-3589.

Collins AR (2009) Investigating oxidative DNA damage and its repair using the comet assay. Mut Res-Rev Mut 681:24-32.

Cosa P, Vlietinck AJ, Berghe DV, Maes L (2006) Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J Ethnopharmacol 106:290-302.

Ellof JN (2000) Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves. S Afr J Sci 96:116-118.

Fabri RL, Nogueira MS, Moreira JR, Bouzada ML, Scio E (2011) Identification of Antioxidant and Antimicrobial Compounds of *Lippia* Species by Bioautography. J Med Food 14:840-846.

Garcia-Hermoso D, Janbon G, Dromer F (1999) Epidemiological Evidence for Dormant *Cryptococcus neoformans* Infection. J Clin Microbiol 37:3204-3209.

Gruszecki WI, Gagos M, Herec M, Kernem P (2003) Organization of antibiotic amphotericin B in model lipid membranes. A mini review. Cell Mol Biol Lett 8:161-170.

Hoeprich PD (1995) Antifungal chemotherapy. Prog Drug Res 44:88-127.

Jurberg P, Cabral-Neto JB, Schall VT (1985) Molluscicide activity of the avelos plant (*Euphorbia tirucalli* L.) on *Biomphalaria glabrata*, the mollusc vector of schistosomiasis. M I Oswaldo Cruz 80:423-427.

Kobayashi CCB, Souza LKH, Fernandes OFL, Brito SCA, Silva AC, Souza ED, Silva MRR (2005) Characterization of *Cryptococcus neoformans* isolated from urban environmental sources in Goiânia, Goiás state, Brazil. Rev I Med Trop 47:203-207.

Komali A S, Zheng Z, Shetty K (1999) A mathematical model forthe growth kinetics and synthesis of phenolics in oregano (*Origanum vulgare*) shoot cultures inoculated with Pseudomonas species. Process Biochem 35:227-235.

Macheix JJ, Fleuriet A, Billot J (1990) Fruit Phenolics. CRC Press, Inc., Boca Raton, 392 pp.

Madureira AM, Ferreira MJ, Gyemant N, Ugocsai K, Ascenso JR, Abreu PM (2004) Rearranged jatrophane-type diterpenes from *euphorbia* species. Evaluation of their effects on the reversal of multidrug resistance. Planta Med 70:45-49.

Makkar HPS, Becker K, Sporer F, Wink M (1997). Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agr Food Chem 45:3152-3157.

Manoj GS, Murugan K (2012) Phenolic profiles, antimicrobial and antioxidant potentially of methanolic extract of a liverwort, *Plagiochila beddomei* Steph. Indian J Nat Prod Res 3:173-183.

Mitchell TG, Perfect JR (1995) Cryptococcosis in the era of AIDS - 100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 8:515-548.

Moller JKS, Madsen HL, Altonen T, Skibsted LH (1999) Dittany (*Origanum dictamnus*) as a source of water-extractable antioxidants. Food Chem 64:215-219.

Morrisson IM, Asiedu EA, Stuchbury T, Powel AA (1995) Determination of Lignin and Tannin Contents of Cowpea Seed Coats. Ann Bot London 76:287-290.

Muchuweti M, Ndhlala AR, Kasiamhuru A (2006). Analysis of phenolic compounds including tannins, gallotannins and flavanols of *Uapaca kirkiana* fruit. Food Chem 94:415-419.

Paschoal RC, Hirata MH, Hirata RC, Melhem MSC, Dias ALT, Paula CR (2004) Neurocryptococcosis: Diagnosis by PCR method. Ver I Med Trop 46:203-207.

Perea S, Patterson TF (2002) Antifungal resistance in pathogenic fungi. Clin Infect Dis 35:1073-1080.

Perfect JR, Cox GM (1999). Drug resistance in *Cryptococcus neoformans*. Drug Res Updates 2:259-269.

Rangasamy O, Raoelison G, Rakotoniriana FE, Cheuk K, Urverg-Ratsimamanga S, Quetin-Leclercq J, Gurib-Fakim A, Subratty AH (2007) Screening for anti-infective properties of several medicinal plants of the Mauritians flora. J Ethnopharmacol, 109:331-337.

Santos Montagner GFF, Sagrillo M, Machado MM, Almeida RC, Mostardeiro CP, Duarte MMMF, Cruz IBM (2010) Toxicological Effects Of Ultraviolet Radiation On Lymphocyte Cells With Different Manganese Superoxide Dismutase Ala16val Polymorphism Genotypes. Toxicol In Vitro 24:1410-1416.

Schofield P, Mbugua DM, Pell AN (2001) Analysis of condensed tannins: A review. Anim Feed Sci Tech 91:21-40.

Silva PR, Rabelo RAS, Terra APS, Teixeira DNS (2008) Susceptibility to antifungal agents among *Cryptococcus neoformans* varieties isolated from patients at a university hospital. Rev Soc Bras Med Trop 41:158-162.

Singh N, McCoy M, Tice R, Schneider, E (1995) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184-191.

Singh UP, Suman A, Sharma M, Singh JN, Amitah S, Maurya S (2008) HPLC analysis of the phenolic profiles in different parts of Chilli (*Capsicum annum*) and Okra (*Abelmoschus esculentus* L.) Moench. Internet J Alt Med http://ispub.com/IJAM/5/2/7183#.

Valadares MC, Carrucha SG, Accorsi W, Queiroz MLS (2006) *Euphorbia tirucalli* L. modulates myelopoiesis and enhances the resistance of tumour-bearing mice. Int Immunopharmacol 6:294-299.

Woisky RG, Slatino A (1998) Analysis of propolis: Some parameters and procedures for chemical quality control. J Apic Res 37:99-105.

Yadav R, Srivastava VK, Chandra R, Singh A (2002) Larvicidal activity of latex and stem bark of *Euphorbia tirucalli* plant on the mosquito *Culex quinquefasciatus*. J Com Dis 34:264-269.

Zu Y, Li C, Fu Y, Zhao C (2006) Simultaneous determination of catechin, rutin, quercetin, kaempferol and isorhamnetin in the extract of sea buckthorn (*Hippophaerhamnoides* L.) leaves by RP-HPLC with DAD. J Pharmaceu Biomed 41:714-719.