Short communication

Plants used in folk medicine: The potential of their hydromethanolic extracts against *Candida* species

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ABSTRACT

Currently, opportunistic fungal infections are considered a serious problem regarding public health. Despite the advances towards the synthesis of new antifungal agents, an increasing incidence of drug-resistant microorganisms has been observed. In this sense, other alternatives are necessary. In the present work, the antifungal activity of extracts from ten different plants, commonly used in folk medicine, were evaluated against nineteen *Candida* strains, including *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* species. Although the majority of the extracts had no antimicrobial effect, *Juglans regia* extract was very effective, exerting an inhibitory effect against all the tested *Candida* strains, while *Eucalyptus globulus* was effective against seventeen of them. *Pterospartum tridentatum* and *Rubus ulmifolius* presented similar antifungal effects, being effective against six *Candida* strains. The diameter of halo ranged, respectively, between 9–14 mm and 9–21 mm to the mentioned plant extracts, and the MIC_{50} values evidenced mainly a fungistatic activity. Both extracts showed similar MIC_{50} values for *C. albicans* strains, while *C. parapsilosis* and *C. glabrata* were more sensible to *E. globulus*. Otherwise, all the *C. tropicalis* strains were more sensible to *J. regia*. Overall, hydromethanolic plant extracts could constitute promissory alternatives to the traditional antifungal agents.

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1. Introduction

Medicinal plants have been widely used, since pre-historic era, not only to improve health and well-being, but also to treat some specific diseases/disorders (Agarwal et al., 2010; Bakkali et al., 2008; Sher, 2009). In the last years, those natural matrices have sparked an increasing interest for scientific researchers, who have proved their extremely richness in natural biomolecules, conferring a multitude of biological properties. Their phytochemical potential, synergistic effects and mechanisms of action have been studied, in different areas of knowledge (Alves-Silva et al., 2013; Shojaii and Fard, 2012; Singh et al., 2010). However, many extracts/compounds from plant origin remain unstudied.

In parallel with these advances on plant products research, microbiological area has been subjected to severe modifications. A wide variety of microorganisms exist in the commensal flora of healthy population, providing several benefits to the host. How-ever, in the last two decades, some species have presented an abnormal overgrowth and they become harmful, affecting directly not only the welfare, but also the life of individuals (Kim and Sudbery, 2011; Mayer et al., 2013; Tsai et al., 2013).

Opportunistic fungal infections, namely *Candida* species, comprise the most common deep pathogenic infections, being observed not only in immunocompromised patients, but also around the hospital and even in the rest of the population (Abi-Said et al., 1997; Eggimann et al., 2003; Li et al., 2006; Raman et al., 2013). *Candida albicans* has been considered the most relevant species in the mentioned infections, nevertheless, other *Candida* species have been, currently, pointed out, such as *Candida tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*, *C. krusei*, *C. famata*, *C. guilliermondii* and *C. lusitaniae* (Brunke and Hube, 2013; Ferreira et al., 2013; Kim and Sudbery, 2011; Mayer et al., 2013; Sardi et al., 2013).

Concomitantly, increasing rates of drug-resistant pathogenic microorganisms have been observed, in part due to the indiscriminate use of some antimicrobial agents (Kanafani and Perfect, 2008; Sangamwar et al., 2008; Sanglard and Odds, 2002; Sanglard, 2002; White et al., 1998). Some advances in pharmaceutical industries have been achieved, towards the synthesis and/or preparation of

Table 1
Antifungal activity of hydromethanolic extracts from different plant origin, against several *Candida* species.

Species	Strains	Origin	Inhibition zones (mm)									
			Echinacea purpurea	*Eucalyptus globulus*	*Foeniculum vulgare*	*Juglans regia*	*Matricaria recutita*	*Melissa officinalis*	*Pterospartum tridentatum*	*Rosa canina*	*Rubus ulmifolius*	*Tabebuia impetiginosa*
C. albicans	ATCC 90028	Reference	–	10	–	13	–	–	10	–	10	–
	575541	Urinary	–	12	–	13	–	–	–	–	9	–
	557834	Vaginal	–	12	–	12	–	+	10	++	10	–
	558234	Vaginal	–	11	–	12	–	–	9	–	15	++
C. glabrata	ATCC 2001	Reference	–	21	–	14	–	–	11	–	19	–
	D1	Oral	–	13	–	12	–	–	11	–	++	–
	513100	Urinary	–	11	–	10	–	–	9	–	16	–
C. parapsilosis	ATCC 22019	Reference	++	10	++	12	–	–	++	–	–	–
	AM2	Oral	–	9	–	12	–	–	++	–	++	–
	AD	Oral	–	18	–	12	–	–	–	–	+	+
	491861	Vaginal	–	+	++	11	+++	–	–	–	++	+++
	513143	Vaginal	–	12	–	10	+	–	–	–	–	–
C. tropicalis	ATCC 750	Reference	–	13	–	11	–	–	+	–	–	–
	AG1	Oral	–	11	++	12	–	–	+++	–	–	–
	75	Vaginal	–	12	–	10	–	–	–	–	++	–
	12	Vaginal	–	+++	–	9	–	–	–	–	–	–
	544123	Urinary	–	9	–	10	–	–	–	–	–	–
	519468	Urinary	–	10	–	10	–	–	–	–	–	–
	T2.2	Oral	–	9	–	10	–	–	–	–	–	–

(−) Absence of antifungal effect, (+) cell growth inhibition, (++) cell density reduction, (+++) cell density reduction and growth inhibition.

new antifungal drugs more effective and selective than the previous (Perlin, 2014, 2009; Sangamwar et al., 2008), but, some crucial aspects need to be considered, such as their safety, tolerability and even side effects. Recent studies have been carried out accessing the antifungal potential of some plant species, but essential oils are the main used extract preparations. Despite the existence of other solvents that have also been used in extraction procedures (*i.e.,* ethanol, alcohol, methanol, ethyl acetate, dichloromethane, water, among others), some evidences show that when a small portion of water is added to the organic solvent, the phenolic extraction is improved. Therefore, considering that natural extracts/compounds from plant origin could play an important role in the development of antifungal agents, the aim of the present work is to identify and highlight the potential of hydromethanolic extracts prepared from ten different plants commonly used in folk medicine against *Candida* species.

2. Materials and methods

2.1. Samples

A total of ten medicinal plants, commonly used in folk medicine, were studied; four were wild harvested in Trás-os-Montes – Bragança, North-Eastern Portugal: flower buds and fully opened flowers of *Rubus ulmifolius* Schott (elm-leaved blackberry), petals of *Rosa canina* L. (rose hips/dogrose), and leaves of *Juglans regia* L. (walnut). The other seven were commercial samples: leaves of *Melissa officinalis* L. (lemonbalm), aerial parts of *Foeniculum vulgare* Miller (fennel), *Matricaria recutita* L. (chamomile) and *Echinacea purpurea* (L.) Moench (purple coneflower), and leaves of *Eucalyptus globulus* Labill. (blue gum), *Tabebuia impetiginosa* (Mart. ex DC) Standley (pau d'arco) and flowering parts of *Pterospartum tridentatum* (L.) Willk. (carqueja). Plant scientific nomenclature according to The Plant List (2013), version 1.1 (2013).

2.2. Standards and reagents

Methanol was of analytical grade purity and supplied by Pronalab (Lisbon, Portugal). RPMI 1640 medium was purchased from Sigma Chemical Co., (St. Louis, MO, USA). Sabouraud Dextrose Broth (SDB) and Agar were from Merck (Darmstadt, Germany). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Preparation of the hydromethanolic extracts

Hydromethanolic extracts were obtained by extracting the sample (1 g) with 30 mL of methanol:water (80:20, *v/v*) at 25 °C and 150 rpm for 1 h, and filtering through Whatman No. 4 paper. The final residue was then extracted with an additional 30 mL portion of the hydromethanolic mixture. The combined extracts were evaporated at 35 °C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The lyophilized hydromethanolic extracts, were re-dissolved in water, performing stock solutions with concentrations of 50 mg/mL, from which several dilutions were prepared.

2.4. Antifungal activity

2.4.1. Disc diffusion assay

Nineteen *Candida* strains were used during this study (Table 1), four from the American Type Culture Collection (ATCC), and fifteen clinical isolates from vaginal and urinary tracts and oral cavity. The clinical isolates were obtained from the archive collection of the biofilm group of the Centre of Biological Engineering, University of Minho, Braga – Portugal. Before each experiment, all strains were grown in Sabouraud Dextrose Agar (SDA) for 24 h at 37 °C. After that time, one loop of each colony of cells was transferred to SDB liquid medium and incubated under stirring at 37 °C during 24 h. An aliquot of each species (300 μL), containing approximately 1×10^5 cells/mL was spread in SDA Petri dishes. Then, an aliquot (25 μL) of each hydromethanolic plant extracts, with a known concentration (50 mg/mL), was placed on a sterile blank disc. Sterile water was used as negative control. The plates were incubated at 37 °C, during 24–48 h. The evaluation of inhibitory properties was performed measuring the corresponding zone of inhibition. However, when there was no evident halo, but some inhibition of growth, the effect was also classified as: (+) cell growth inhibition, (++) cell density reduction, (+++) cell density reduction and growth inhibition.

2.4.2. Minimal inhibitory concentrations (MIC)

Minimal inhibitory concentrations were determined for the plant extracts that demonstrated the most pronounced positive results in the disc diffusion test, according with the guidelines from the Nature Protocols (Wiegand et al., 2008), with some modifications. Afterwards, a colony recovered from the SDA was suspended in 5 mL of sterile saline solution (0.85% NaCl) and vortexed for 15 s. The resulting suspension was adjusted by adding saline solution to reach the value of 0.5 in McFarland scale. Successive dilutions of each plant extract (0.1875, 0.375, 0.75, and 1.5 mg/mL) were prepared in RPMI 1640 medium at pH 7. Aliquots of each plant extract (100 μL), were dispensed into a 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and further incubated with aliquots (100 μL) of the tested *Candida* species. Sample and yeast-free controls were also included. The 96-well plates were incubated at 37 °C for 48 h. After visualization of the resultant plate, the MIC values were correspondent to the antifungal concentration where there was no growth, or even fungistatic effect, by comparison with the control (cells grown without extracts). The number of viable cells was assessed by the determination of number of colony forming units (CFUs), through several dilutions, after 24 h of incubation at 37 °C and the number of colonies formed were counted. The results were presented as the total of CFUs (Log CFUs) and the experiments were repeated in triplicate on three different occasions.

2.5. Statistical analysis

The results were analyzed using one-way analysis of variance (ANOVA) and means were compared using Tukey's honestly significant difference (HSD) multiple comparisons test and further coupled to Welch to verify the equality of means. All statistical tests were performed using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

3. Results and discussion

The results of the antifungal activity screening of the tested hydromethanolic extracts, against nineteen *Candida* strains are presented in Table 1. No evident halo formation was observed for *Echinacea purpurea*, *Foeniculum vulgare*, *Matricaria recutita*, *Melissa officinalis*, *Rosa canina* and *Tabebuia impetiginosa*, while in *Eucalyptus globulus*, *Juglans regia*, *Pterospartum tridentatum* and *Rubus ulmifolius* plant extracts, the halo diameters ranged between 9–21 mm. *Juglans regia* extract was the most effective one, presenting an evident halo for all the tested *Candida* strains (varying between 9–14 mm). *Eucalyptus globulus* extract also presented a significant antifungal potential, being effective against seventeen *Candida* strains (diameter of halo ranging between 9–21 mm). *Pterospartum tridentatum* and *Rubus ulmifolius* presented similar antifungal effects, being effective against six *Candida* strains,
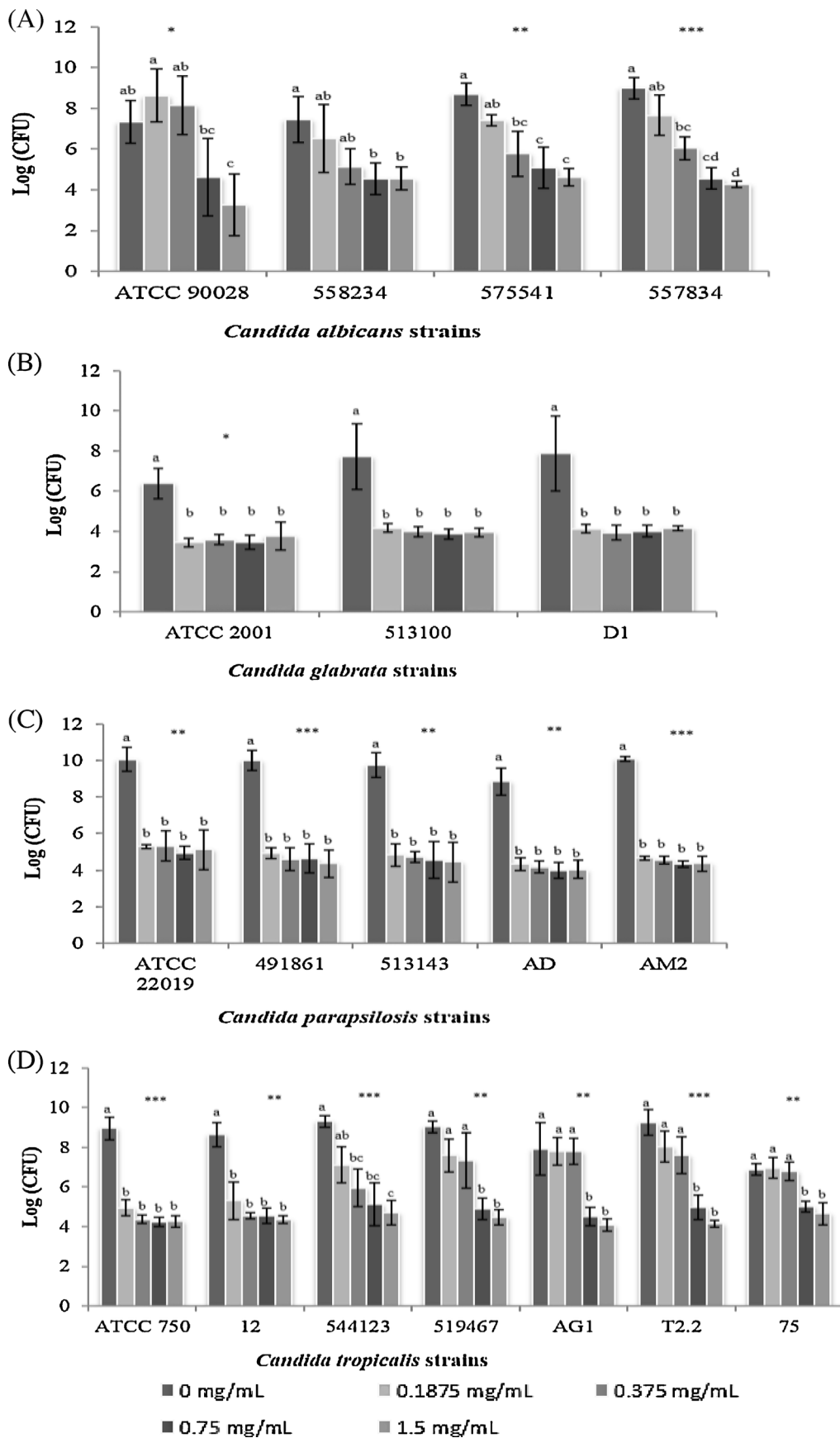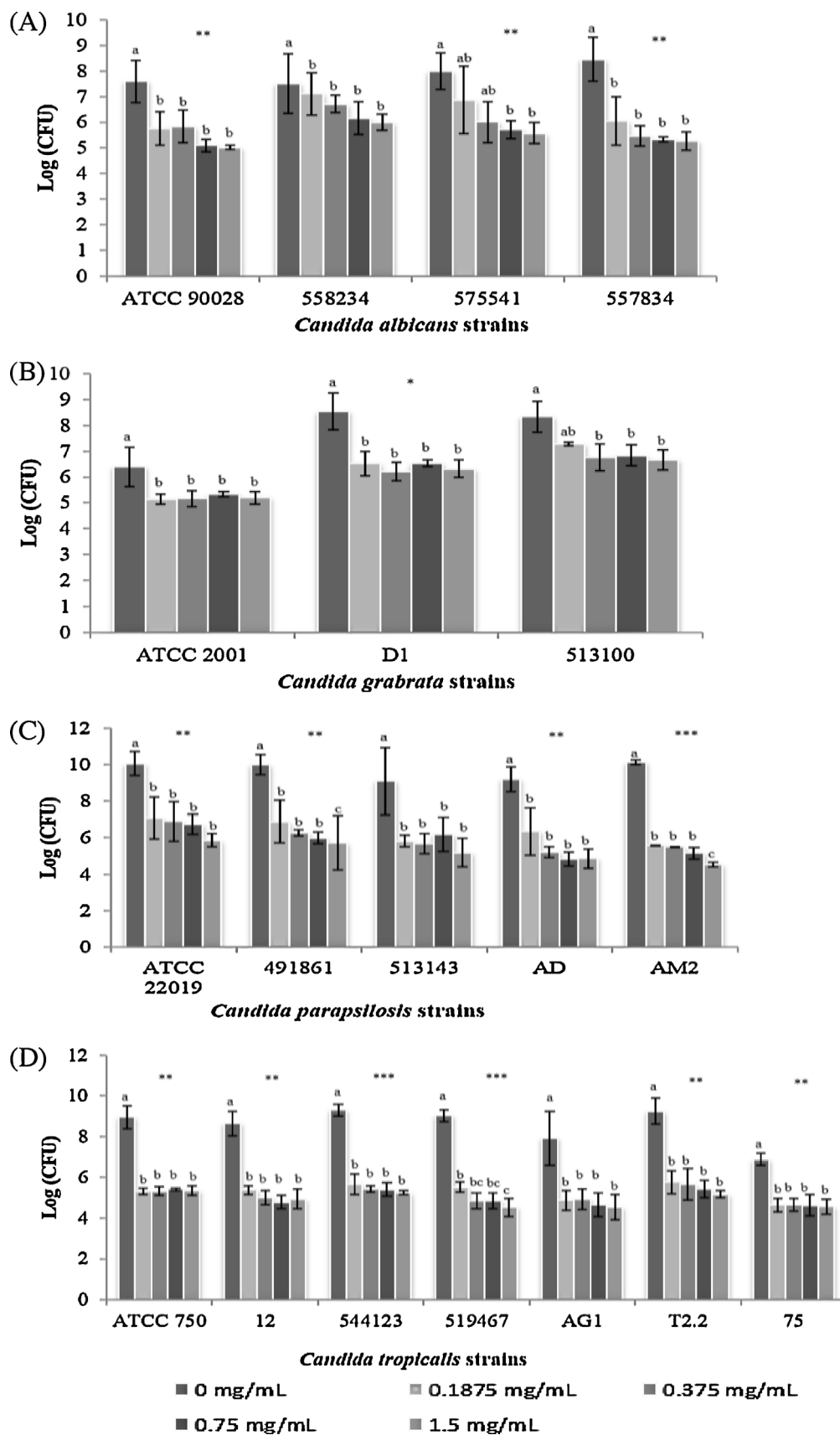
Fig. 1. Logarithm of number of colony forming units (CFUs) of different strains of *C. albicans* (A), *C. glabrata* (B), *C. parapsilosis* (C) and *C. tropicalis* (D) cultured within different concentrations of the hydromethanolic extract of *Eucalyptus globulus*. Error bars represent standard deviations (SD). (*$P < 0.05$, **$P < 0.005$ and ***$P < 0.001$), extract concentrations results that are significantly different. In each strain different letters mean significant differences ($P < 0.05$).

Fig. 2. Logarithm of number of colony forming units (CFUs) of different strains of *C. albicans* (A), *C. glabrata* (B), *C. parapsilosis* (C) and *C. tropicalis* (D) cultured within different concentrations of the hydromethanolic extract of *Juglans regia*. Error bars represent standard deviations (SD). (*$P < 0.05$, **$P < 0.005$ and ***$P < 0.001$), extract concentrations results that are significantly different. In each strain different letters mean significant differences ($P < 0.05$).

belonging to *C. albicans*, *C. glabrata* and *C. parapsilosis* species. Moreover, the majority of *C. tropicalis* strains were resistant to both tested extracts. No evident antifungal activity was also observed for *Rosa canina*. This work revealed that, in general, *C. glabrata* strains were the most susceptible *Candida* species, followed by *C. albicans*, *C. parapsilosis* and, lastly, *C. tropicalis* strains.

Some previous reports have described the antimicrobial properties of those medicinal plants, namely *Foeniculum vulgare* essential oil (Roby et al., 2013), *Melissa officinalis* ethanol extract (Ertürk, 2006; Uzun et al., 2004), *Rosa canina* aqueous extract (Orhan et al., 2012), and *Juglans regia* methanol, ethyl acetate and acetone extracts (Noumi et al., 2011, 2010), remaining unstudied the hydromethanolic extract preparations. In fact, there are some evidences confirming the high diversity of chemical compounds solubility and, therefore, the extraction solvent affect the extracts bioactivity. In this sense, and in order to understand the effects of hydromethanolic extract preparations, minimal inhibitory concentrations were determined for the plant extracts that demonstrated positive results in the initial screening (Table 1).

Thus, the MIC values were determined for *Eucalyptus globulus* and *Juglans regia* extracts (Figs. 1 and 2, respectively) against *C. tropicalis* strains ($n = 7$), *C. parapsilosis* strains ($n = 5$), *C. glabrata* ($n = 3$) and *C. albicans* ($n = 4$). The obtained results revealed that both extracts exerted more fungistatic than fungicidal effect, since they only caused a considerable CFUs reduction, without evidences of a full growth inhibition. Moreover, all the obtained results are in accordance with the previous assay (disc diffusion assay). For example, *E. globulus* showed a growth reduction between 2 and 5 Log (CFUs), in all the tested *C. parapsilosis* ($MIC_{50} = 0.1875$ mg/mL), followed by *C. glabrata* ($MIC_{50} = 0.1875$ mg/mL) and some *C. tropicalis* strains, namely ATCC 750, 12, 544123, 519467 and T2.2 ($0.1875 < MIC_{50} < 1.5$ mg/mL). While those *Candida* species were the most sensible to *E. globulus* extract, *C. albicans* strains were less sensible ($MIC_{50} \geq 1.5$ mg/mL). Otherwise, for *J. regia*, the tested *C. parapsilosis* ($0.1875 < MIC_{50} < 1.5$ mg/mL) and *C. tropicalis* ($MIC_{50} \approx 0.1875$ mg/mL) strains were the most sensible. The Log CFUs reduction ranged between 3 and 5, while for *C. albicans* ($MIC_{50} \geq 1.5$ mg/mL) and *C. glabrata* ($MIC_{50} \geq 1.5$ mg/mL) strains ranged between 0.5 and 2.5 Log (CFUs), corresponding to a higher resistance.

Overall, and despite the need of further studies to elucidate the mechanisms of action of the tested plant extracts, as well as to test their *in vivo* efficacy, it is possible to conclude that hydromethanolic extracts of *E. globulus* and *J. regia* could constitute promissory alternatives to the current antifungal agents.

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