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ANTIFUNGAL EFFECT OF ETHANOL PLANT EXTRACT ON *CANDIDA* SP

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ABSTRACT

In this study, we investigated the *in-vitro* antimicrobial activity of some medicinal plants in the Arabian peninsula, including *Rhamnus globosa*, *Ocimum basilicum*, *Tecoma stans* and *Coleus forskohlii*. Our results showed high inhibitory growth in yeast after treatment with *R. globosa* and *O. basilicum. C. tropicalis* was shown to be a sensitive strain with an inhibition of 29, 28, 35, 25 and 27 mm after treatment with *R. globosa*, *R. globosa** “leaf with thorns,” *O. basilicum*, *Tecoma stans* and *Coleus forskohlii*, respectively. Thus, our results confirmed the fungicidal effect of *O. basilicum* and *R. globosa* with a 20 and 30% reduction in CFU compared with the starting inoculums in the time-kill.

Keywords: Antifungal, Medicinal Plant Extracts, *Candida* sp., MIC, Effect

1. INTRODUCTION

Candida albicans is a common microflora in humans and an opportunistic fungal infection, which is often found in compromised immune systems and the overgrowth of this yeast causes candidiasis. In addition, these fungal pathogens have become increasingly important over the past 20 years; with the success of modern medical practices, this provides hope for the survival of weakened and immunosuppressed patients (Kourkoumpetis *et al*., 2010; Kothavade *et al*., 2010; Tanushree *et al*., 2010; Dalirsani *et al*., 2011). Treatment of pathogenic fungi involves antifungal medications, which include several groups. However, fungal and human cells are similar at the molecular level and thus it is difficult to identify medicines that target fungi without affecting human cells. Consequently, the side effects of these drugs include allergic reactions, liver damage and altered estrogen levels. Moreover, improper use of antifungals can be life threatening (McMichael and Hordinsky, 2008). Thus, new antifungal drugs that are safer and more effective are needed.

Natural products derived from medicinal plants are among the safest sources of new medications and antifungal drugs. Several studies have investigated natural products and essential oils with antimicrobial and antifungal effects. For example, in a study of alcoholic curry leaves, a maximum zone of inhibition on *Candida albicans* following treatment with aqueous tea leaves was observed. However, other plant extracts such as alcoholic onion leaves, alcoholic tea leaves, alcoholic onion bulb, alcoholic aloe vera and alcoholic mint leaves inhibited the growth of *Candida albicans*, but to a lesser extent (Doddanna *et al*., 2013). *Salvia officinalis* (L.) demonstrated less inhibitory effects against *C. albicans* or *C. tropicalis* and the root extracts of *Labisia pumila* showed a higher activity in response to *Candida* sp. compared with leaf extracts (Celi Garcia *et al*., 2012; Karimi *et al*., 2013). In a study on Chinese medicinal plants, water, ethanol, acetone and n-hexane extracts for each plant were tested on *C. albicans*, as well as extracts of *Pseudolarix kaempferi* Gord., acetone extract of *Sophora flavescens* Ait., ethanol, acetone and hexane extracts of *Pogostemon cablin* (Blanco) Benth. and *Alpinia officinarum* hance, hexane extract of *Eugenia caryophyllata* Thunb. ethanol and acetone extracts of *Melia toosendan* Sieb. et Zucc. and *Polygonum hydropiper* L., which showed an inhibition of more than

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50% of *C. albicans* growth and was comparable to miconazole in some cases (Liu *et al*., 2012).

In this study, the objective was to investigate the antifungal activity of ethanol extracts of some medicinal plants in the Arabian Peninsula. These medicinal plants included *Rhamnus globosa*, *Ocimum basilicum*, *Tecoma stans* and *Coleus forskohlii*.

2. MATERIALS AND METHODS

2.1. Fungi Studies

The examined fungi included *Candida albicans* ATCC CA 10231 and *C. tropicalis* ATCC CT 2697, which were prepared as inocula by obtaining 100 µL of each yeast containing 1×10^5-5×10^5 (cfu) and inoculating onto Mueller-Hinton agar (OXOID CM 337).

2.2. Plant Studies and Extract Preparation

The plants were collected from different locations in the Arabian Peninsula. They were identified in the Botany section at the biology department, faculty of science, KAU as *Rhamnus globosa*, *Ocimum basilicum*, *Tecoma stans* and *Coleus forskohlii*. The plant leaves, except *R. globosa,* which consisted of two types of extracts (leaves and leaves with thorns) were washed several times with distilled water, spread onto plates and dried at 40°C. After drying, the samples were grounded and solubilized with ethanol solvent at 10 mg mL^{-1}. The mixtures were maintained on a shaker at 120 rpm at 30°C for 24 h and then filtered using Whatman No. 1 filter paper. The samples were dried under a reduced pressure at 40°C and the thick deposits obtained were used as crude extracts (Vijayakumar *et al*., 2013).

2.3. Antifungal Assays

The antimicrobial activity of each crude plant extract was determined *in vitro* in response to the *Candida* species. The activities were measured using disc diffusion and broth dilution methods, as previously described by the Clinical and Laboratory Standards Institute (CLSI; formerly known as the National Committee for Clinical Laboratory Standards) (NCCLS, 2004; Fothergill, 2011).

Each thick deposit extract was dissolved in Dimethylsulfoxide (DMSO) at 50 µg mL^{-1} and filtered through a 0.22 µm pore filter (Millipore, Billeria, MA). The antibacterial activities of each extract were investigated by disc diffusion using filter paper discs (1-mm diameter impregnated with 100 µL), which were then placed on the pre-inoculated agar surface. Negative controls were prepared with the same solvent. Plates were then incubated at 35°C for 48 h and the inhibitory zones of each disc were measured. All tests were performed in triplicate.

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

Extracts that inhibited the growth of bacteria were investigated to determine the MIC and MFC using a broth-microdilution method. The yeast were cultured overnight on mueller-hinton agar and then resuspended in 1 mL mueller-hinton broth (OXOID CM 405) to obtain a final concentration of 100 cfu mL^{-1}. Each extract was serially diluted with mueller-hinton broth using methods approved by the National Committee for Clinical Laboratory Standards (M27-A) (NCCLS, 1997). After incubation, the MIC was determined as the lowest concentration of extract for which there was no visible growth compared with the control (CLSI, 2008; 2007). The MFC was determined by inoculating 0.1 mL of negative growth in MIC onto sterile SDA (OXOID) plates. The plates were incubated at 35°C for 48 h. The lowest concentration of plant extract that did not demonstrate growth of the tested organisms was considered the MFC; the negative control was a plate grown with media only (Ernst *et al*., 2002; Wiegand *et al*., 2008).

2.5. Time-Kill Determination

Liquid cultures (1 mL) were diluted to an initial inoculum of 2×10^5-5×10^5 CFU/mL in Mueller-Hinton broth and *R. globosa*, *O. basilicum*, *T. stans* and *C. forskohlii* were added at one-half and one and two times the MICs. The cultures were incubated for 0, 2, 4, 8, 12 and 48 h at 30°C. After each time point, 50 µL aliquots were obtained from the cultures, plated onto the SDA and incubated at 35°C for 48 h. Visible colonies were read using an Interscience scan 500 colony counters and each treatment was performed in triplicate (Nostro *et al*., 2000; Lewis *et al*., 2002).

2.6. Statistical Analysis

The results were analyzed by paired-samples *t*-test using the IBM SPSS 20 statistical software to compare the mean values of each treatment. The results are expressed as means ± SE. Probability levels of less than 0.01 were considered highly significant.

3. RESULTS

Investigation of new antifungal agents from natural sources is a major field of research and the results of these studies have identified several new sources of plant medicines and their synthetic compounds. As shown in **Table 1**, high inhibitory growth of the tested yeast was observed after treatment with *R. globosa* and *O. basilicum and C. tropicalis* was shown to be a sensitive strain with an inhibition of 29, 28, 35, 25 and 27 mm after treatment with *R. globosa*, *R. globosa** “leaf with thorns,” *O. basilicum*, *Tecoma stans* and *Coleus forskohlii*, respectively. The ethanol extract of the *R. globosa* leaf and *R. globosa* leaf with thorns showed that there was no significant difference between these bacteria on yeast growth inhibition and the highest inhibitory effect was observed in *C. tropicalis,* with an inhibition of 29 mm for *R. globosa*.

The MIC and MFC values are shown in **Table 2 and 3.** According to these results, the highest MICs were 8 µL mL^{-1} and 4 µL mL^{-1}, which were obtained by treatment with *O. basilicum* on *C. albicans* and *C. tropicalis*, respectively. In contrast, the lowest MICs were obtained by treatment with *Tecoma stans* extracts on the tested yeast. The most sensitive yeast was *C. tropicalis* with MICs of 4, 8, 4, 16 and 8 µL mL^{-1} after treatment with *R. globosa*, *R. globosa** “leaf with thorns,” *O. basilicum*, *Tecoma stans* and *Coleus forskohlii*, respectively. The MFCs were approximate in most plant extracts. Most fungicidal concentrations were affected by treatments with *O. basilicum* on *C. albicans* and *C. tropicalis* with values of 32 and 16 µL mL^{-1}, respectively and the fungicidal extract concentrations of *Tecoma stans* increased to 128 and 128 µL mL^{-1}, for *C. albicans* and *C. tropicalis*, respectively.

The concentration of MICs and MBCs reflected the kill-times of the tested yeast. As shown in **Fig. 1 and 2**, the kill-time of one-half MIC, MIC and two MIC of each plant extract had no significant differences. The tested yeast had an endpoint of kill-times at a concentration of two MICs within 4 h except *T. stans* and *C. forskohlii,* which had an endpoint of kill-time of 12 h and 8 h, respectively. The CFUs of the tested bacteria decreased after 2 h and continued to decrease until they reached the kill-time. The fungicidal endpoint was not reached at a concentration of one-half MIC and extracts of R. *globosa*, *O. basilicum* and *C. forskohlii* resulted in 20, 30 and 10% reduction in CFU from the starting inoculums, respectively.

Table 1. Inhibition of *candida* sp. growth (mm) after 48 h of incubation with 100 µL plants ethanol extracts

	Mean ± SE				
	Rhamnus globosa	*R. globosa*[1]	*Ocimum basilicum*	*Tecoma stans*	*Coleus forskohlii*
Candida albicans	26±0.11667**	25±0.17638**	32±0.09280**	24±0.23154*	25±0.22850*
C. tropicalis	29±0.11667**	28±0.08333**	35±0.10408**	25±0.06009**	27±0.32830*

*R. globosa**: The leaf and thorns

Table 2. MIC (µL/m) of *Candida* growth after 48 h of treated with serial concentrations of plants methanol extracts

	Rhamnus Globosa	*R. globosa**	*Ocimum basilicum*	*Tecoma stans*	*Coleus forskohlii*
Candida albicans	16	16	8	64	32
C. tropicalis	4	8	4	16	8

*R. globosa**: The leaf and thorns

Table 3. MFC (µL/m) of *Candida* growth after 48 h of incubation in SDA

	Rhamnus globosa	*R. globosa**	*Ocimum basilicum*	*Tecoma stans*	*Coleus forskohlii*
Candida albicans	32	64	32	128	64
C. tropicalis	32	64	16	128	64

*R. globosa**: The leaf and thorns

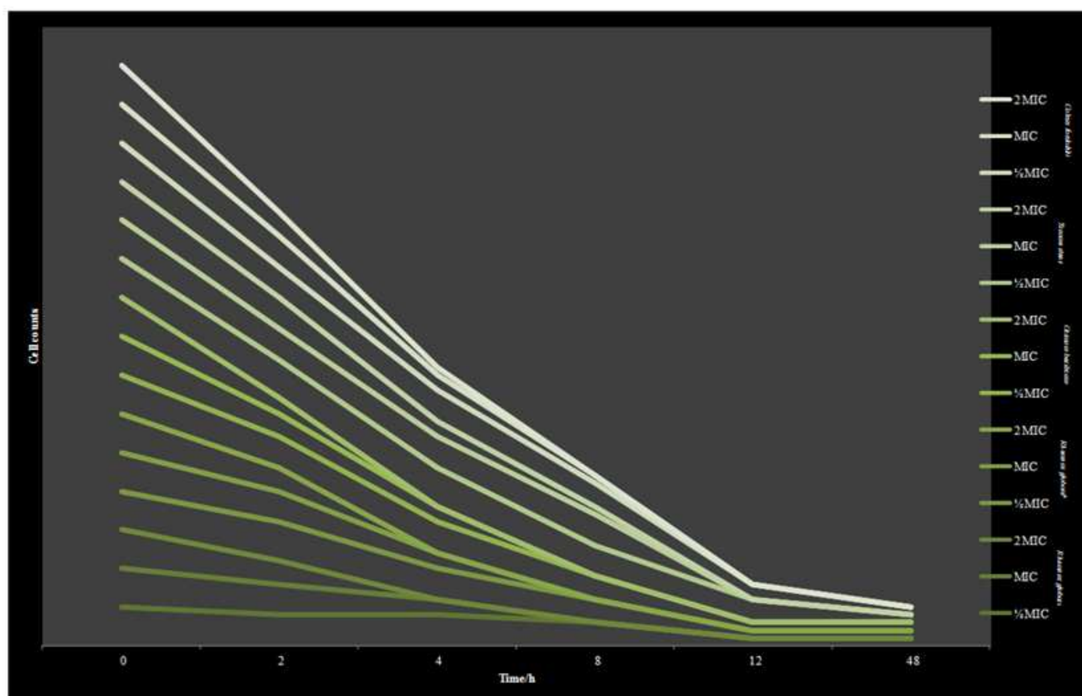

Fig. 1. Time-kill of *Candida albicans* within incubation in variety periods with plant extracts

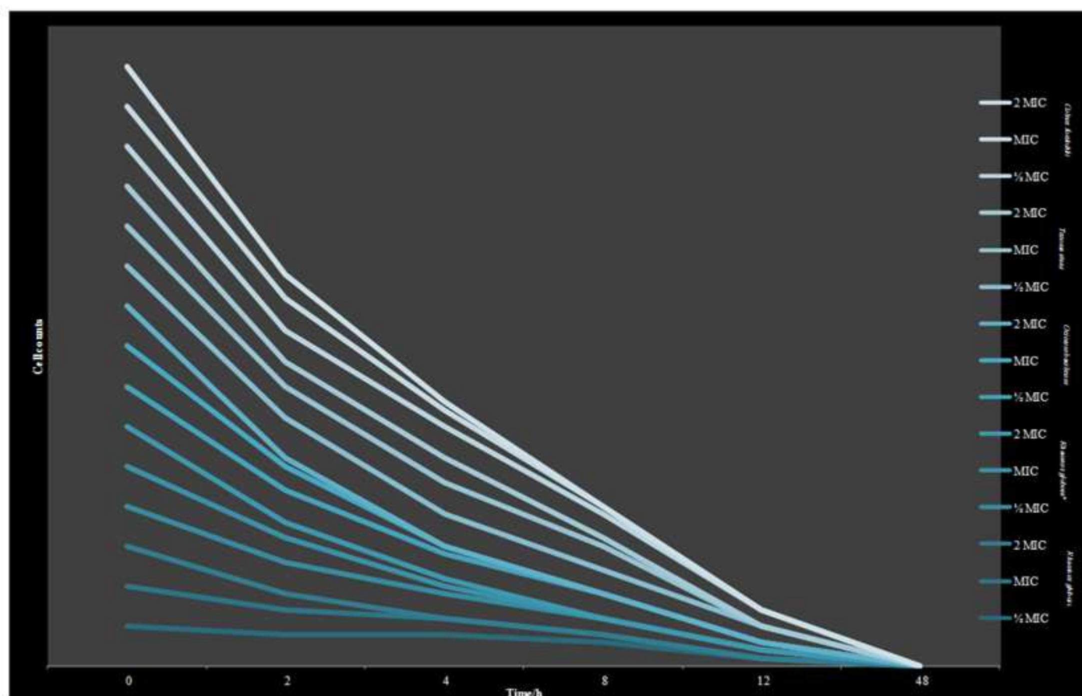

Fig. 2. Time-kill of *Candida tropicalis* within incubation in variety periods with plant extracts

4. DESSCUSION

Previous studies have performed screens on the antimicrobial activity of extracts from plants. On the basis of these studies, it was concluded that diethyl ether extracts were the most efficient antimicrobial compounds and this activity was more pronounced against gram-positive bacteria (Nostro *et al*., 2000). Our results on ethanol extracts were consistent with the results of several studies on alcoholic and non-alcoholic plant extracts. A previous study of alcoholic curry leaves demonstrated the strongest growth inhibition of *C. albicans* followed by aqueous tea leaves, alcoholic onion, tea, mint leaves extracts and alcoholic onion bulb and aloe vera extracts (Duarte *et al*., 2005). Moreover, a study of Traditional Chinese Medicine (TCM) and Chinese folk medicine demonstrated antifungal activity against *Candida albicans*; however, out of 58 extracts examined, two plant extracts, *Codonopsis pilosula* and *Tussilago farfara*, showed high inhibitory effects against *C. albicans* (Karimi *et al*., 2013; Zhang *et al*., 2013). In addition, a recent study on six natural commodities and four commercial medicines against *C. albicans* revealed that Mayaca extracts could act as a potential antifungal agent for oral thrush caused by *C. albicans* (Reena *et al*., 2013). Furthermore, extracts of *Althaea officinalis* and *Matricaria recutita* and *Combretum molle*, *Piper capense*, *Solanum aculeastrum*, *Syzygium cordatum* and *Zanthoxylum davyi* have a fungicidal effect on *C. albicans* (Steenkamp *et al*., 2007; Shakib *et al*., 2013). However, a study on essential oils and ethanolic extracts from the leaves and roots of 35 medicinal plants commonly used in Brazil, were tested for an antifungal effect on *C. albicans* and essential oils from 13 plants showed antifungal activity, including *Aloysia triphylla*, *Anthemis nobilis*, *Cymbopogon martini*, *Cymbopogon winterianus*, *Cyperus articulatus*, *Cyperus rotundus*, *Lippia alba*, *Mentha arvensis*, *Mikania glomerata*, *Mentha piperita*, *Mentha* sp., *Stachys byzantina* and *Solidago chilensis*. Moreover, the ethanol extract was not effective at any of the concentrations tested (Duarte *et al*., 2005).

Several studies have been performed to determine the MICs and MFCs and time-kill of medicinal plant extracts, including studies investigating the MICs and MFCs of medicinal plant extracts, including *Pinus monticola*, *Taxus baccata, Phyllanthus debilis* and *Plectranthus amboinicus*, *Catharanthus roseus, Nerium oleander*, *Tabernaemontana divaricata*, *Etlingera elatior*, *Rhizoma coptidis, Radix stemonae, Radix sophorae flavescentis*, *Ajuga pseudoiva* (L.), *Foeniculum vulgare*, *Trachyspermum ammi*, *Cuminum cyminum*, *Syzygium aromaticum*, *Cinnamomum tamala* and 54 species of Orchidaceae from Brazil on species of *Candida* and other fungi. Their results were consistent with our results (Vaz *et al*., 2009; Zamany *et al*., 2011; Ben Mansour *et al*., 2013; Khan *et al*., 2013; Khodavandi *et al*., 2013; Wankhede *et al*., 2013).

The high antimicrobial effect of medicinal plant extracts may be due to the compensation between the secondary metabolic compounds in plant tissues. Results of the phytochemical screening on medicinal plants showed that the antimicrobial activity was most likely due to the compounds reduced from plant metabolism, such as flavonoids, terpenes, alkaloids, tannins, the hydroxyl group and phenol; 1,8-cineole, geranial, germacrene-D, limonene, linalool, fatty acids, esters and menthol; and essential oils, such as yarrow, carvacrol, thymol, glycosides, tannins, saponins and steroids (Gregory *et al*., 2009; Choudhury *et al*., 2013; Jadha *et al*., 2013; Joshua and Takudzwa, 2013; Mulyono *et al*., 2013).

5. CONCLUSION

In conclusion, we highlighted the antifungal activity of ethanol extracts of *Rhamnus globosa*, *Ocimum basilicum*, *Tecoma stans* and *Coleus forskohlii*. These results confirmed the traditional uses of *R. globosa* as medicinal plants. In addition, extracts of *O. basilicum* primarily produced fungicidal effects, with a limited number of observed growths, which was consistent with the MIC and MBC and kill-time of both plant extracts.

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