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Original Article

In vitro and *in vivo* antifungal activity, liver profile test, and mutagenic activity of five plants used in traditional Mexican medicine

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ABSTRACT

Berberis hartwegii Benth., Berberidaceae, *Hamelia patens* Jacq., Rubiaceae, *Dendropanax arboreus* (L.) Decne & Planch., Araliaceae, *Erythrina herbacea* L., Fabaceae, and *Zanthoxylum caribaeum* Lam., Rutaceae, acetone extracts were selected on the basis of their use in traditional Mexican medicine to treat scabies or skin diseases. Anti-dermatophyte activity *in vitro* was evaluated using the agar dilution assay, and the therapeutic efficacy of *B. hartwegii* and *Z. caribaeum* were tested against experimental *tinea pedis*. The infected animals were treated intragastrically daily for seven days with 2.5 and 5 mg/kg of acetone extracts. The acetone extract of *H. patens* exhibited 100% growth inhibition against *T. mentagrophytes* and *E. floccosum* at 100.0 and 50.0 μg/ml, respectively, and *B. hartwegii* inhibited growth of *M. canis* and *T. mentagrophytes* at 100.0 μg/ml. Effective treatments with 2.5 mg/kg of *Z. caribaeum* and *B. hartwegii* extract were comparable with 1 mg/kg of clotrimazole in mice. Liver profile tests and histological analyses did not exhibit any signs of toxicity and the Ames test indicated that both extracts were safe when evaluated in strains TA98, TA100 and TA102. Our results suggest the potential for the future development of new antifungal drugs from *B. hartwegii* or *Z. caribaeum*.

Introduction

Skin, hair and nail fungal infections are common and widespread. These infections are caused by three groups of fungi: dermatophytes, yeasts and molds (Schwartz, 2004). Dermatophytes are the primary cause of these infections, particularly *Trichophyton rubrum* (Moreno and Arenas, 2010). Dermatophytes can invade several keratinized tissues and use the keratin as a carbon, nitrogen and sulfur source. In humans and other animals, these fungi can produce an infection known as dermatophytosis (Vermout et al., 2008; Weitzman and Summerbell, 1995).

Superficial mycoses are not life-threatening but are a serious problem for the quality of life because these infections can cause serious discomfort and cosmetic deformities (Koga et al., 2012). These diseases are observed worldwide and affect 20–25% of the world's population (Havlickova et al., 2008). The incidences of these infections have not changed within the last three decades (Borman et al., 2007; Havlickova et al., 2008), although many antifungal drugs with potent action against this species have been introduced into the market (Brennan and Leyden, 1997; Kumar and Kimball, 2009). Current treatment is based on the use of topical antifungal agents, or systemic treatments in the case of extended or recurrent infections (Bell-Syer et al., 2012; Dik et al., 2003). The drugs that are most commonly used against these diseases include amphotericin B, ketoconazole, fluconazole, terbinafine and clotrimazole (Khan and Ahmad, 2011). These drugs are sometimes inefficient and often present nephrotoxicity and hepatotoxicity (Andriole, 1999; Somchit et al., 2004; Zapata-Garrido et al., 2003). In addition, the dermatophyte resistance to antifungal drugs has resulted in the need for novel antimycotic agents with a broad spectrum and fewer side effects (Duraipandiyan et al., 2012; Girois et al., 2006; Khan and Ahmad, 2011). Thus, a more potent therapeutic drug that is capable of shortening treatment duration and improving patient adherence rate would be beneficial (Koga et al., 2012).

Medicinal plants are a source of bioactive compounds used to treat many diseases. Many plants possess antimicrobial agents and

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exhibit antifungal activity (Lemos et al., 2005). Several *in vitro* and *in vivo* studies using plant products traditionally used in ethnomedicine have demonstrated promising antifungal activity without any side effects (Bajpai et al., 2009; Park et al., 2007; Sokovic and Griensven, 2006).

The aim of this study was to evaluate Mexican plants for activity against dermatophyte strains, particularly *T. rubrum*. Plants were selected on the basis of their use in traditional medicine to treat scabies or diseases with related symptoms, such as skin diseases. Acetone extracts from five plant species were evaluated for antifungal activity using the agar dilution assay and "experimental *tinea pedis*" model in BALB/c mice. In addition, experimental toxicology studies, including liver profile tests and the Ames test, were performed to determine the risk to benefit ratio and the mutagenic activity of the extracts.

Materials and methods

Plant material

We select five medicinal plants used in traditional Mexican medicine. *Berberis hartwegii* Benth., Berberidaceae, *Hamelia patens* Jacq., Rubiaceae, *Dendropanax arboreus* (L) Decne & Planch., Araliaceae, *Erythrina herbacea* L., Leguminosae, and *Zanthoxylum caribaeum* Lam., Rutaceae, were collected by Cynthia Ordaz-Pichardo Ph.D. in 2010 in Gómez Farías, Tamaulipas state, Mexico. Plant identifications were performed by M.C. Patricia Jácquez-Ríos at the "IZTA Herbarium" at the Iztacala campus of the National Autonomous University of Mexico (UNAM). A specimen of each plant was deposited in the herbarium with voucher numbers: *B. hartwegii* (1759), *D. arboreus* (1652), *E. herbacea* (1650), *H. patens* (1649) and *Z. caribaeum* (1651). Plants were air-dried and powdered.

Extract preparations

Acetone extracts from the leaves of *B. hartwegii* and *H. patens*, barks of *D. arboreus* and *Z. caribaeum*, and roots from *E. herbacea* were produced by direct maceration of dry and powdered plant material (25 g) for five days at room temperature in 250 ml of acetone five times. The extracts were filtered and evaporated under low pressure to dryness at 40 °C. The collected extracts were stored at −20 °C until further use.

Dermatophytes species

Dermatophytes (*Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Trichophyton tonsurans*) are well-characterized clinical isolates from the strain collection of the Hospital Juárez de Mexico and were donated by Q.F.B. Misael González Ibarra, the chief of the Laboratory of Immunology and Medical Mycology.

Inoculum preparation for biological assay

All of the strains were cultured in Sabouraud dextrose broth (Bioxon) at 28 °C until the maximal numbers of conidia were formed (15–20 days). The conidia were recovered by centrifugation at 2795 × *g* for 20 min at 4 °C. The pellet was suspended in sterilized distilled water and was adjusted to 10^5 conidia (*in vitro*) or 10^6 conidia (*in vivo*) per ml for use as the inoculum. The conidia were quantified using a Neubauer quantification chamber under a microscope.

Agar dilution assay

The assay was carried out according to the method of Hufford with some modifications (Hufford et al., 1975). Agar plates were prepared by adding 10 ml of Sabouraud dextrose agar in Petri dishes (9 cm in diameter). Next, 100, 50 or 25 μg of extract were added in 100 μL of dimethyl sulfoxide (DMSO) and control agar plates with 1% of DMSO and 100 μg of clotrimazole were included. The plates were incubated for 10 min at room temperature and were subsequently inoculated with each of the test fungi in triplicate. The plates were incubated at 28 °C for 12 days. These results were expressed as the percentage of inhibition of hyphal elongation compared to the negative controls.

Determination of therapeutic efficacy against experimental tinea pedis in BALB/c mice

Male BALB/c eight-week-old mice weighing 25 ± 5 g were used and ten mice were assigned to each group. The mice were placed in stainless steel cages, which were maintained at 25 °C in a 12 h dark–light cycle and provided with food and water *ad libitum*. All of the experiments were performed according to the Mexican Official Norm for Animal Care Handing (NOM-062, 1999).

Procedures for infection

The assay was carried out according to the method of González-Ávila (González-Ávila, 2002). One side of a paper disk (6 mm in diameter, Antibiotic Assay Paper, Whatman #1) was covered with a piece of aluminum foil, and the other side was free to carry the inoculum suspension. The discs were inoculated with 10^6 conidia of *T. rubrum* and were fixed to the soles of the feet of animal with adhesive elastic tape. The discs were removed on days 7, 14 and 21, and each sole was examined for clinical signs of dermatophytosis. The right leg of each animal was infected, and the left leg served as the uninfected control.

Macroscopic observation

The skin lesions caused by *T. rubrum* were observed macroscopically on days 7, 14 and 21 and were assessed using the following score system: 0, no signs or normal; 1, a small number of pink erythematous papules and less than 25% of desquamation at the site; 2, moderate intense pink erythema spread over the entire site accompanied by 25–50% desquamation an inflammatory response; 3, patches of intense red erythema with 50–80% desquamation and crusting; and 4, severe carmine red erythema with 80–100% desquamation and severe crusting. The average score for each group was calculated to determine the average lesion score.

Treatment

From day 14 post-infection, the mice were treated intragastrically (IG) daily for seven days with 100 μL DMSO:H_2O 1:3 serving as a vehicle. All groups were kept under constant observation to detect any changes in behavior or changes in water and food intake.

The animals were divided into five groups: group A was the non-treated infected control, group B was the positive control (treated with 1 mg/kg of clotrimazole IG), groups C and D were the infected groups treated with 2.5 and 5 mg/kg of acetone extract of *B. hartwegii* or *Z. caribaeum*, respectively, and group E consisted of non-infected animals treated with 10 mg/kg of extract to determine the potential toxic effects of extracts.

After treatment, the mice were anesthetized with 60 mg/kg of intraperitoneal sodium pentobarbital and sacrificed by cardiac puncture to collect 2–3 ml of blood. Serum from non-heparinized

Table 1
In vitro activity against *E. floccosum*, *M. canis*, *T. mentagrophytes*, *T. rubrum* and *T. tonsurans* (percentage of growth inhibition).

	μg/ml	*Epidermophyton floccosum*	*Microsporum canis*	*Trichophyton mentagrophytes*	*Trichophyton rubrum*	*Trichophyton tonsurans*
Berberis hartwegii	25	20.0 ± 0.3	85.0 ± 0.0	6.7 ± 0.4	26.7 ± 0.0	26.7 ± 2.1
	50	36.7 ± 6.7	88.3 ± 5.9	26.7 ± 2.1	46.7 ± 0.0	78.3 ± 2.2
	100	78.3 ± 6.8	100.0 ± 0.0	100.0 ± 0.0	65.0 ± 0.0	85.0 ± 5.8
Dendropanax arboreus	25	23.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.3 ± 0.6	40.0 ± .0.0
	50	36.7 ± 0.0	20.0 ± 0.0	23.3 ± 1.8	56.7 ± 3.8	60.0 ± 0.0
	100	50.0 ± 0.0	28.3 ± 1.1	77.0 ± 0.0	75.0 ± 0.0	90.0 ± 0.0
Erythrina herbacea	25	20.0 ± 0.0	0.0 ± 0.0	3.3 ± 0.2	16.7 ± 1.1	40.0 ± 0.0
	50	20.0 ± 0.0	75.0 ± 0.0	13.3 ± 0.9	36.7 ± 3.3	70.0 ± 0.0
	100	46.6 ± 5.1	85.0 ± 0.0	33.3 ± 2.9	78.3 ± 5.8	95.0 ± 0.0
Hamelia patens	25	50.0 ± 0.0	70.0 ± 0.0	80.0 ± 0.4	10.0 ± 0.0	73.0 ± 8.7
	50	100.0 ± 0.0	83.3 ± 5.4	90.0 ± 0.3	26.7 ± 1.1	90.0 ± 0.3
	100	100.0 ± 0.0	97.0 ± 2.9	100.0 ± 0.0	46.7 ± 5.0	92.7 ± 5.9
Zanthoxylum caribaeum	25	13.3 ± 0.9	1.7 ± 0.1	10.0 ± 0.0	6.7 ± 0.4	80.0 ± 0.0
	50	56.7 ± 5.8	26.7 ± 4.2	40.0 ± 0.0	20.0 ± 0.0	90.0 ± 0.0
	100	75.0 ± 0.0	55.5 ± 5.8	60.0 ± 0.0	75.0 ± 0.0	93.0 ± 0.0
Clotrimazole	100	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
DMSO	100 μL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

blood was carefully collected for a liver profile test. The mice were euthanized according to the official Mexican standard for the humane killing of animals (NOM-033, 1995). All animals were euthanized, and the vital organs were macroscopically analyzed.

Histological analyses

Representative fragments of the kidney, lungs and liver (to observe the toxic effects of extracts) from three mice were fixed in a 10% solution of formalin and enclosed in paraffin. Paraffin blocks were prepared after completing tissue processing in different grades of alcohol and xylenes. Sections (5 μm) were prepared from paraffin blocks using a microtome, and the sections were stained with hematoxylin–eosin (H&E).

Mutagenicity assay

A *Salmonella* mutagenicity test using the incorporation method was performed as previously described (Maron and Ames, 1983), using the *Salmonella typhimurium* strains TA98, TA100 and TA102 and the S9 fraction obtained from the liver of a male Wistar rat induced with Aroclor 1254 (New England Nuclear, North Haven, Conn) as the metabolic activation system. *S. typhimurium* strains were cultured for 16 h at 37 °C in a shaking water bath set at 90 rpm. In a sterile tube containing 2 ml of soft agar (at 45 °C), the bacterial cultures (100 μL) were exposed to final extract concentrations of 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, 50.0, 100 and 200.0 μg/ml, or to 10 μl of DMSO as a negative control. Subsequently, the suspension mixture was placed in a Petri dish containing Vogel–Bonner minimal medium. After incubation for 48 h at 37 °C, the revertant colonies (His^+) were quantified. The mutagenesis positive controls used for each strain were picrolonic acid, methyl-*N*-nitro-*N*-nitrosoguanidine, mitomycin C, 2-aminoanthracene and cyclophosphamide, as previously described (Maron and Ames, 1983).

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software, Inc.) was used for statistical analysis. The average lesion score on the last day of macroscopic observation was analyzed using one-way ANOVA non-parametric test and Dunnett *post hoc* analysis. The differences of the means of the groups were compared with the non-treated group and were considered significant when $p < 0.05$.

The mutagenicity was evaluated using a two-way ANOVA test and Bonferroni *post hoc* analysis. The differences of the means of the groups were compared with the corresponding positive control and were considered to be significant when $p < 0.05$.

Results

The acetone extracts from *B. hartwegii*, *D. arboreus*, *E. herbacea*, *H. patens* and *Z. caribaeum* (yields of 4.1, 2.5, 2.7, 3.7 and 2.1% respectively) were evaluated for activity against *E. floccosum*, *M. canis*, *T. mentagrophytes*, *T. rubrum* and *T. tonsurans* (Table 1). The acetone extract of *H. patens* exhibited 100% growth inhibition against *T. mentagrophytes* and *E. floccosum* at 100.0 and 50.0 μg/ml, respectively, and *B. hartwegii* inhibited growth of *M. canis* and *T. mentagrophytes* at 100.0 μg/ml. None of the extracts inhibited 100% growth of *T. tonsurans*, but all of the extracts exhibited good activity, with 85–95% growth inhibition at 100.0 μg/ml. The 1% DMSO did not affect the growth of the fungi.

The macroscopic observation results in the *tinea pedis* model are shown in Fig. 1. At day 7 post-infection, all of the groups exhibited a small number of pink erythematous papules and less than 25% of desquamation. At day 14 post-infection, the disease progressed uniformly in all groups, with patches of intense red erythema and 50–80% of scales and crusting. At day 21 post-infection (7 days of treatment), the mice treated with *B. hartwegii* (2.5 mg/kg) or *Z. caribaeum* (2.5 and 5 mg/kg) showed improvement in the soles (Fig. 2), with a level of damage similar to day 7 post-infection, while the mice treated with *B. hartwegii* (5 mg/kg) showed an improvement of 100% with no signs of disease. Compared to the non-treated group, there was a statistically significant improvement in the lesions treated with *B. hartwegii* and *Z. caribaeum* acetone extracts.

The non-infected animals treated with 10 mg/kg of *B. hartwegii* or *Z. caribaeum* did not exhibit signs of toxicity, such as piloerection, alteration in locomotor activity or mortality. The internal organs did not exhibit any gross morphological lesions.

The results of the liver profile test showed that the values of serum glutamic pyruvic transaminase (56.1 ± 9.9 UI/l), serum glutamic oxaloacetic transaminase (160.2 ± 64.1 UI/l), total bilirubin (0.74 ± 0.25 mg/dl), direct bilirubin (0.67 ± 0.28 mg/dl), and indirect bilirubin (0.90 ± 0.56) did not present any significant difference between the treated groups and control group, indicating an absence of liver damage caused by treatment.

Kidney sections from animals treated with acetone extracts of *B. hartwegii* or *Z. caribaeum* (10 mg/kg for seven days) showed a normal renal cortex with a normal appearance of the glomerulus, tubules and renal papilla. The lung sections displayed normal alveolar geometry, and the alveolar septum appeared normal. The liver sections showed normal hepatic architecture, normal hepatocytes, a normal portal triad and a normal central vein.

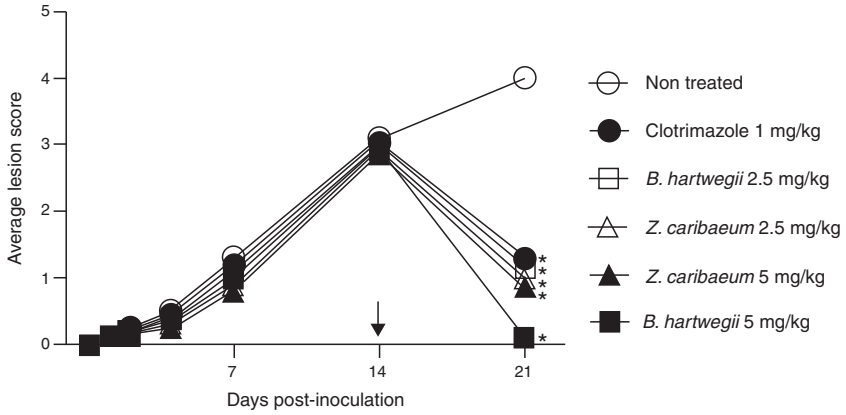

Fig. 1. The macroscopic improvement of skin lesions in experimental *tinea pedis* in Balb/c mice treated with 2.5 and 5 mg/kg of *B. hartwegii* or *Z. caribaeum* acetone extracts. Intragastric treatment was started on day 14 post-inoculation (↓) and was continued once daily for seven consecutive days. $^{*}p < 0.05$ *versus* the non-treated group (One-way ANOVA nonparametric test and Dunnett *post hoc* analysis).

Mutagenicity studies by the incorporation method showed that the acetone extracts of *B. hartwegii*, *D. arboreus*, *E. herbacea*, *H. patens* and *Z. caribaeum* at concentrations between 0.78 and 200 μg/ml (only 0.78, 12.5, 25.0, 50.0 and 200.0 μg/ml are showed) with or without a metabolic system (S9 mixture) did not produce frame-shift mutations in *S. typhimurium* His^{-} strain TA98. The acetone extracts did not induce mutations by base-pair substitutions in strain TA100 and did not produce mutations due to oxidative damage in strain TA102 (Table 2).

Discussion

The anti-dermatophyte *in vitro* activities of several herbal extracts have been examined in previous reports (Machado et al., 2009; Ponnusamy et al., 2010; Sule et al., 2012). However, the extracts displayed much weaker activity than the commonly used synthetic anti-dermatophyte drugs, and only a small number of these extracts have been evaluated in experimental models, which limits the use of these extracts as potential therapeutic agents (Evron et al., 1990; Mikaeili et al., 2012).

In our experiments, we found that *B. hartwegii* and *H. patens* extracts have the most optimal therapeutic potential because these extracts exhibit comparable or even higher efficiencies than the reference drugs against *E. floccosum*, *M. canis* and *T. mentagrophytes*.

Other plant extracts used in traditional medicine have shown similar activities; for example, the hydroalcoholic extract of *Berberis aristata* has activity against ten species of yeasts and molds, including *T. rubrum* (MIC of 2.5 μg/ml), *Candida albicans* (MIC 1.25 μg/ml), *Cryptococcus albidus* (MIC 1.25 μg/ml), *Blastoschizomyces capitatus* (MIC 22 μg/ml), *Fusarium moniliforme* (MIC 12 μg/ml), and *Fusarium semitectum* (MIC 15 μg/ml), and against five species of *Aspergillus* with MIC values between 0.3 and 10 μg/ml (Sharma et al., 2008; Singh et al., 2007). The hydroalcoholic extracts of species, such as *Berberis asiatica*, have reported activity against *C. albicans* and *Aspergillus flavus* (MIC = 1.25 and 0.62 μg/ml, respectively); *Berberis chitira* against *Aspergillus nidulans*, *C. albidus* and *A. flavus* (MIC = 1.25 μg/ml); and *Berberis lycium* against *Aspergillus terreus* and *Aspergillus spinulosus* (MIC = 0.31 and 0.62 μg/ml, respectively) (Singh et al., 2007).

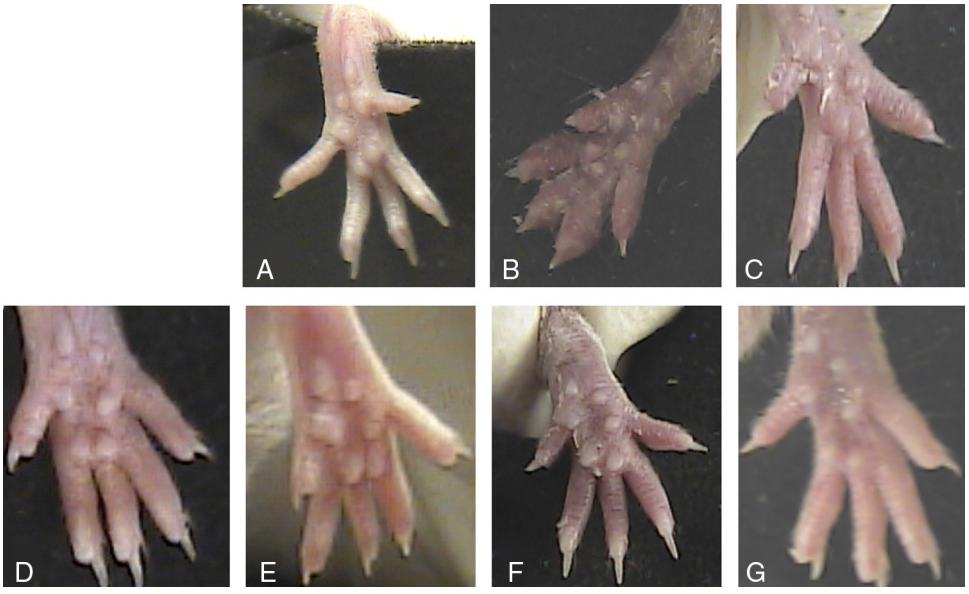

Fig. 2. Experimental *tinea pedis*. The soles of animals treated for 7 days (day 21 post-infection). A, the uninfected, non-treated control; B, The infected, non-treated control showed severe carmine red erythema with 80–100% desquamation and severe crusting; C, D, F and G, The infected groups, treated with clotrimazole 1 mg/kg, *B. hartwegii* 2.5 mg/kg or *Z. caribaeum* 2.5 and 5 mg/kg, respectively, showed a small number of pink erythematous papules and less than 25% of desquamation at the site; and E, The treated group showed no signs of toxicity.

Table 2
Mutagenicity of acetone extracts on *S. typhimurium* using the Ames test (plate incorporation assay), in presence or absence of metabolic activation.

Test substance	Conc/ml	Strains and metabolic system (S9)					
		TA98		TA100		TA102	
		−S9	+S9	−S9	+S9	−S9	+S9
B. hartwegii	12.5 μg	23.9 ± 1.9	25.8 ± 1.3	167.6 ± 14.3	194.7 ± 9.3	313.0 ± 25.4	356.9 ± 39.4
	25.0 μg	25.4 ± 1.2	27.0 ± 1.6	204.1 ± 12.4	200.8 ± 9.3	322.6 ± 21.5	350.4 ± 23.5
	50.0 μg	22.0 ± 0.9	23.6 ± 1.8	175.6 ± 12.5	199.7 ± 8.2	271.9 ± 18.1	320.4 ± 28.1
D. arboreus	12.5 μg	30.8 ± 3.3	33.9 ± 4.5	184.6 ± 13.6	177.1 ± 13.0	366.9 ± 19.5	382.8 ± 72.5
	25.0 μg	30.1 ± 6.0	33.4 ± 3.7	199.9 ± 13.0	165.0 ± 10.2	384.2 ± 23.2	422.2 ± 67.5
	50.0 μg	35.3 ± 4.6	31.2 ± 4.2	183.1 ± 9.3	188.8 ± 13.5	398.1 ± 5.0	315.6 ± 62.5
E. herbacea	12.5 μg	26.3 ± 1.4	30.1 ± 1.9	199.3 ± 13.1	173.3 ± 9.5	312.3 ± 36.7	352.8 ± 17.4
	25.0 μg	26.4 ± 1.5	32.2 ± 1.8	214.2 ± 5.2	163.3 ± 9.5	394.6 ± 36.9	374.2 ± 22.9
	50.0 μg	31.6 ± 2.1	33.3 ± 1.8	199.7 ± 8.2	175.9 ± 3.8	283.7 ± 19.8	402.9 ± 26.4
H. patens	12.5 μg	25.9 ± 1.7	31.7 ± 2.5	186.0 ± 5.6	159.0 ± 4.2	334.2 ± 43.7	361.7 ± 37.0
	25.0 μg	26.4 ± 1.5	30.6 ± 1.7	183.4 ± 11.9	164.3 ± 6.8	294.9 ± 21.5	452.7 ± 47.5
	50.0 μg	24.6 ± 3.5	30.4 ± 2.2	189.7 ± 7.7	194.8 ± 11.4	285.8 ± 27.6	362.6 ± 35.9
Z. caribaeum	12.5 μg	23.9 ± 1.8	33.8 ± 2.1	213.4 ± 7.4	179.9 ± 6.9	333.2 ± 24.8	352.0 ± 25.8
	25.0 μg	22.7 ± 1.3	34.2 ± 1.0	197.3 ± 7.3	178.1 ± 12.1	357.8 ± 20.6	429.7 ± 22.3
	50.0 μg	24.3 ± 2.3	35.0 ± 2.2	200.3 ± 4.3	201.9 ± 12.6	352.4 ± 26.1	516.7 ± 16.7
PA	82.0 μg	422.2 ± 30.9*					2055.1 ± 347.6*
2AA	10.0 μg		4910.4 ± 187.8*				
MNNG	10.0 μg			13,248.3 ± 1036.6*			
CP	500 μg				558.8 ± 21.5*		
Mit C	2.0 ng					4386.9 ± 248.7*	
DMSO	10.0. μl	24.0 ± 2.1	33.7 ± 1.4	207.0 ± 5.6	195.2 ± 13.7	343.0 ± 33.7	417.3 ± 19.9

Data represent the mean ± SD of the number of histidine revertant colonies by plate in each treatment; three independent experimental series carried out in triplicate were performed. Incubations were made in presence (+S9) and absence (−S9) of rat liver microsomal mix. PA, picrolonic acid; 2AA, 2-amino-anthracene; MNNG, methyl-*N*-nitro-*N*-nitrosoguanidine; CP, cyclophosphamide; Mit C, mitomycin C and DMSO, dimethyl sulfoxide.

* $p < 0.05$ (two-way ANOVA test and Bonferroni *post hoc* analysis).

This study is the first report describing the *in vitro* antifungal activity of *H. patens* and is the first to report this activity in a species of the genus *Hamelia*. However, other biological activities have been previously reported, including the antibacterial activity of the methanolic extract from the leaves of *H. patens* against *E. coli* and *Pseudomonas aeruginosa* with MIC = 2.5 mg/ml (Camporese et al., 2003). The cytotoxic activities of this specie have been reported against human epithelioid cervix carcinoma cells (HeLa), CI_{50} = 13.3 μg/ml; human prostate cancer cells (PC-3), CI_{50} = 41 μg/ml; breast cancer human cells (MCF7 and MDA-MB-231), CI_{50} = 63 and 45 μg/ml; breast cancer mouse cells (4T-1), CI_{50} = 31 μg/ml; colon carcinoma cells (HT-29), CI_{50} = 34 μg/ml; and mouse leukemic monocyte macrophage cells (RAW-267), CI_{50} = 9 μg/ml (Mena-Rejon et al., 2009; Taylor et al., 2013). *H. patens* displayed anti-inflammatory activity against Croton oil-induced ear edema (Sosa et al., 2002) and increased the breaking strength in a double incision wound healing bioassay model (Gomez-Beloz et al., 2003). A variety of alkaloids that are potentially responsible for these biological activities have been described, and the most abundant alkaloids are isopteropodine, rumberine, palmirine, maroquine, (−)-hameline, aricine, pteropodine, uncarine F and speciophylline (Paniagua-Vega et al., 2012; Reyes-Chilpa et al., 2004). Many alkaloids have antifungal properties (Cimanga et al., 1998; Hu et al., 2014; Zhou et al., 2003), and alkaloids can constitute up to 90% of the metabolites in *H. patens* extracts; thus, we propose that this type of compound is also responsible for the observed antifungal activity.

To determine the activity against *T. rubrum* in the experimental model, we chose to study the species *B. hartwegii* because this plant showed good activity against the five dermatophytes evaluated, and we examined *Z. caribaeum* due to the wide use of this plant in traditional medicine for the treatment of skin diseases. Moreover, both species were selected due to the high availability of these plants.

B. hartwegii and *Z. caribaeum* acetone extracts administered at different concentrations improved the skin lesions of experimental *tinea pedis*. Doses of 2.5 mg/kg of *Z. caribaeum* and *B. hartwegii* extracts had comparable efficacy to 1 mg of clotrimazole. Furthermore, treatment with *B. hartwegii* (5 mg/kg) extract completely healed the mycological infection after 7 days of treatment; thus, this natural extract exhibited better activity than the reference drug.

Currently, there have been no reports of any study on the species *B. hartwegii*; however, other species of the same genus, such as *B. aristata*, *B. asiatica*, *Berberis criteria*, *B. lycium*, *Berberis thunbergii*, *Berberis heterophylla* and *Berberis aetnensis*, have demonstrated activity against a large number of pathogenic strains of the genera *Aspergillus* and *Candida* (Freile et al., 2003; Iauk et al., 2007; Li et al., 2007; Sharma et al., 2008; Singh et al., 2007). Berberine, the compound responsible for this antifungal activity (Freile et al., 2003; Soffar et al., 2001; Zhang et al., 2013a), has a synergistic effect with ketokonazole (Zhou et al., 2012), miconazole (Wei et al., 2011), fluconazole (Iwazaki et al., 2010; Quan et al., 2006) and amphotericin B (Han and Lee, 2005). This synergistic activity is due to the up-regulation of CDR1 mRNA and the transport function of Cdr1p induced by fluphenazine (Zhu et al., 2014). Recent studies have found that berberine azoles exhibited good antimicrobial activity against *Candida mycoderma* (Zhang et al., 2013c), andberberine triazoles has good activity against *C. mycoderma* and *C. albicans*. This activity was comparable or better than the reference drugs berberine, norfloxacin and fluconazole (Zhang et al., 2013b). Thus, the antifungal activity observed in *B. hartwegii* could be due to the presence of berberine.

There have been no reports on the antifungal activity of *Z. caribaeum*, but there have been reports on the activity of the genus *Zanthoxylum*. The methanolic extract of *Zanthoxylum alatum* exhibited potent antifungal activity against *Alternaria alternata*, *Alternaria brassicae*, and *Curvularia lunata* (Guleria et al., 2013), and *Zanthoxylum capense* exhibited antifungal activity against *Aspergillus fumigatus* (Adamu et al., 2012). Extracts from the leaf, fruit, stem, bark and root of *Zanthoxylum americanum* demonstrated antifungal activity against *C. albicans*, *Cryptococcus neoformans* and *A. fumigatus* in a disk diffusion assay (600 μg/disk)

(Bafi-Yeboa et al., 2005). In other cases, alkaloids have been demonstrated to be responsible for the antifungal activity of *Zanthoxylum* plants; for example, three alkaloids with antifungal activity have been isolated from *Zanthoxylum nitidum* (dictamnine, gamma-fagarine, and 5-methoxydictamnine) (Yang and Chen, 2008), and eight active alkaloids have been isolated from *Zanthoxylum rhetsa* and *Zanthoxylum chiloperone* (Krohn et al., 2011; Soriano-Agaton et al., 2005). Alkaloidal extract of *Z. chiloperone* var. *angustifolium* has activity against *C. albicans*, *A. fumigatus* and *T. mentagrophytes*, being responsible for the biological activity two alkaloids (canthin-6-one and 5-methoxycanthin-6-one). The canthin-6-one has a broad antifungal spectrum against twelve species (MICs between 12.8 and 56.1 μM/l) (Thouvenel et al., 2003). Thus, alkaloids must be responsible for the biological activity observed in *Z. caribaeum.*

Medicinal plants and their derivatives have been used as alternatives in allopathic medicine in many countries. Despite the widespread use of medicinal plants, few scientific studies have been performed to examine the safety and efficacy of traditional remedies or pure compounds (Silva et al., 2011). Several drugs for dermatophytosis, such as ketokonazole, itraconazole and terbinafine treatment, can cause hepatotoxicity (Andriole, 1999; Somchit et al., 2004; Zapata-Garrido et al., 2003); thus, it is important to evaluate the potential toxic effects of extracts and natural compounds.

The results of the liver profile test did not demonstrate any significant differences between the groups treated with *B. hartwegii* or *Z. caribaeum* and the control group, indicating an absence of liver damage caused by treatment. Liver histological sections showed normal hepatic architecture, normal hepatocytes, normal portal triad and normal central vein. These observations, as well as the serum levels of bilirubin and transaminases, established the absence of hepatocellular damage induced by the extracts. Histological analysis and biochemical parameters are essential for evaluating the toxicological risk of substances with biological activity because any changes in one of these parameters in animal studies can provide a high predictive value of potential damage in humans (Carvalho et al., 2011; Olson et al., 2000; Silva et al., 2011).

Mutagenic compounds are potentially hazardous to humans. Substances that are mutagenic for bacteria are likely to be carcinogenic in animals. Thus, all substances must be analyzed to establish the risk-benefit ratio. A useful procedure to detect mutagenic substances is the standard plate incorporation test (Ames et al., 1973). Certain substances are not mutagenic by themselves; thus, the Ames test includes a metabolic system (mixture of liver enzymes S9).

None of the tested strains (TA98, TA100 and TA102) revealed mutagenic potential. All of the results were significantly lower than those of the corresponding positive control (Two-way ANOVA test and Bonferroni *post hoc* analysis). Moreover, the number of revertant colonies per plate containing the tested substance must be at least more than twice the number of spontaneous revertants (Maron and Ames, 1983) for a substance to be considered mutagenic, which was never observed in the tests with the acetone extracts of *B. hartwegii*, *D. arboreus*, *E. herbacea*, *H. patens* and *Z. caribaeum*. In addition, no dose-dependent effects were demonstrated.

In summary, *B. hartwegii* and *Z. caribaeum* might be useful agents against *Trichophyton* infections because these extracts exhibited good activity in *in vitro* and *in vivo* evaluations with no signs of genotoxicity or hepatotoxicity.

Our findings justify the use of *B. hartwegii* and *Z. caribaeum* in Mexican traditional medicine for the treatment of fungal skin infections. These results in combination with the absence of hepatotoxic or mutagenic effects indicated that these two species have significant potential for the development of new anti-dermatophyte drugs.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

JCG contributed to analysis the data, drafted the paper and biological studies. MSS and ADL contributed to biological studies. PJR contribute in plant identification and herbarium confection. MAA contribute to Ames test analysis. COP as head of the research team organized and supervised the research project. All the authors have read the final manuscript and approved the submission.

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