Antifungal effect of *Arnica montana* and *Hamamelis virginiana* against *Candida* species

Atividade antifungica da Arnica montana *e* Hamamelis virginiana *contra espécies de* Candida

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Abstract

Objective – To evaluate the antifungal activity of *Arnica montana* and *Hamamelis virginiana* glycolic extracts against *Candida* strains. **Methods** – The antifungal activities of glycolic extracts were investigated by determination of the minimum inhibitory concentration (MIC) according to protocol M27-S3 of Clinical and Laboratory Standards Institute (2008). **Results** – *A. montana* glycolic extract showed the strongest antifungal activity against *C. tropicalis*, with a minimum inhibitory concentration (MIC) of 10% v/v and a minimum fungicidal concentration (MFC) of 80% v/v, then *C. krusei* and *C. glabrata*, with MIC and MFC values of 20% v/v. *H. virginiana* glycolic extract exhibited stronger activity against *C. albicans* and *C. tropicalis*, with MIC and MFC values of 10% v/v, than against *C. glabrata*, *C. krusei*, and *C. parapsilosis*, with MIC and MFC values of 20% v/v. Moreover, we evaluated the toxicity of the two glycolic extracts in the *Galleria mellonell*a model using the survival curves of larvae treated with the two extracts. Our results demonstrated that the glycolic extracts of *A. montana* and *H. virginiana* exhibited no toxicity against *G. mellonella* larvae and demonstrated antifungal activity against *Candida* species. **Conclusion** – Thus, both extracts are promising candidates for the development of novel antifungal agents.

Descriptors: *Candida*; Glycolic extract; *Arnica*; *Hamamelis*; Antifungal

Resumo

Objetivo – Avaliar a atividade antifúngica dos extratos glicólicos de *Arnica montana* e *Hamamelis virginiana* contra cepas de *Candida spp*. A candidíase é uma infecção fúngica comum, portanto, a pesquisa de novos agentes antifúngicos tem sido um alvo interessante. Várias plantas apresentaram atividades biológicas e, portanto, podem ser fontes promissoras de produtos naturais com atividades antifúngicas. **Métodos** – As atividades antifúngicas dos extratos glicólicos foram avaliadas por meio da determinação da concentração inibitória mínima (CIM) de acordo com o protocolo M27-S3 do *Clinical and Laboratory Standards Institute* (2008). **Resultados** – O extrato glicólico de *A. montana* apresentou a atividade antifúngica mais forte contra *C. tropicalis*, com concentração inibitória mínima (CIM) de 10% v/v e concentração fungicida mínima (MFC) de 80% v/v, seguido por *C. krusei* e *C. glabrata*, com valores de MIC e MFC de 20% v/v. Além disso, avaliamos a toxicidade dos dois extratos glicólicos no modelo *Galleria mellonella* usando as curvas de sobrevivência de larvas tratadas com os extratos. Nossos resultados demonstraram que os extratos glicólicos de *A. montana* e *H. virginiana* não exibiram toxicidade contra larvas de G. mellonella e demonstraram atividade antifúngica contra espécies de Candida spp. Conclusão – Assim, ambos os extratos são candidatos promissores para o desenvolvimento de novos agentes antifúngicos.

Descritores: *Candida*; Extrato glicólico; *Arnica*; *Hamamelis*; Antifúngicos

Introduction

Candida species are serious opportunistic organisms, which are part of the human microbiota.[1] In HIV patients, *Candida* infections are responsible for more than 30% of the death cases and lead to several serious nosocomial infections.[2,3] Candidiasis can manifest as systemic, subcutaneous, or cutaneous infections.[1]

Antimycotic treatments involve a limited number of drugs, being the most common classes the echinocandins, polyenes and azoles. These drugs can be used in different forms and dosage, depending on the type of infection. However, resistance to the usual antifungals is a common problem, which arouses the need for new treatments with greater efficacy and less toxicity than the current ones.[4-7]

The investigation of plants and plant constituents is promising since plants are sources of diverse active compounds. *Arnica montana* is a plant found all over the world that grows mainly in nutrient-poor soils, is commonly used in topical anti-inflammatory preparations[8]. In addition, other biological activities of A. montana, such as analgesic, antioxidant and antimicrobial, have also been reported.[9-11] Another plant called Hamamelis virginiana is widely used in the treatment of wounds and inflammatory skin diseases, in addition to having antioxidant and astringent effects. These biological activities are attributed to the chemical composition of the plant.[12]

In this study, we evaluated the antifungal activities of glycolic extracts of *A. montana* and *H. virginiana* against *Candida* species and investigated their toxicity in an alternative model.

Methods

Strains

The following *Candida* strains were used in our study: *Candida albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 750, and *C. glabrata* ATCC 90030. These strains were cultured in Sabouraud Dextrose Agar at 30 °C, 24h before the experiments.

Glycolic extracts

Glycolic extracts of *A. montana* and *H. virginiana* were purchased commercially (Florien Fitoativos, Piracicaba, Brazil). The *A. montana* extract was obtained from the flowers while the *H. virginiana* extract was obtained from the bark. Concentrations of 10 to 80% v/v of the glycolic extracts were evaluated in our experiments. All glycolic extracts were sterilized by filtration through a 0.22-μm filter (Millipore, Billerica, USA) to ensure the absence of contamination.

Minimum inhibitory concentration determination

The antifungal activities of the two glycolic extracts against the aforementioned *Candida* strains were investigated for the determination of the minimum inhibitory concentration (MIC) according to protocol M27-S3 of Clinical and Laboratory Standards Institute (2008)[13]. Serial dilutions of the glycolic extracts at concentrations of 80, 40, 20, and 10% v/v were placed in 96-well plates. Inocula of the strains were prepared in RPMI-1640 (Sigma-Aldrich, St Louis, USA), buffered to pH 7 with MOPS (Sigma-Aldrich), and added to the plate at a final concentration of 1×10^4 CFU/mL. The plates were incubated at 36 °C for 24 and 48h, and then fungal growth was evaluated by visual inspection. For the confirmation of these results, 10 μL of MTT solution (5 mg/mL) (Sigma-Aldrich) was added to evaluate the viability of the yeasts treated with the extracts. The positive control wells contained fungal growth without treatment and the negative control wells contained RPMI without *Candida* inocula.

Minimum fungicidal concentration determination

To better evaluate the activity of the glycolic extracts against *Candida* yeasts, the viability of the fungal cells after treatment with different concentrations of the glycolic extracts was investigated. An aliquot of 10 μL of MIC assay cultures was transferred to plates with Sabouraud Dextrose Agar and incubated at 36 °C for 48h. Then, the minimum fungicidal concentration (MFC), the lowest extract concentration that killed the fungal cells was determined by growth absence in visual inspection.

Toxicity assay using Galleria mellonella

The toxicity of the glycolic extracts was evaluated in vivo using G. mellonella larvae according to Sangalli-Leite et al.[14]. These larvae were maintained and grown in the laboratory at 28 °C and fed with wax and pollen. Larvae weighing 150 mg and displaying no color alterations were selected for this assay and were distributed into the following groups (six larvae per group): negative control, 80 and 40% v/v of A. montana extract, 80 and 40% v/v of H. virginiana extract, and diluent control (10 mM phosphate-buffered saline (PBS), pH 7.2). First, larvae were sterilized with 70% ethanol and then they were injected with 10 μL of diluted glycolic extracts in the proleg region using Hamilton syringe. Larvae were maintained at 36 °C for 7 d and were observed daily for melanization and locomotion. Dark and unresponsive larvae were considered dead.

Results

Antifungal activity of A. montana and H. virginiana glycolic extracts

In order to evaluate the antifungal effect of *A. montana* and *H. virginiana* extracts, strains of different *Candida* species were treated with different concentrations of the two extracts to determine their MIC and MFC values. The MIC is the concentration that inhibits the yeast growth and the MFC is the concentration that kills the yeast cells (Table 1).

A. montana extract concentrations that inhibited the growth of different Candida species (MIC) were 10% v/v for *C. tropicalis*, 20% v/v for *C. glabrata* and *C. krusei*, and 40% v/v for *C. albicans* and *C. parapsilosis*. Its MFC against C. tropicalis was 80% v/v, whereas for the other species, the MFC was equal to the MIC (Table 1, Figure 1).

Regarding *H. virginiana* extract, its MIC values were 10% v/v for *C. tropicalis* and *C. albicans*, and 20% v/v for *C. glabrata*, *C. krusei*, and *C. parapsilosis*, and were the same as the MFC values for the respective species (Table 1, Figure 1). According to the presented results, we can conclude that the antifungal activity of H. virginiana extract against Candida species is stronger than that of A. montana extract.

Toxicity study of A. montana *and* H. virginiana *glycolic extracts*

The toxicity of the glycolic extracts was evaluated in vivo using the *G. mellonella* model. Results are shown in Figure 2 as the survival curves of larvae receiving different treatments during 7 d. Data obtained demonstrate that neither of the two glycolic extracts caused death of a significant number of larvae. In conclusion, the two extracts showed no toxicity in the *G. mellonella* model.

Discussion

This work was carried out with the aim of identifying natural antifungal substances that may be employed in the treatment of candidiasis. Natural substances constitute an interesting source of active compounds against *Candida* species that act by different mechanisms such as inhibition of germination or biofilm formation,

Table 1. Antifungal activities of *A. montana* and *H. virginiana* glycolic extracts. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were determined against different *Candida* species

Candida spp	*A. montana*		*H. virginiana*	
	MIC	**MFC**	**MIC**	**MFC**
C. albicans	40% v/v	40% v/v	10% v/v	10% v/v
C. parapsilosis	40% v/v	80% v/v	20% v/v	20% v/v
C. glabrata	20% v/v	20% v/v	20% v/v	20% v/v
C. krusei	20% v/v	20% v/v	20% v/v	20% v/v
C. tropicalis	10% v/v	10% v/v	10% v/v	10% v/v

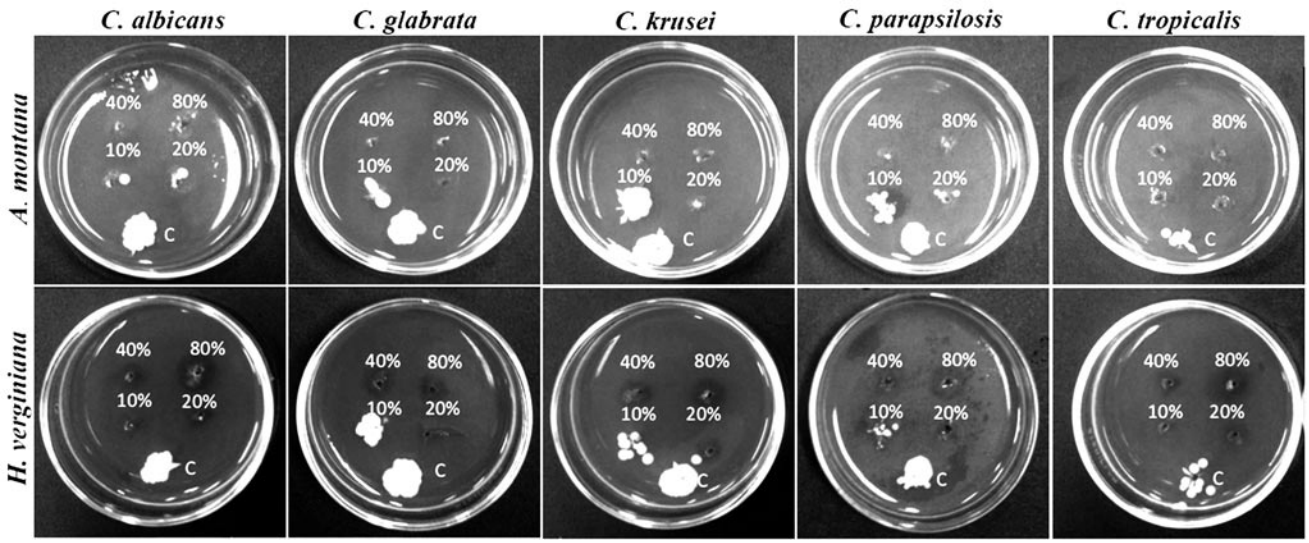

Figure 1. Determination of MFC values of *A. montana* and *H. virginiana* glycolic extracts against *Candida* species. Aliquots of MIC assay cultures were grown on Sabouraud Dextrose Agar and incubated at 36 °C for 48 h. The concentrations 10, 20, 40, and 80% v/v are identified in the image. Letter C corresponds to positive control for assessment of cellular viability without treatment.

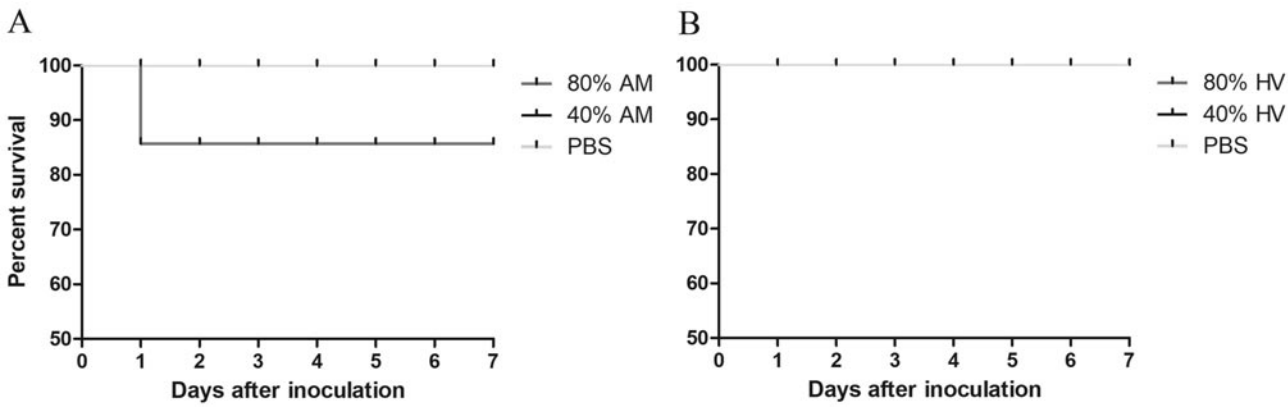

Figure 2. Toxicity assay of the two extracts using survival curve of *G. mellonella* larvae. Larvae were distributed into the following groups: negative control, 80% and 40% v/v of the two extracts, and diluent control (PBS). (A) Survival curve after treatment with A. montana extract (AM). (B) Survival curve after treatment with H. virginiana extract (HV).

impairment of cell metabolism or cell wall integrity, and induction of apoptosis or cell membrane plasticity.[5] Therefore, in this study, the antifungal effects and toxicities of *A. montana* and *H. virginiana* extracts were evaluated.

Glycolic extracts of *A. montana* and *H. virginiana* demonstrated similar antifungal activities. However, there was a difference in the sensitivity of the tested *Candida* species to both extracts. Corroborating with our results, Arendrup and Patterson showed in a

literature review that different *Candida* species may present different sensitivity levels depending on the type of the antimycotic used. They demonstrated that C. *albicans* and *C. tropicalis* are sensitive to amphotericin B, echinocandins, and fluconazole; however, alterations in species sensitivity to fluconazole could take place, leading to development of resistance. *C. glabrata* exhibits intermediate resistance to fluconazole, while *C. krusei* shows strong resistance to the drug. Although *C. parapsilosis* is sensitive to fluconazole; it possesses intermediate resistance to echinocandins.[4]

The obtained results were expected in light of previous studies. Studies on the biological activity of *A. montana* extracts have demonstrated that their effect is attributed to the presence of the following chemical components: lactones, flavonoids, essential oils, carotenoids, alkaloids, polyacetylenes, phenolic acids, lignins, and dicaffeoylquinic derivatives.[15] Among these compounds, phenolic acids like gallic acid and caffeic acid were previously found to exhibit anticandidal activity.[6,16] In addition, A. montana belongs to family Asteraceae, which is known to include other plants exhibiting antimicrobial activities.[17]

Studies on *H. virginiana* biological activities in the literature are few compared to studies on *A. montana*. The chemical composition of *H. virginiana* includes tannins, which are important chemical compounds with promising antimicrobial activities.[18] Results obtained from studies on mouthwashes containing *H. virginiana* extract show the effectiveness of this plant in reducing the dental plaque index, which is associated with biofilm formation.[19]

With the exception of a few natural substances that already had their toxicity tested[5], it is extremely important to assess the toxic potential of any natural substance before proposing its antifungal potential. Therefore, we investigated the toxicity of the extracts in the *G. mellonella* model, a well-established model for toxicity testing during the development of new drugs.[7] Our results showed that both glycolic extracts were non-toxic to *G. mellonella* larvae, thereby favoring their potential use in the treatment of fungal diseases.

Conclusions

In this study, the glycolic extracts of *A. montana* and *H. virginiana* were found to exhibit antifungal activities against *Candida* species and to be non-toxic in an in vivo model. Therefore, these glycolic extracts can be beneficial in the development of antifungal products against *Candida* species.

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The authors declare that they have no conflict of interests.

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