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Phytotherapeutic potential of *Artemisia ludoviciana* and *Cordia boissieri* extracts against the dermatophyte *Microsporum canis*

José Pablo Villarreal Villarreal[1], Beatriz A. Medina Soto[1], Michel Stéphane Heya[2], Sergio A. Galindo-Rodríguez[3], Uziel Castillo Velázquez[4], Kevin A. Cárdenas Noriega[4], Romario García-Ponce[1,3]✉

[1]Department of Microbiology, [4]Department of Immunology,
Faculty of Veterinary Medicine and Zootechnics, Universidad Autónoma de Nuevo León,
Nuevo León C.P. 66455, Mexico
[2]Faculty of Public Health and Nutrition, Universidad Autónoma de Nuevo León,
Nuevo León C.P. 66455, Mexico
[3]Analytical Chemistry Department, Biological Sciences Faculty,
Universidad Autónoma de Nuevo León, Nuevo León C.P. 66455, Mexico
qbpromario@gmail.com

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Abstract

Introduction: *Microsporum canis* is a dermatophyte that mainly affects dogs and cats. However, it can be transmitted to humans by direct contact. This makes it one of the most frequent causative agents of dermatophytosis in humans, reflecting the frequent human close relationships with pets. Conventional treatment relies on antifungal pharmacological agents. However, errors in application have led to the occurrence of fungal resistance and toxic effects. Consequently, new therapeutic alternatives are needed for *M. canis* infections. Plant extracts have been explored as phytotherapeutics for the treatment of dermatophyte infections, which prompted an attempt to apply extracts of the ethnopharmacologically important plants *Artemisia ludoviciana* and *Cordia boissieri*. **Material and Methods:** Methanolic extracts of these two plants were obtained using a Soxhlet method and were characterised by phytochemical screening. Extracts were evaluated against a *M. canis* commercial strain (ATCC-11621) using the microdilution method described in the Clinical and Laboratory Standards Institute protocol M38-A, determining its minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). Subsequently, these concentrations were tested in a human keratinocyte human cell line. **Results:** *Artemisia ludoviciana* and *C. boissieri* extracts showed MIC values of 2,500 and 1,250 µg/mL, and MFC values of 5,000 and 2,500 µg/mL against *M. canis*, respectively. These extracts did not inhibit HaCaT cell proliferation *in vitro*. **Conclusion:** The evaluated extracts showed potential for the treatment of *M. canis* fungal infections. However, further studies on their phytochemical characterisation, purification, clinical safety and formulation are required.

Keywords: antifungal resistance, dermatophytosis, *Microsporum canis*, plant extracts.

Introduction

Dermatophytosis is a prevalent health issue in developing countries, affecting all age groups and different parts of the body. It estimated that between approximately 30% and 70% of adults are asymptomatic carriers of dermatophytes, while at least 10% of people develop dermatophytosis during their lifetime (41, 28). One causative agent of the disease is *Microsporum canis*, a zoophilic dermatophyte primarily afflicting dogs and cats (39). This zoonotic fungus can be transmitted through direct contact, making it one of the main species of keratinocytic and keratinolytic filamentous fungi that causes superficial infections in humans worldwide, by reason of the close relationships many humans have with pets (17). Associated pathologies include multifocal alopaecia and desquamation or tinea, marking *M. canis* infections as a significant public health issue (7). Conventional treatments include the use of topical and oral antifungal pharmacological agents such as amphotericin B, griseofulvin, terbinafine, itraconazole, flucytosine and

fluconazole, either alone or in combination (1, 21). However, errors in use, including application, excessive or inadequate use and treatment interruption, have led to the occurrence of fungal resistance and adverse health effects such as nephrotoxicity, hepatotoxicity and neurotoxicity (21, 33, 27). In this context, natural products such as plant extracts are considered potential alternatives for developing new antifungal strategies or even for improving the existing ones (21). Various studies have explored the use of plant extracts as phytotherapeutic alternatives for the treatment of dermatophyte infections (7). *Artemisia ludoviciana* and *Cordia boissieri* are plants of considerable ethnopharmacological importance. Despite this, evidence regarding their antimycotic activity against *M. canis* remains elusive. *Artemisia ludoviciana* extracts have shown antiprotozoal, antioxidant, ixodicidal, antimicrobial and anti-inflammatory activity (34, 14, 30, 15). Similarly, *C. boissieri* has exhibited antimicrobial, antifungal, antioxidant, cytotoxic and ixodicidal properties (35, 38). Therefore, this study aimed to evaluate the antifungal activity of extracts from *C. boissieri* and *A. ludoviciana* against *M. canis*, and the extracts' cytotoxicity to human epithelial cells, with the objective of identifying their therapeutic potential against *M. canis* infections.

Material and Methods

Plant collection. The aerial parts of *A. ludoviciana* were collected from Jaumave, Tamaulipas, Mexico (23°24′22.36, −99°22′50.59) and from *C. boissieri* in Guadalupe, Nuevo León, México (25°71′53.09, −100°22′52.36). Plant identification was performed by García-Ponce *et al.* (15) in connection with earlier research.

Extraction and characterisation of extracts. Methanolic extracts were obtained using a Soxhlet extractor, by placing 70 g of plant material and 700 mL of methanol in the apparatus for 48 h. The liquid was removed from the obtained product using a rotary evaporator (Laborota 4003-control; Heidolph Instruments, Schwabach, Germany) at 30°C and under reduced pressure. The resultant extract was dried at 25°C and was stored at 4°C. Phytochemical screening, as per the methods described by Verde-Star *et al.* (37), identified the presence of sterols and triterpenes, sesquiterpene lactones, coumarins, saponins, flavonoids, alkaloids, aromatic compounds, carbonyls and phenol oxides in the extracts.

Antifungal activity. *Microsporum canis* (American Type Culture Collection 11621) colonies were inoculated on oatmeal agar and incubated for 10 d at 32°C. Subsequently, a concentrated suspension of conidia was obtained by washing the colonies with 10 mL of sterile distilled water. The suspension was adjusted to a concentration of 3×10^4 colony-forming units/mL. A modified microdilution method was used, described in the Clinical and Laboratory Standards Institute protocol M38-A (11). A working solution of 20,000 µg/mL of extract diluted in methanol was prepared and stored at 6°C until use. Dilutions were made in 96-well microplates of the working solution (312.5 to 10,000 µg/mL), using Mueller–Hinton broth (Sigma-Aldrich, St. Louis, MO, USA) and phenol red as a growth indicator. A colour change to red signalled the growth of the dermatophyte through the release of alkaline metabolites. Subsequently, 100 µL of adjusted conidial suspension was added. A conventional fungicide, Imidazole (Sigma-Aldrich), was used as a positive control (1,000 µL/mL) and methanol served as a vehicle control. Regarding the growth control, 100 µL of each strain was inoculated into 100 µL of medium. The obtained solutions were incubated for 120 h at 32°C. Antifungal activity was determined by visual observation to obtain the minimum inhibitory concentration (MIC), which was the concentration that inhibited 80% of fungal growth relative to the growth control. In addition, the minimum fungicide concentration (MFC) was determined as the concentration that managed to inhibit 100% of fungal growth.

Cytotoxic evaluation. To assess cytotoxicity, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using a healthy human keratinocyte cell line (HaCaT). Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine foetal serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) and incubated in a sterile atmosphere with 4% CO_2 for 5 d at 37°C until confluence was reached. After that, cells were cultured in 96-well microplates at a density of 5×10^3 cells/well. The cells were treated with extracts at the MIC and MFC obtained from the antifungal activity assays and with the extracts at a lower concentration diluted in dimethyl sulfoxide (DMSO) for 24 h. Growth control cells were given 100 µL of DMEM. After that, cells were washed with phosphate-buffered saline and 150 µL of MTT reagent at a concentration of 0.4 mg/mL. The cells were incubated for 3 h, the medium was removed and 100 µL of DMSO was added. The assay was performed in triplicate. Absorbance was measured by spectrophotometry at 560 nm. Additionally, trypan blue cell staining was performed using the same cell line and treatments. A cell quantity of 5×10^3 was cultured in each well of 96-well microchambers. The cells were incubated for 24 h in a sterile atmosphere with 4% CO_2 at 37°C and then exposed to different concentrations of the methanolic extracts for 24 h. Finally, a cell count was performed adding trypan blue dye and using a hemacytometer.

Statistical analysis. A randomised block design statistical model was employed, and mean comparisons were performed using Tukey's test. In all analyses, P-values ≤ 0.05 were considered statistically significant. Data were analysed using Minitab 17 software (Minitab, State College, PA, USA).

Results

Phytochemical yield and characterisation of extracts. Methanolic extraction of *A. ludoviciana* and *C. boissieri* yielded 37.85% and 11.42%, respectively. Phytochemical characterisation revealed the presence of secondary metabolites such as sterols and terpenes, phenolic oxides, aromatic compounds, carbohydrates, flavonoids and saponins in both extracts. The presence of coumarins was only observed in the *A. ludoviciana* methanolic extract (Table 1).

Table 1. Phytochemical characterisation of methanolic extracts of *Artemisia ludoviciana* and *Cordia boissieri*

Phytochemical group	*A. ludoviciana*	*C. boissieri*
Sterols and triterpenes	+	+
Sesquiterpene lactones	+	+
Coumarins	+	−
Saponins	+	+
Flavonoids	+	+
Alkaloids	−	−
Aromatics	+	+
Carbonyls	−	−
Phenolic oxides	+	+

+ - presence; − - absence

Table 2. Inhibition activity of methanolic extracts of *Artemisia ludoviciana* and *Cordia boissieri* against *Microsporum canis*

Extract	Concentration	% H
A. ludoviciana	1,250 µg/mL	85.92 ± 7.34[a]
	2,500 µg/mL	94.30 ± 5.84[a]
	5,000 µg/mL	99.63 ± 1.24[a]
C. boissieri	625 µg/mL	77.11 ± 4.32[b]
	1,250 µg/mL	94.12 ± 3.56[a]
	2,500 µg/mL	100.00 ± 0.00[a]
Imidazole	1,000 µg/mL	90.25 ± 3.44[a]

n = 3; % H – inhibition percentage; a-b indicate significant differences; P-value ≤ 0.05

Table 3. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of methanolic extracts of *Artemisia ludoviciana* and *Cordia boissieri* against *Microsporum canis*

Extract	MIC	MFC
A. ludoviciana	2,500 µg/mL	5,000 µg/mL
C. boissieri	1,250 µg/mL	2,500 µg/mL

Antifungal activity. *Artemisia ludoviciana* and *C. boissieri* methanolic extracts exhibited inhibitory effects on *M. canis* growth (Table 2). The *M. canis* strain was inhibited by *A. ludoviciana* at an MIC value of 2,500 and by *C. boissieri* at 1,250 µg/mL. The dermatophyte was killed by *A. ludoviciana* at an MFC value of 5,000 and by *C. boissieri* at 2,500 µg/mL (Table 3).

Cytotoxic activity. *Artemisia ludoviciana* and *C. boissieri* methanolic extracts were tested at their MIC and MFC against *M. canis* in a HaCaT cell line in an MTT assay. These concentrations did not act cytotoxically to significantly different extents than the negative control (DMEM) (P-value ≤ 0.05). The cell viability percentages are shown in Fig. 1A. The effect of these concentrations was also evaluated using trypan blue dye, and again no significant viability differences were observed between HaCaT cells exposed to the extracts and those only exposed to the negative control (DMEM) (P-value ≤ 0.05), as shown in Fig. 1B.

Discussion

In recent years, extensive research has been dedicated to exploring the therapeutic effects of plant extracts, particular emphasis falling on research regarding the study and exploitation of their antibacterial and antifungal activity. In this study, a partial phytochemical characterisation of methanolic extracts from *A. ludoviciana* and *C. boissieri* was performed. The analysis revealed that the *C. boissieri* extract contained sterols, triterpenes, saponins and flavonoids, which was consistent with the results reported by Alves *et al.* (4), who emphasised the abundance of these compounds in plants of the *Cordia* genus. Extracts obtained from this plant additionally exerted antibacterial (26), antioxidant (38) and antifungal activity against pulmonary mycosis–inducing pathogens (3). Extracts of *A. ludoviciana* were found to contain sterols, triterpenes, saponins, and flavonoids similarly to extracts of *C. boissieri*, additionally sesquiterpene lactones and coumarins were noted as present.

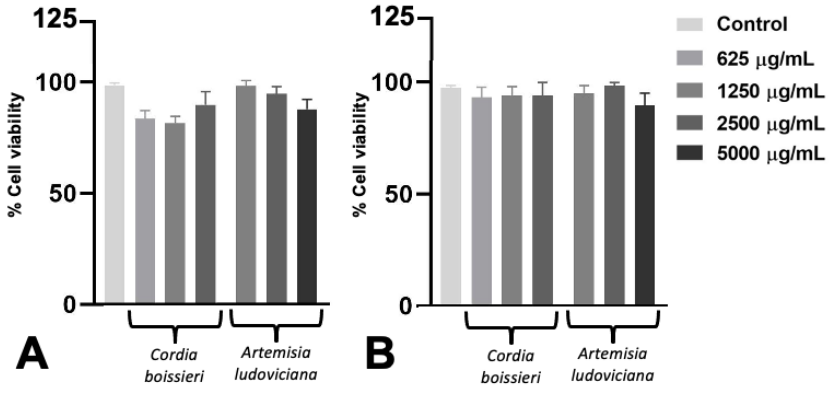

Fig. 1. Cell viability of HaCaT human keratinocyte cells exposed to different concentration of extracts of *Artemisia ludoviciana* and *Cordia boissieri*. Determination of viability was by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (A) and trypan blue dye (B) Control – DMSO; P-value ≤ 0.05

These results corroborated findings previously reported by several authors (22, 32, 8). Extracts from *A. ludoviciana* were previously associated with a spectrum of pharmacological activities, such as antioomycete (12), antinociceptive (5), hypoglycaemic (6), antioxidant (19), antimicrobial, cytotoxic, anti-inflammatory (14), antiparasitic (34) and antifungal (23) effects. Our research specifically evaluated the antimycotic activity of *A. ludoviciana* and *C. boissieri* methanolic extracts against the *M. canis* ATCC-11621 strain, using imidazole as a control, revealing MIC values of 2,500 and 1,250 µg/mL and MFC values of 5,000 and 2,500 µg/mL, respectively. Previous studies indicated that *A. ludoviciana* extracts inhibited *Microsporum cookei* and *Microsporum gypseum*, with halos of 10–15 mm assessed by a disc diffusion method (26). Similarly, Damián-Badillo *et al.* (13) reported inhibitory effects of flower, leaf and root extracts of *A. ludoviciana* against strains of *Candida albicans*, *Colletotrichum lindemuthianum*, *Mucor circinelloides*, *Saccharomyces cerevisiae* and *Sporothrix schenckii*. Moreover, *C. boissieri* hydroalcoholic extracts exhibited antifungal activity with a lower MIC value of 1,000 µg/mL against *Candida albicans*, *Aspergillus fumigatus* and *Coccidioides immitis* strains; and an even lower one of 125 µg/mL against *Histoplasma capsulatum* (3). A *C. boissieri* flower methanolic extract was also evaluated against species of the *Candida* genus and was found to be effective at a MIC value of 125 µg/mL against *Candida glabrata* (35). Our results show that the evaluated *A. ludoviciana* and *C. boissieri* extracts had antifungal activity against *M. canis* ATCC-11621, which may be considered to be directly dependent on the secondary metabolites contained in the extracts. Some studies suggest that tannins, flavonoids, triterpenes, sterols, phenolic compounds, terpenes and monoterpenes can be highly active antimicrobial agents (24, 10, 31). Other studies propose that some dermatophytes, including *M. canis*, are sensitive to terpenes and monoterpenes, particularly those from *Euphorbia tirucalli* L. (18, 9). It is worth noting that the extracts' antifungal activity against *M. canis* may be attributed to the presence of sterols, terpenes and sesquiterpene lactones, compounds identified through the phytochemical screening of the extracts. However, it is essential to conduct phytochemical composition studies to identify and quantify the compounds present in the extracts used in this work. This step will enable us to attribute the observed biological activity to the major compounds found in the extracts. Additionally, it will aid in identifying any possible synergistic or individual effects they may exhibit.

To assess the safety profile of the methanolic extracts, cytotoxicity tests were conducted on a human keratinocyte cell line (HaCaT) using the MTT assay and trypan blue dye. The MIC and MFC of both extracts were tested in these assays, assessing the cell viability percentage. No statistically significant differences were observed when compared to the cell growth control, demonstrating that these extracts did not inhibit cell growth. While cytotoxic studies on *A. ludoviciana* and *C. boissieri* methanolic extracts have not previously been reported for this cell line, literature on related plant extracts from the *Artemisia* genus, specifically *A. princeps*, indicated cytoprotective effects, aiding the survival of HaCaT cells exposed to ultraviolet B radiation and reducing radiation-induced DNA damage (20). Other beneficial effects on human keratinocyte cells have also been described: Yang *et al.* (42) noted that ethanolic extracts from *A. apiacea* reduced proinflammatory chemokines in HaCaT cells *in vitro*; and Wang *et al.* (40) discussed the hydrating and antioxidant activity of an *A. argyi* extract in this cell line. In the case of plants from the *Cordia* genus, extracts from the species *C. myxa* were evaluated in the human L929 fibroblast cell line, and were reported to exert no antiproliferative effect (2). Similarly, Geller *et al.* (16) observed a *C. americana* extract at a concentration of 1 g/mL to increase cell proliferation and migration in albino Swiss 3t3 mice fibroblasts by 19.8% in a scratch assay. When loaded onto electrospun fibres applied in wound healing, *C. myxa* and *C. curassavica* extracts potentiated human fibroblast proliferation and significantly improved *in vivo* wound healing (36, 29). These findings support our results in cytotoxicity assays, which showed that methanolic extracts of *A. ludoviciana* and *C. boissieri* did not exhibit toxicity in HaCaT cells at the evaluated concentrations.

Conclusion

Methanolic extracts of *A. ludoviciana* and *C. boissieri* demonstrated antifungal activity against the ATCC-11621 strain of *M. canis*, with the *C. boissieri* extract having a higher inhibition percentage. Importantly, these extracts did not inhibit the proliferation of HaCaT cells at the evaluated concentrations, suggesting a favourable safety profile for human epithelial cells. The antifungal efficacy of plant extracts is considered to be related to the presence of secondary metabolites, and according to our results, these extracts contain metabolites responsible for antifungal activity which inflict no cytotoxic effects on human epithelial cells. This supports the extracts' therapeutic potential for treating *M. canis* fungal infections. Nonetheless, further studies encompassing phytochemical characterisation, isolation, clinical safety and formulation are imperative for a comprehensive understanding of their therapeutic application.

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