

SUSCEPTIBILITY PROFILE OF *CANDIDA SPP.* ISOLATED FROM HUMANS AND DOGS WITH STOMATITIS TO THE ESSENTIAL OIL OF *THIMUS VULGARIS*

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Candida spp. form a part of human and animal oral cavity flora. However Candida spp. is the main cause of dental related stomatitis in humans and stomatitis in dogs. Stomatitis treatment implies the use of azoles and polyenes to which yeasts build up resistance. The research is directed to the use of natural compounds such as essential oils.

The aim of this paper is to define the antifungal activity of thyme oil on 15 clinical strains of Candida spp., isolated from humans and dogs and to determine if there is a difference in susceptibility between human and dog isolates.

Sampling in patients with stomatitis was done by swabbing the denture or oral mucosa swab while sampling in dogs was done by swabbing the oral cavity mucosa after stomatitis has been diagnosed. In order to investigate the antifungal activity of thyme oil in vitro, microdilution method was used.

Thyme oil expressed antifungal effects on all investigated strains. Also, our data show that the values of minimum fungicide concentration (MFC) and minimum inhibitory concentration (MIC) are lower in human strains. Explanation is that in most cases, stomatitis in humans is asymptomatic and thus not treated, so Candida strains have not developed resistance. On the other hand, stomatitis in dogs is followed by a marked clinical picture and treated is by antimicrobics (mostly by azoles), therefore resistant Candida strains are more likely to occur.

Key words: Candida, dogs, essential oil, stomatitis, Thymus vulgaris

INTRODUCTION

Yeasts of genus *Candida* are residential microorganisms which very often can be found in both human (Carvalho, 2012) and animal oral cavity. Apart from the oral cavity, *Candida* is present in some other human and animal moist areas such as: skin, inner ear, conjunctival canal, mouth, digestive system and perianal area. *Candida* yeasts along with other microorganisms are microbes of healthy living beings, but in specific conditions they can cause opportunistic infections (Cleff *et al.*, 2005).

Candida albicans (*C. albicans*) is the main etiological factor of oral candidiasis in humans and other small animals. Apart from *C. albicans*, other kinds of oral yeasts may be included in various pathogenic processes. In the available literature, the following are found: *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis* in humans or *C. parapsilosis*, and *C. guilliermondi* and *C. tropicalis* in dogs (Moreti *et al.*, 2006; Cleff *et al.*, 2007; Pozzatti, 2007; Yurayart *et al.*, 2011).

There are different clinical manifestations of oropharyngeal candidiasis in humans, including pseudomembranous candidiasis (thrush) and erythematous forms such as denture related stomatitis, which is often recurrent (Cannon *et al.*, 1995). Wearing prosthetic appliances is commonly associated with denture stomatitis, which is characterized by inflamed mucosa covered by denture, more often under the upper denture (Wilson, 1998). Patients with denture related stomatitis may complain of burning sensation, discomfort, or bad taste. However patients very often do not have any symptoms. There are many pre-dilutional factors that influence denture stomatitis, including age of denture, denture cleanliness, continuous constant denture wearing, denture trauma which appears as a consequence of wearing dentures, smoking, dietary factors, pH of denture plaque (Soysa *et al.*, 2004, 2006). Denture stomatitis is a disease of fungal and bacterial origin, and *C. albicans* is most frequently isolated yeast from the patients' oral cavities (Baena-Monroy *et al.*, 2005). Denture is more often a reservoir of microorganisms from which they can spread and cause diseases to other organs.

Stomatitis in dogs is characterized by erythematous oral mucous membrane which is ulcerous and covered with pseudo membranes, with halitosis and hypersalivation (McKcever and Klausener, 1986).

Opportunistic infections in dogs develop as a consequence of deteriorating defense mechanisms, immunosuppression and prolonged antibiotic treatment (Moreti *et al.*, 2006; Cleff *et al.*, 2007; Ong *et al.*, 2010; Yurayart *et al.*, 2011). Different *Candida* species are very important pathogenic factors in small animals and they are linked to urinary infections, (Ozawa *et al.*, 2005) skin lesions (Moretti *et al.*, 2004), otitis and systematic infections (Brown *et al.*, 2005).

Today candidiosis is a problem of public health due to resistance to conventional antifungal medicines and there are some reports on relapsing disease especially in immunocompromised patients (Sanglard and Kvota, 2002; Kolombo and Guimaraes, 2003; Pozzatti *et al.*, 2010).

In general, oral *C. albicans* isolates have high levels of susceptibilities to a range of antifungal agents (Kuriyama *et al.*, 2005), but some studies have shown high levels of azoles resistance in *C. albicans* strains isolated from the throat and mouth (Paniaqua *et al.*, 2002; Manfredi *et al.*, 2006). Owing to their antibacterial effects, essential oils are the alternative for antibiotics in the pharmaceutical industry (Bakkali, 2008). The increase of *C. albicans* resistance to antifungal medicines encourages the research of new therapeutic alternatives such as essential oils (Giordani *et al.*, 2004; Cavaleiro *et al.*, 2006).

Studies on essential oils have a goal to examine active compounds with a wide spectrum of effects, low toxicity and low prices (Rochette *et al.*, 2003). Treatment of fungal infection in veterinary medicine is limited to conventional polyenes and triazoles. Additionally, these medicines have adverse effects and relatively high prices, especially when they are used for animals (Sanglard, 2002; Mondelo *et al.*, 2003; Pressler *et al.*, 2003; Rochette *et al.*, 2003; Lukman *et al.*, 2007).

The aim of this study is to:

1. determine antifungal activity (minimum inhibitory concentration – MIC and minimum fungicide concentration – MFC)
2. determine if there is a difference in susceptibility profile of *Candida* spp. isolated in both humans and dogs

MATERIAL AND METHODS

Essential oils

In the research, the essential thyme oil used (Citychem, Belgrade, Serbia), was obtained by water vapour distillation under of *Thymus vulgaris* flowers. The percentage of particular components of thyme oil is given in Table 1.

Table 1. Components of thyme oil

Substance	%
Thymol	46.0
p-cymene	27.5
γ-terpinene	9.0
Linalool	6.5
Carvacrol	2.5
β-myrcene	2.4
Terpinen-4-ol	1.5

Fungal strains

The study has included the research of 15 clinical isolates of *Candida* spp. isolated in seven patients with diagnosed dental related stomatitis (5 *C. albicans*,

2 *C. glabrata*, 2 *C. krusei* and 1 *C. tropicalis*) and five dogs with stomatitis (3 *C. albicans*, 1 *C. parapsilosis* and 1 *C. krusei*).

The sampling in patients was done by swabs taken from their dentures (in patients with dental stomatitis) or by oral mucosa swabs. The sampling in dogs was done by mucosa swabs of the oral cavity when stomatitis has been diagnosed.

Swabs were transported to the microbiological laboratory in a period no longer than 30 minutes from the moment of sampling. Material was seeded on CHROMagar *Candida* plates (CHROMagar, Paris, France) and incubated at 37°C for 48 hours. The identification of *Candida* strains was done according to the specific appearance of the colonies as defined by the manufacturer.

In vitro antifungal activities of thymes oil

Susceptibility of strains to *thyme* oil has been investigated by the microdilution method according to the standardized protocol by The European Committee on Antimicrobial Susceptibility Testing – EUCAST (EUCAST). Serial oil dilutions were prepared in RPMI 1640 with 2% of glucose, in the concentration range 0.1 = 16 $\mu\text{L mL}^{-1}$.

Before testing, 15 *Candida* isolates, together with two reference strains (*C. albicans* ATCC 24433 and *C. krusei* ATCC 6258) were cultivated on Sabouraud dextrose agar plates (Oxoid, Basingstoke, United Kingdom) at 37°C during 24 hours. Yeast suspension has been prepared in sterile 0.9% NaCl and adjusted to 0.5 *McFarland* standard of turbidity (which fits the size of inoculums of 1-5 $\times 10^6$ CFU/mL).

Each chamber of the microtitration plates was inoculated by adding 100 μL of yeast suspension and 100 μL of corresponding concentration of *thyme* oil. The test has also included three controls: positive control/growth control (fungi in RPMI 1640 without *thyme* oil), negative control (fungi in solution 2 $\mu\text{g/mL}$ of amphotericin B) and sterility control (only RPMI 1640 without yeast suspensions).

Microtitration plates were incubated at 37°C. The results have been read visually after 24 and 48 hours on the basis of chamber's content turbidity. MIC is defined as the lowest concentration of solution which causes a significant decrease in turbidity comparing to the positive control.

Determination of Minimum fungicide concentration (MFC) is done by seeding 20 μl from all clear wells on SDA incubated at 37°C for 24-48 hours.

RESULTS

Essential *thyme* oil has manifested antifungal effects to all tested strains. The susceptibility results presented as MIC and MFC values are shown in Table 2 for *Candida* strains isolated from humans and Table 3 for strains isolated from dogs.

Strains of the species *C. albicans* have shown the greatest susceptibility to *thyme* oil, while between other *Candida* species difference in MIC/MFC values has not been noticed.

Table 2. The results of *Candida* spp. susceptibility isolated from humans

Strain number	Species	MIC ($\mu\text{L mL}^{-1}$)	MFC ($\mu\text{L mL}^{-1}$)
36A	<i>C.albicans</i>	0.5	1
62A	<i>C.albicans</i>	0.2	0.5
80A	<i>C.albicans</i>	0.2	0.5
82A	<i>C.albicans</i>	0.2	0.5
87A	<i>C.albicans</i>	0.2	0.5
90T	<i>C.tropicalis</i>	0.5	1
49G	<i>C.glabrata</i>	0.5	1
60G	<i>C.glabrata</i>	0.5	1
33K	<i>C.krusei</i>	0.5	1
43K	<i>C.krusei</i>	0.5	1

Table 3. Susceptibility results of *Candida* spp. isolated from dogs

Strain number	Species	MIC ($\mu\text{L mL}^{-1}$)	MFC ($\mu\text{L mL}^{-1}$)
A12	<i>C.albicans</i>	5.0	5.0
A2	<i>C.albicans</i>	2.5	5.0
A8	<i>C.albicans</i>	2.5	5.0
K4	<i>C.krusei</i>	5.0	10
P15	<i>C.parapsilosis</i>	1.25	5.0

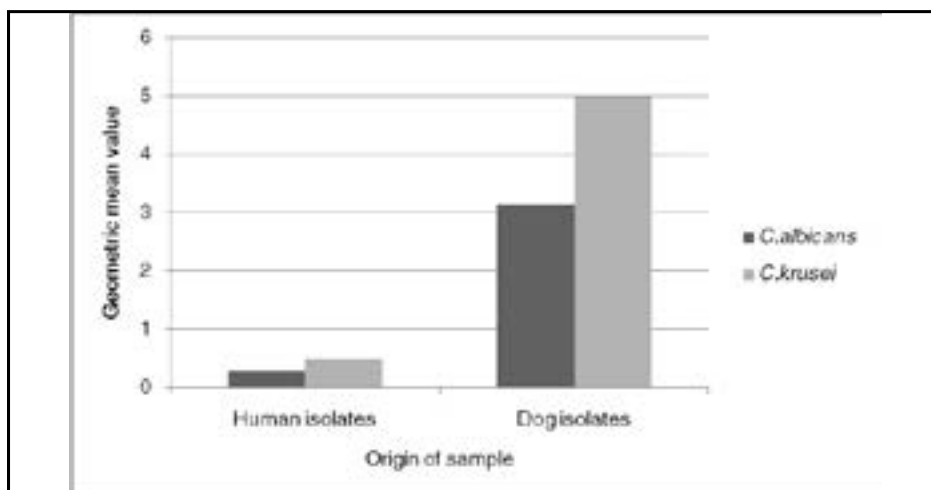


Figure 1. Comparison of Gm values for human and dog isolates

On the basis of our results it may be concluded that *Candida* spp. isolated from humans shows higher susceptibility to thyme oil compared to *Candida* spp. isolated from dogs. Comparison of geometric mean (GM) values for human and dog isolates is given in Figure 1.

DISCUSSION

The effect of essential oils and their main components on *Candida* spp. have been confirmed in *in vitro* and *in vivo* studies (Giordani *et al.*, 2004; Tamperi *et al.*, 2005; Duarte *et al.*, 2005; Pinto *et al.*, 2006; He *et al.*, 2007; Palmeira- de Oliveira *et al.*, 2009). Their activity can usually be presumed on the basis of their chemical content. Essential oils have antifungal activity since they contain high content of phenol. *Thymus* spp. is very rich in phenol (thymol). *Thymus* zygis is classified as a fungicide due to its powerful effect on *C.albicans* (Pina - Vaz, 2004). Various studies have shown that thyme oils manifest the highest antifungal activity compared to other essential oils (Omran, 2009).

Scanning by SEM, Braga and del Sasso (2008) have shown that the effect of thymol causes the change of susceptibility of the cell membrane, degradation of cellular wall, leaking of cell content, coagulation and cell lysis (Bakkali *et al.*, 2008; Palmeira de Olivera *et al.*, 2009). Concentrations below the MIC values significantly inhibited germ tube formation, an important virulence factor (Pina - Vaz, 2004).

Hammer (1999) has also defined MIC of thyme oil for *C.albicans* from 0.03% (0.3 $\mu\text{L mL}^{-1}$) by using the microdilution method. In this study the value was 0.02% (0.2 $\mu\text{L mL}^{-1}$) for human isolates of *C.albicans*.

In the study of Omran *et al.* (2010) where antifungal activity of thyme oil was investigated by disc diffusion method, MIC for *Candida* spp. was 2 mg/mL=0.02%. *C.albicans* showed the highest susceptibility, followed by *C.krusei* and *C.glabata* (Omran *et al.*, 2009). In our research, *C.albicans* strains also expressed the highest susceptibility. *C. krusei* is less susceptible to thyme oil and to other essential oils due to its cellular structure, as well as the intensified action of cell pumps which disturb the entrance of essential oils into the cell (Mondelo *et al.*, 2003; Pozzatti, 2007).

Our study also demonstrated higher susceptibility of human *Candida* spp. comparing to the strains *Candida* spp. isolated from dogs, which could be explained in a way that dental related stomatitis in patients very often is not treated. Therefore, human strains of *Candida* spp. have not developed resistance. On the other hand, in dogs, stomatitis has been accompanied by a stronger clinical picture, and is treated by antimicrotics (mostly azoles) therefore resistant profiles of *Candida* spp. are more likely to occur.

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OSETLJIVOST SOJEVA *CANDIDA* SPP. IZOLOVANIH KOD ČOVEKA I PSA SA STOMATITISOM NA ETARSKO ULJE TIMIJANA

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SADRŽAJ

Candida spp. je sastavni deo mikrobioma usne duplje čoveka i psa. Među-
tim *Candida* spp. predstavlja glavni uzročnik proteznog stomatitisa kod čoveka i
stomatitisa kod psa. Terapija stomatitisa podrazumeva korišćenje azola i poliena
na koje poslednjih godina gljivice razvijaju otpornost. Istrživanja se usmeravaju
ka primeni prirodnih preparata kao što su etarska ulja. Cilj rada je utvrdi anti-
fungalnu aktivnost ulja timijana na kliničke izolate *Candida* spp. čoveka i psa, i
utvrdi da li postoji razlika u osetljivosti sojeva *Candida* spp. izolovanih kod čoveka
i psa. Uzorkovanje kod pacijenata sa stomatitisom je vršeno brisom proteze ili
brismom sluzokože. Kod pasa je uzorkovanje vršeno brisom sluzokože usne duplje
kada je dijagnostikovao stomatitis. Zasejani sojevi su *in vitro* tretirani korišćen-
jem mikrodilucione metode da bi se utvrdilo da li ulje timijana deluje na kliničke
izolate *Candida* spp. čoveka i psa. Rezultati pokazuju da su vrednosti minimalne
fungicidne koncentracije (MFC) i minimalne inhibitorne koncentracije (MIC) man-
je kod čoveka. Stomatitis je kod ljudi najčešće je asimptomatski, pacijenti se ne
podvrgavaju lečenju, pa sojevi *Candida* spp. nisu razvili rezistenciju na antifungi-
cidne preparate. Kod pasa stomatitis je praćen burnijom kliničkom slikom, leči se
antimikoticima (uglavnom azolima) pa je verovatnoća pojave rezistentnih sojeva
Candida spp. veća.