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Antimicrobial activity of Brazilian plants of the genera *Leguminosae* and *Myrtaceae*

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Five Brazilian plant extracts from *Pimenta pseudocaryophyllus*, *Erythrina speciosa*, *Tephrosia toxicaria*, *Inga marginata* and *Cassia leptophylla* found in Paraná State and currently used in folkloric medicines, were assayed for antibacterial, antifungal and toxic activities. The antibacterial and anti-yeast activities were assessed by a diffusion assay on Gram (+) bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), Gram (-) bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and the yeasts (*Candida albicans*, *Candida krusei,* and *Candida tropicalis*). The extracts were also tested against the phytopathogenic fungi (*Lasiodiplodia theobromae*, *Botryosphaeria ribis*, *Botryosphaeria rhodina,* and *Fusarium verticillioides*) by growth inhibition using Captan™ as control. The toxicity was evaluated in *Artemia salina* through LC_{50} (50% median lethal concentration) using Probit analysis. *P. aeruginosa* was inhibited by *P. pseudocaryophyllus* stems, *E. speciosa* and *C. lephophylla* leaves. *E. coli* by *P. pseudocaryophyllus* stems, *B. subtilis* by *C. leptophylla* leaves, and *T. toxicaria* roots. *C. albicans* by *P. pseudocaryophyllus* stems and *E. speciosa* leaves, while *C. krusei* by *P. pseudocaryophyllus* stems, and *C. tropicalis* by *P. pseudocaryophyllus* leaves, *E. speciosa* leaves and *I. marginata* leaves. *E. speciosa* and *P. pseudocaryophyllus* leaf extracts also inhibited all the phytopathogenic fungi examined. *T. toxicaria* roots showed stronger toxicity towards *A. salina*.

Key words: Antibacterial activities, antifungal activities, *Candida* species, phytopathogenic fungi, medicinal plant extracts, *Artemia salina*.

INTRODUCTION

The interest in plant natural products with various therapeutic applications has been increasing considerably because of the biological properties detected in different medicinal plant extracts. These plants are widely used as folkloric medicines in rural and urban areas, as infusion or cold macerated homogenates (Rates, 2001).

Pimenta pseudocaryophyllus (Gomes) L. R. Landrum

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belongs to the Myrtaceae family, and is a native species of the Brazilian flora with a clove-like taste. It has been used for culinary and medicinal purposes such as flavour enhancer in cooking, tea preparations, refreshing drinks, for treatment of fatigue, colds and menstrual problems, and as agents to treat diuretic and digestive problems (Landrum and Kawasaki, 1997; Lima et al., 2006; Paula et al., 2008, 2010). Pentacyclic triterpenes, glycosylated flavonoids, gallic acid, and ellagic acid have been isolated from ethanolic leaf extracts (Paula et al., 2012).

Erythrina speciosa Andrews, *Tephrosia toxicaria*, *Inga marginata* and *Cassia leptophylla* belong to the Fabaceae (Leguminosae) family of plants. *E. speciosa* is found in the Atlantic Rainforest of Brazil (Lorenzi, 1998). The analgesic, anti-inflammatory and antibacterial activities were previously reported by Cruz (1979) to treat microbial and parasitic diseases. This plant has also been described to treat ailments, dysentery, asthma, indigestion, and female infertility as folk medicine (Mitscher et al., 1987; Soto-Hernandez and Jackson, 1994; Saidu et al., 2000; Kone et al., 2011). Alkaloids and flavonoids are plant secondary metabolites described in various species of this genus (Sarragiotto et al., 1981; Nkengfack et al., 1997; Faria et al., 2007; Amir et al., 2011).

The *Tephrosia* genus presents some bioactive metabolites such as rotenoids and flavonoids. The species *Tephrosia calophylla*, *Tephrosia purpurea* and *Tephrosia maxima* of this genus was described in the treatment of various ailments, including cancer, in the Indian subcontinent (Subhadra et al., 2011), piscicides and insecticides (Cronquist, 1981).

The *I. marginata* fruit has been used for the treatment of vaginal ulcers, and the cooked stem works as an astringent and a vasoconstrictor (Lopez et al., 1987). Activities such as antibacterial, antifungal (Alvarez et al., 1998), anti-inflammatory and anti-diarrheic (Silva et al., 2007) have been described in various species of this genus. The secondary metabolites include phenolic compounds, saponins and non-protein amino and imino acids, and an over expression of protein amino acids, and galloyl tyrosine (Alvarez et al., 1998; Lokvam et al., 2004, 2006, 2007; Dias et al., 2010).

Cassia species have been described to present laxative, purgative, antimicrobial, antipyretic, antiviral and anti-inflammatory properties, and have been used as folk medicines in some regions of India, Asia and Africa (Agarkar and Jadge, 1999; Viegas-Junior et al., 2006). The pharmacological potential of the aforementioned plants may be due to the presence of a broad range of secondary metabolites such as phenolic compounds, anthraquinones, steroids and stilbenoids, and piperidine alkaloids.

Considering the resistance of microorganisms to antibiotics and the biological activities of some plants, the aim of the present work was to screen five Brazilian medicinal plant extracts collected in the region of Londrina-Paraná State, in order to identify the most active to be submitted for further studies related to the control of human or plant diseases. The phytochemical analysis of all the plants cited earlier were previously reported by Custódio (2009), Faria et al. (2007), Martinez et al. (2012), Rieger (2011), and Burgo (2010). All the results obtained herein are described for the first time with *P. pseudocaryophyllus* stems, *E. speciosa* leaves, *T. toxicaria* roots, and *C. leptophylla* leaf extracts, as well as the toxicity and antifungal assays using *Lasiodiplodia theobromae*, *Botryosphaeria ribis*, *Botryosphaeria rhodina* and *Fusarium verticillioides* with the *P. pseudocaryophyllus* and *I. marginata* leaf extracts are also described.

MATERIALS AND METHODS

Plant

P. pseudocaryophyllus leaves and stems were collected on a private property in São Jerônimo da Serra, Paraná-Brazil (latitude 23° 43' 30" S, and longitude 50° 43' 47" W), with the owner's permission in December 2007, under the supervision of a botanist.

E. speciosa, *C. leptophylla*, and *I. marginata* leaves were collected in August 2007, March 2009 and April 2010, respectively, on the Campus of Universidade Estadual de Londrina (UEL), Londrina-Paraná, Brazil (latitude 23° 19' 25" S, and longitude 51° 12' 65"W), under an UEL botanist supervision.

T. toxicaria was donated by Instituto Agronômico de Campinas (IAC), Campinas – São Paulo (SP), Brazil, in the 1970's to Instituto Agronômico do Paraná (IAPAR), Londrina-Paraná, Brazil (latitude 23°22' S, and longitude 51°10' W), where it was cultivated and collected with authorization in July of 1987. Specimens voucher of these plants were deposited at UEL and IAC Herbaria (Table 1).

Preparation of the different plant extracts

Air-dried plant materials were ground in a knife mill, and the powder extracted exhaustively with different organic solvents [ethyl acetate (EA), and ethanol (ET)], and filtered through filter papers. The organic solvents were removed by vacuum distillation at 55 and 60°C using a rotary evaporator. The roots from *T. toxicaria* were directly and exhaustively extracted with EA to obtain the EA extract. Then it was fractionated by chromatography on a column of silica gel (50 cm × 10 cm diameter), and was eluted with hexane (HE) and methanol (ME). Parts of these fractions were used to develop the biological tests of this work. The results of all extracts and the yields are presented in Table 1.

Microorganisms

Bacterial, yeast and filamentous fungal isolates

Two Gram (+) bacterial strains, *Bacillus subtilis* (ATCC 8272) and *Staphyloccocus aureus* (ATCC 25923), and two Gram (-) bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922), were kindly donated by Dr. R. M. B. Quesada (Department of Microbiology, University Hospital, UEL, Londrina, PR-Brazil), and were also used to develop the screening tests. The bacterial strains were grown on Müeller-Hinton agar (MHA) medium (Oxoid), and the yeasts on Sabouraud Dextrose Agar (SDA) medium (Difco). After growth, bacteria were maintained in

Table 1. Method of extraction and yield (%) from Brazilian plant extracts.

Scientific name and voucher specimen	Popular name	Method used in the extractions	Yields (%, w/w)[b]
Pimenta pseudocaryophyllus (FUEL nº 43025)[a]	"Cataia", "Craveiro" "Lourodo-Mato", "pau-cravo, craveiro"	The leaves and stems were dried, pulverized and exhaustively extracted with EA at room temperature.	6.2 (LE) 1.67 (SE)
Erythrina speciosa (FUEL nº 3513)[a]	"Mulungu", "mulungu do litoral" and "eritrina candelabro"	The dried leaves were pulverized and exhaustively extracted with ET at room temperature.	9.4 (LE)
Tephrosia toxicaria (IAC 17211)[a]	"timbó de caiena"	The roots were dried, ground in a knife mill, and then submitted to exhaustive extraction with EA. This extract was fractionated using HE and ME.	1.1 (RE)
Inga marginata (FUEL nº 40918)[a]	"Ingá-feijão"	The dried leaves were pulverized and exhaustively extracted with EA at room temperature.	6.0 (LE)
Cassia leptophylla (FUEL nº 46129)[a]	"falso-barbatimão"	The dried leaves were pulverized and exhaustively extracted with EA at room temperature.	2.4 (LE)

[a]FUEL:Herbarium Universidade Estadual de Londrina. IAC: Instituto Agronômico de Campinas. [b](W1*100)/W2; W1 is the weight of the extract after dried; W2 is the weight of the plant powder. EA: Ethyl acetate; ET: ethanol; HE fr.: hexane fraction; ME fr.: methanol fraction; LE: leaf extract; SE: stem extract; RE: root extract.

Eppendorf tubes on MHA, and yeast on SDA at 4°C. The strains were activated by subculture at 37±1°C over 18 to 24 h on an appropriate freshly poured agar plate prior to any of the antimicrobial tests.

Candida albicans, *Candida krusei* and *Candida tropicalis* used to develop the antimicrobial assays were isolated at UEL in the microbial laboratory of the University Hospital (HU), Londrina, PR-Brazil, where these strains were deposited.

Four filamentous phytopathogenic fungi: *B. rhodina* MAMB-05 [ACCESSION: AY612337-isolated from eucalypt canker (Australia)], *B. ribis* EC-01 [ACCESSION: DQ852308-isolated from *Eucalyptus citriodora* (Brazil)], and *L. theobromae* MMPI [ACCESSION: DQ852315-isolated from Pinha (Brazil), that had the RNA gene sequences, deposited in GenBank, were used for antifungal tests (Saldanha et al., 2007), and a *F. verticillioides* isolated from a contaminated animal feed, identified in the Science University of Tokyo, Japan (Morey et al., 2009). The filamentous fungi were preserved on Potato Dextrose Agar (PDA) at 4°C, and subcultured on PDA (Difco) plates at 28± 2°C for 5 to10 days prior to use.

Antimicrobial activities

Two different methods were employed to determine *in vitro* antimicrobial activities from the Brazilian plants extracts.

Antimicrobial activity: Assays by the well-plate diffusion method

Plant extracts were prepared at a concentration of 5 to 7 mg/ml in dimethyl sulfoxide (10%; DMSO, Merck) as solvent, and sterilized by filtration (0.20 µm Millipore filters). Extracts were screened for antimicrobial activity using the well-plate diffusion method (NCCLS, 1997, 2001). Cell suspensions of 2.0 × 10^8 CFU/ml (24 h cultures) were used as inocula. Aliquots of a 50 µl suspension containing the tested microorganism were overlaid on Petri dishes containing 14 ml of Müeller-Hinton agar for bacteria, or Sabouraud Dextrose agar for yeasts. Wells (6 mm diameter) were cut out using a cork borer, and were individually impregnated with 30 µl of extracts dissolved in DMSO (10%, v/v) and 0.5% Tween 80. Wells prepared under the same condition with the same volume of 10% DMSO solution and 0.5% Tween 80 were used as a negative control. Thirty (30 µg) of anfotericin B (Fungizon) and 50 µg of the tetracycline (tetracycline hydrochloride), penicillin (Penicillin G,crystalline) and Nystatin (Mycostatin) were prepared in 0.5% (v/v) Tween 80 in 1 ml of water. The antimicrobial references referred to earlier were used as positive controls to determine the sensitivity of one microbial strain/isolate in each of the species tested. Inoculated plates were incubated at 37°C for 24 h for bacterial strains, and 48 h for yeasts. After the incubation period the inhibition zones were measured (mm) with a ruler, against the test organisms. All the tests were performed in duplicate.

Test for antifungal activity

The *in vitro* biological activity of ethanolic extracts was assessed based on the radial hyphal growth rate of the fungi tested in the presence and absence of the plant extracts. Agar plugs of 7-mm diameter colonized with five filamentous phytopathogens were used as inocula. Plant extracts of concentrations of 1.48 to 1.54 mg/ml were dissolved in DMSO containing three drops of Tween 80. Aliquots (0.8 ml) of each extract were added to the assay in flasks containing 17 ml of sterile growth medium (PDA). After vortexing,

17.8 ml was poured into Petri dishes (60 × 15 mm). Plates containing only PDA with DMSO (0.8 ml) were used as negative controls. Positive controls contained the fungicide Captan™ [4-cyclohexene-1, 2-dicarboximide, N-(trichloromethyl)thiol] (0.18%). The assay was performed by placing a 7-mm diameter agar plug containing the growing fungal mycelium at the center of a Petri dish, and left to growth. Three replicates were run simultaneously (Quiroga et al., 2001). In all the plates, the radial mycelia growth was measured after 7 to 10 days, which was the time required for the micro-organisms to grow in the culture medium containing only PDA at 28 ± 2°C. Each data point represented the mean of at least four measurements of a growing colony (MGC: mycelial growth control; MGPE: mycelial growth with the plant extract). Growth inhibition (GI) was calculated from the expression:

GI (%) = MGC – MGPE/MGC. All experiments were conducted in replicates of three.

Toxicity to *Artemia salina*

A. salina (brine shrimp) assay was performed according to Meyer's method (Meyer et al., 1982) with some modifications. An amount of *A. salina* eggs (Maramar, Rio de Janeiro) was incubated in fresh artificial sea water. All plant extracts and the positive control using potassium dichromate solution (PDS, $K_2Cr_2O_7$) were dissolved in three drops of Tween 80, 2 ml of dimethyl sulfoxide (DMSO), nine to twelve nauplii larvae were deposited in each glass tube, and the volume was completed to a final 5 ml with artificial sea water.

After 24 h incubation, the number of survivors and dead-hatched, brine shrimp larvae, (nauplii) in each one of the tubes were noted and registered. LC_{50} (50% median lethal concentration values) and 95% confidence limits were estimated using Finney's statistical method by Probit analysis (Finney, 1952). Two control samples, one of which contained fresh artificial sea water, and other with PDS, were run simultaneously under the aforementioned conditions. The experiments were performed in quadruplicate and developed according to Sam (1993).

Statistical analysis

The filamentous antifungal data were expressed as mean ± standard error of mean (SEM), and data evaluated by statistical analysis (ANOVA) to determine the significance level of the differences ($p<0.05$). The statistical significance for the differences between extracts was detected by ANOVA, followed by the Tukey test. The *p* values under 0.05 ($p<0.05$) were considered significant. Data analysis was performed by R software (R Core Team (2013), http:/www.R-project.org/). A pairwise comparison of each treatment was performed using Tukey's multiple-range comparison tests to identify the significant differences between the fungal bioassay results. Finney's statistical method by Probit analysis (Finney, 1952) was used to analyse the toxicity to *A. salina*.

RESULTS AND DISCUSSION

The phytochemical analysis of the ethanolic extracts was obtained from the leaves and stems of *P. pseudocaryophyllus,* and was previously described for having triterpenes by Custódio (2009). Paula et al. (2008), by contrast reported phenolic compounds, flavonoids and tannins to be present in ethanolic extracts of the leaf, collected in the state of Minas Gerais (Brazil). More recently, however, pentacyclic triterpenes, glycosylated flavonoids, gallic acid, and ellagic acid were also isolated (Paula et al., 2012).

The alkaloid nororientaline was isolated from *E. speciosa* leaves (Faria et al., 2007), and the extract was studied in this work. A further two alkaloids, erysotrine and erythartine, were described by Faria et al. (2007), and were isolated from the flowers of this plant. Moreover, other species showed the presence of alkaloids (Folkers and Koniuszy, 1940; Folkers et al., 1941; Soto-Hernandez and Jackson, 1994; Chacha et al., 2005; Cui et al., 2009).

The ethyl acetate extract of *T. toxicaria* roots used in this work was evaluated for antimicrobial and toxic activities, and was previously described by Martinez et al. (2012) to contain rotenoids, flavonoids, a biflavonoid, flavanols and coumarins, and also possessed antioxidant activity. In the same way, the extract of *I. marginata* leaves used herein, were reported by Rieger (2011) to contain triterpenes and antraquinones. On the other hand, Alvarez and collaborators (1998) showed the presence of saponins, tannins, phytosterols, and triterpenes in the stem extracts of this last plant.

The ethyl acetate extract of *C. leptophylla* leaves used in this work had the phytochemistry studied by Burgo (2010), who reported the presence of sitosterol and antraquinones. Phenolic compounds, anthraquinones, steroids, triterpenoids, saponins, stilbenoids, and piperidine alkaloids have been described in different *Cassia* species by Bolzani et al. (1995) and Yang et al. (2003).

Antimicrobial activities: Bacteria and yeast

According to the results shown in Table 2, the Gram-negative bacterium, *P. aeruginosa,* was sensitive to three extracts tested. The *P. pseudocaryophyllus* stem extract was active in two Gram-negative bacteria. Only *T. toxicaria* (fraction methanol) and *C. leptophylla* extracts, showed antibacterial activities against Gram-positive *B. subtilis*, and neither extract inhibited *S. aureus*.

P. aeruginosa is responsible for a wide variety of acute and chronic human infections, including patients with severe burn wounds, urinary tract infections, AIDS, lung cancer, chronic obstructive pulmonary disease, and cystic fibrosis (Balasubramanian et al., 2013). This pathogen exhibited an inhibition zone of 12 mm using extracts of *E. speciosa* and *C. leptophylla*.

The Gram-negative bacterium, *E. coli*, was susceptible only to the stem extract of *P. pseudocaryophyllus*. This pathogen can cause serious intestinal or extra-intestinal diseases in humans and animals (Leimbach et al., 2013). This activity may be explained by the presence of triterpenes in this extract. Literature data indicated that betulinic, ursolic and oleanolic acids and derivatives showed antimicrobial activities (Silva et al., 2012). Also, amyrins, ursolic acid and their 3-*O*-fatty acid ester chains were active against a series of Gram-positive and Gram-

Table 2. Antimicrobial activity from different extracts by the well-plate diffusion method.

Plant species	Plant parts	Extracts or fractions (µg/well)	Inhibition zone (cm)						
			Gram-positive		Gram-negative		Yeast		
			Bacillus subtilis	*Staphyloccocus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*	*Candida krusei*	*Candida tropicalis*
Pimenta pseudocaryophyllus	Leaves	EA (168.0)	-	-	-	-	-	-	1.1
	Stem	EA (165.0)	-	-	1.2	1.2	0.9	1.3	-
Erythrina speciosa	Leaves	ET (159.0)	-	-	1.2	-	0.9	-	1.1
Tephrosia toxicaria	Roots	HE fr. (199.0)	-	-	-	-	-	-	-
	-	ME fr. (198.0)	0.8	-	-	-	-	-	-
Inga marginata	Leaves	ET (195.0)	-	-	-	-	-	-	0.9
Cassia leptophylla	Leaves	EA (216.0)	0.8	-	1.0	-	-	-	-
Nystatin	-	0.0015	nt	nt	Nt	nt	2.0	-	2.0
Amphotericin B	-	0.0009	nt	nt	Nt	nt	nt	3.0	nt
Tetracycline	-	0.0015	-	-	1.5	2.0	nt	nt	nt
Penicillin	-	0.0015	2.5	2.0	Nt	nt	nt	nt	nt

-: Inactive; nt: not tested; EA: ethyl acetate; ET: ethanol; HE fr.: hexane fraction; ME fr.: methanol fraction.

negative bacteria (Mallavadhani et al., 2004). The antimicrobial activity of the ethanolic extract of *P. pseudocaryophyllus* leaves collected in different Brazilian states have been described to be active against Gram-positive bacteria and the yeast *C. albicans* (Paula et al., 2008, 2009, 2012).

Antimicrobial results on Gram-positive bacteria showed the efficacy of *C. leptophylla* and *T. toxicaria* (fraction methanol) extracts against *B. subtilis*. In addition, *C. fistula* is considered a medicinal plant with pharmacological activity, which includes antibacterial and antifungal activities (Kumar et al., 2006).

Antimicrobial activities: Phytopathogenic filamentous fungi

As shown in Table 3, the Brazilian plant extracts tested were found to be active towards four phytopathogenic fungi at low concentrations. Among the tested extracts, only *P. pseudocaryophyllus* and *E. speciosa* leaves inhibited all of the phytopathogens examined.

The fungus *L. theobromae,* an anamorphic form of *B. rhodina* (Saldanha et al., 2007), has caused serious crop damages in Brazil, particularly to tropical fruits and mango trees. It has been associated with leaf spots, necrosis, gummosis and even death of many host plants (Costa et al., 2010).

The statistical analysis of the colony diameter using *P. pseudocaryophyllus* leaf and stem extracts (Table 3) showed no significant difference at the 5% level to inhibit *L. theobromae* (p=0.4411), and *B. rhodina* (p=0.5173) growth, considering that the inhibitions were 36.63% (leaves), 30.30% (stems), 29.90% (leaves), and 36.85% (stems), respectively. Then both plant extracts can be used to inhibit these fungi. Similar results were obtained for *F. verticillioides* (p=1.0000), considering that the percentage of inhibition was 6.20% (leaves) and 6.98% (stems), respectively. On the other hand, the *P. pseudocaryophyllus* stem extracts did not inhibit *B. ribis*.

The fungicidal activity of *E. speciosa* leaf extract (50.31%) on *F. verticillioides* (Table 3) was superior when compared with Captan™ fungicide (47.85%; p=0.9984). The methanolic fraction from *T. toxicaria* root (28.11%; p=0.0001) also inhibited this phytopathogen, but it was lower than Captan™. On the other hand, there was no statistical significant difference regarding the inhibition percentage for extracts from *P. pseudocaryophyllus* leaves (6.20%; p=0.5750) and stems (6.98%; p=0.6102), the hexanic fraction from *T. toxicaria* roots (9.44%; p=0.1334), *I. marginata* (5.74%; p=0.7442) and *C. leptophylla* leaves (5.04%; p=0.8552), in comparison to the

Table 3. Growth inhibition of plant extracts on phytopathogens filamentous fungi.

Plant species	Plant parts	Extract and fractions (mg/ml)	*Lasiodiplodia theobromae*		*Botryosphaeria Ribis*		*Botryosphaeria rhodina*		*Fusarium verticillioides*	
			Radial growth (cm)	Inhibition (%)	Radial growth (cm)	Inhibition (%)	Radial growth (cm)	Inhibition (%)	Radial growth (cm)	Inhibition (%)
Pimenta pseudocaryophyllus	Leaves	EA (1.48)	$4.24^{a,c}$	36.63	$3.79^{b*\lozenge,c}$	21.14	2.78^{c}	29.90	$4.41^{b,c}$	6.20
	Stem	EA (1.50)	$4.64^{a,c\lozenge,e}$	30.30	4.88^{b*}	-	$2.49^{a,c,d}$	36.85	$4.43^{b,c}$	6.98
Erythrina speciosa	Leaves	ET (1.52)	$4.31^{a,c*,e}$	35.36	$4.27^{b,c}$	7.05	2.81^{c}	28.68	2.35^{a}	50.31
Tephrosia toxicaria	Roots	HE fr. (1.48)	$3.91^{a,c*\lozenge,d}$	41.32	$5.25^{b\lozenge}$	-	$2.51^{c,d}$	36.50	$4.28^{b,c}$	9.44
	-	ME fr. (1.54)	$4.10^{a,c,d}$	38.41	$4.51^{b,c}$	-	2.75^{c}	30.37	3.40^{*}	28.11
Inga marginata	Leaves	ET (1.54)	3.56^{d}	46.40	$5.20^{b\lozenge}$	-	2.91^{c}	26.35	$4.46^{b,c}$	5.74
Cassia leptophylla	Leaves	EA (1.52)	$4.84^{a,e}$	27.37	$4.84^{b\lozenge}$	-	$2.20^{a,d}$	43.81	$4.49^{b,c}$	5.04
Fungicide Captan^{o}	-	1.8×10^{-6}	4.38^{a}	34.33	1.47^{a}	67.96	2.06^{a}	47.79	2.47^{a}	47.85
Negative control$^{\phi}$	-	-	6.68^{b}	-	4.46^{b}	-	3.95^{b}	-	4.73^{b}	-

EA: Ethyl acetate; ET: ethanol; HE fr.: hexane fraction; ME fr.: methanol fraction; -: Inactive; ^{o}: positive control; $^{\phi}$: control used to calculate inhibition: DMSO 10%, three drops of Tween 80; ^{a,b,c,d,e}Values in the same letters did not differ in significance ($P < 0.05$), analysis in the same column indicates significance and not differences between growth zone (cm). $^{*\lozenge}$Values with different significances.

negative control.

Additionally, *E. speciosa* extracts were effective for all of the filamentous fungal species tested (Table 3) in this work, particularly *F. verticillioides*. These results were in accordance with those presented by Soto-Hernández and Miguel-Chávez (2006), which showed that the interaction of the various alkaloids detected in the *Erythrina coralloides* extract could cause growth inhibition of fungi. In contrast, the phytopathogen *B. ribis* was susceptible only to the leaf extracts of *P. pseudocaryophyllus* and *E. speciosa*.

In relation to the results obtained with the fractions of *T. toxicaria* root extracts (Table 3), there was no statistical significant difference against *L. theobromae* (p=0.9816), as the inhibition was 41.32% (HE), and 38.41% (ME), and against *B. rhodina* (p=0.7184) 36.50% (HE), and 30.37% (ME). However, using these same fractions, there were significant differences for *F. verticillioides* (p=0.0001) with 9.44% (HE), and 28.11% (ME) of inhibition. These fractions did not inhibit *B. ribis*.

The analysis of *C. leptophylla* and *I. marginata* leaf extracts revealed that there was significant difference (p=0.0001) on *L. theobromae* inhibition (27.37 and 46.40%, respectively), and similar results were found for *B. rhodina* (43.81 and 26.35%, respectively). However, *F. verticillioides* inhibition did not show significant differences (p=1.000). These extracts did not inhibit *B. ribis*.

Results for antifungal activity in the six plant extracts when considering *L. theobromae*, were statistically similar to the Captan™ fungicide (34.33%). This phytopathogen was powerfully inhibited by *I. marginata* leaf extracts (46.40%), with significant difference compared to the Captan™ control (p=0.0009). On the other hand, the fungus *B. ribis* was only inhibited by extracts of *P. pseudocaryophyllus* leaves (21.14%) and *E. speciosa* leaves (7.05%), and both presented significant differences as compared to Captan™ fungicide (67.96%), with (p=0.0001) for extracts.

Although all the plant extracts examined inhibited the fungus *B. rhodina*, the extract activities from *P. pseudocaryophyllus* stems (36.85%; p=0.0567), and *C. leptophylla* leaves (43.81%; p=0.9829) was statistically similar to the

Table 4. Toxic effects of Brazilian plant extracts using the *A. salina* lethality test.

Plant name	Used parts	Solvent	LC_{50} (µg/ml)	95% Confidence interval (µg/ml)
Pimenta pseudocaryophyllus	Leaves	EA	141.41	133.56 - 149.43
	Stem	EA	710.64	662.48 - 765.41
Erythrina speciosa	Leaves	ET	476.41	425.11 - 535.09
Tephrosia toxicaria	Roots	HE fr.	241.74	253.71 - 231.29
	-	ME fr.	1.71	1.70 - 1.72
Inga marginata	Leaves	ET	285.84	271.04 - 300.98
Cassia leptophylla	Leaves	EA	1,243.94	1,314.98 - 1,180.80
Potassium dichromate	-	-	16.24	15.89 - 16.58
Distilled water	-	-	-	<1000

EA: Ethyl acetate; ET: ethanol; HE fr.: hexane fraction; ME fr.: methanol fraction.

positive control Captan™ (47.79%; *p*=0.0567; p=0.9829). Furthermore, these extracts also exhibited good antifungal activity towards *L. theobromae*. In addition, there was significant antifungal activity difference for *F. verticillioides* between extracts with 6.20, 28.11 and 50.31% of inhibition (p=0.0001) for *P. pseudocaryophyllus* leaves; the methanolic fraction of *T. toxicaria* and *E. speciosa*, respectively. However, *P. pseudocaryophyllus* and *E. speciosa* extracts showed no significant difference towards *L. theobromae* and presented 36.63 and 35.3% activity (p=0.9999), *B. ribis* 21.14 and 7.05% activity (p=0.8528), and *B. rhodina* 29.90 and 26.68% activity (p=0.9999).

Among all of the plant extracts evaluated within this work, *I. marginata* ethanolic extract showed the highest growth inhibition for *L. theobromae* (46.40%), while *C. leptophylla* ethyl acetate extract was the highest for *B. rhodina* (43.81%). Results suggest that the antifungal activity can be correlated with the presence of sitosterol in the *C. leptophylla* extract, and stigmasterol in *I. marginata*. These compounds according to Haraguchi et al. (1999) could change the membrane permeability arising from membrane lipid alteration, thus promoting the fungal inhibition.

Brine shrimp toxicity test

The toxicity of the different plant extracts is shown in Table 4. Furthermore, ethanolic extracts obtained from *P. pseudocaryophyllus* leaves were found to be toxic (LC_{50}=141.41 µg/ml) to *A. salina*. These extracts can be explored for future cytotoxic studies based on the literature data that have shown important activities of this plant or genus species. The presence of tannins in extracts from *Pimenta dioica* leaves and cytotoxic assessment showed activity against solid tumors and cancer cells (Marzouk et al., 2007). In addition, the extract of *E. speciosa* leaves (LC_{50}=476.41 µg/ml) was toxic to *A. salina*, while the dichloromethane extracts of *Erythrina crista* bark used in Argentina was also described as being cytotoxic, and presented DNA interaction (Mongelli et al., 2000).

The lethality of the ME fraction from *T. toxicaria* roots was notable on brine shrimp (LC_{50}=1.71 µg/ml), followed by the HE fraction (LC_{50}=241.74 µg/ml). These results are in agreement with data from others species of this genus used as folkloric medicines in the Indian subcontinent: *T. calophylla*, *T. maxima* and *T. purpurea*. Root chloroform extracts from these plants were active to hatchability and lethality of brine shrimp (LC_{50}:70.7 to 100.0 µg/ml) assays, and these extracts were cytotoxic to animal cell-lines (Subhadra et al., 2011). According to these authors, the most active extracts bore high contents of phenolic compounds and flavonoids. It is well known that the various biological properties provided by these metabolites are due to antioxidant activity. Similarly, the powerful and very interesting toxic activity of the ME fraction from *T. toxicaria* roots can be explained by the presence of phenolic compounds, flavonoids along with rotenoids extracted with methanol. However, the activity of the HE fraction was probably due to the presence of the rotenoides (as tephrosin, rotenolone, deguelin) present in the EA extract. The presence of these compounds extracted with polar solvents had previously been described by Andrei et al. (1997). The anticancer (Kim et al., 2008) and insecticide (Khater, 2012) activities of degelin isolated from natural sources have been reported. Furthermore, the cancer chemopreventive properties of *T. toxicaria* were attributed to bioactive metabolites (Jang et al., 2003; Vasconcelos et al., 2009).

I. marginata leaves (LC_{50}=285.84 µg/ml) was toxic to *A. salina*, and the bark extract of this same plant was

ichthyotoxic to the guppy fish, *Poecilia reticulata*, and also inhibited tumor growth (Alvarez et al., 1998).

Conclusion

Antibacterial activity was detected in the plant extracts from *P. pseudocaryophyllus* stems (*P. aeruginosa* and *E. coli*) and *E. speciosa* (*P. aeruginosa*) and antifungal activity (inhibited *Candida* species). Three other plant extracts from *I.marginata*, *C. leptophylla* and *E. speciosa* inhibited *L. theobromae*, *B. rhodina* and *F. verticillioides*. The methanol fraction from *T. toxicaria* showed higher toxicity towards *A. salina* in comparison to the five plants screened. The results suggest future investigations on human cells to search for new drugs, and on the studied fungi to control them in plants diseases.

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Conflict of Interests

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

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