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In Vitro evaluation of antifungal activity of ethanolic propolis extract against *Candida albicans* from patients with urinary tract infections

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The management of patient with urinary tract infections difficult and put into evidence the need of searching for new, effective, safe, low-cost antifungal alternatives against this pathology. Our study aims to assess in vitro antifungal activity of ethanolic propolis extract against Candida albicans isolates from patients with urinary tract infections. urine samples were taken from hospitalized patients inoculated on Cys tine Lactose Electrolyte Deficiency Agar (CLED) at 37ºC for24hr then inoculating colonies on Sabouraud's Dextrose Agar at 37ºC for 24 hr. Antifungal activity of ethanolic propolis extract determined by disc diffusion method. Ethanolic propolis extract showed the strongest antifungal activity against Candida albicans with minimum fungistatic concentrations (MFCs) of 5mg/ml and minimum fungicidal concentration (MFCc) 10mg/ml.

Keywords: fungi static, fungicidal , *Candida albicans* ,ethanolic propolis extract.

INTRODUCTION

Propolis is a resinous hive product produced by bees. The name derives from the Greek words Pro (defence of) and Polis (city), and the name reflects the importance of this substance as a hive protectant. Propolis consists of plant buds that are collected on the hind legs of worker bees and then masticated. Bee salivary enzymes are added through this process and the resulting product is then mixed with wax (Bankova and Marcucci, 2000).

Propolis is characterized by a broad range of biological activities. Propolis has been documented to possess antibacterial, antifungal, antiviral, antiparasitic, antioxidative, anticancer, anti-inflammatory, antiulcer, and antidiabetic effects (Pasupuleti et al., 2017).

Ethanolic extract of propolis exhibited inhibitory effects on the growth of *Candida albicans*. Importantly, it altered the expression of genes coding virulence-associated hyphal adhesion proteins of *Candida albicans,* causing the yeast to lose their characteristic features that enable colonization germination, biofilmformation, and invasion (Pobiega et al., 2019).

Urinary tract candidiasis is known as the most frequent nosocomial fungal infection worldwide. *Candida albicans* is the most common cause of nosocomial fungal urinary tract infections; however, a rapid change in the distribution of *Candida* species is undergoing. Simultaneously, the increase of urinary tract candidiasis has led to the appearance of antifungal resistant *Candida* species (Mayer et al., 2013).

Despite the high rate of morbidity in UTIs

caused by *Candida albicans*, the mortality is low .However, the rate of mortality in patients with systemic candi¬diasis and AIDS is high (Lai et al., 2008).

Although most species of *Candida*, especially *Candida albicans*, are sensitive to routinely used antifungals increasing resistances is observed. In recent years, drug-resistance to antifungal agents and optimizing therapy of Candida infections have been broadly focused (Kareem et al., 2015).

MATERIALS AND METHODS

Candida albicans isolated from urine samples were taken from hospitalized patients at Shaqra hospital- Shaqra governorate- KSA

Study Design:

Experimental research designs

Study Location:

Laboratory of medical microbiology, College of Applied Medical Sciences at Shaqra, Shaqra University – Saudi Arabia

Study Duration:

March 2019 to June 2019.

1-Cystine Lactose Electrolyte Deficiency Agar (CLED) used in the isolation and differentiation of urinary organisms.

2-Sabouraud's Dextrose Agar (Oxoid) used to cultivate *Candida albicans* and other types of fungi

3-Muller Hinton agar (Oxoid) used to determine Antifungal activity of Ethanolic extracts of propolis on *Candida albicans*

4- Sheep serum into a small tube used to Germ Tube Test

5- Gram's stain

6- Ethanolic propolis extracts

Collection of Samples

A total of 30 urine samples were taken from hospitalized patients (Ali et al., 2015)**.**

Samples were collected in sterile screw capped, wide mouthed, Containers was given to patients instructed to collect a mid- stream early morning urine and transferred to the laboratory processed immediately by inoculating each sample on Cystine Lactose Electrolyte Deficiency Agar (CLED) inverted plates were incubated at 37°C for24hr then inoculating colonies on Sabouraud's Dextrose Agar at 37°C for 24 hr.

Identification of isolates.

The suspected colonies were examined for their colonial character, agar, microscopical examination and Germ Tube Test.

Germ Tube Test for identification of Candida *albicans*

1. Put 0.5 ml of sheep or human serum into a small tube.
2. Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum. .
3. Incubated the tube at 37°C for 2 to 4 hours.
4. Transfer a drop of the serum to a slide for examination.
5. Coverslip and examine microscopically under low and high power objectives.

Propolis extraction

Propolis samples were first cut into small pieces and ground several times to get a very fine powder. (Kareem et al., 2015)

The method described to obtain extracts as follows:

1-15 grams of ground propolis was then extracted with 150 ml of 70% ethanol in a beaker and the beaker was covered with an aluminum foil.

2- The beaker was incubated in a shaker incubator at 35°C with 100 rotary/ min for 48 hr.

3- After the completion of the incubation process, the extracted liquid was filtered using 0.2mm bacterial filter to ensure their sterility. Sterile extracts were evaporated to dryness by poured into a petri dish and was allowed to dry.

4- The pure extract was stored at 4°C in amber vials in the dark to prevent photo-isomerization.

Preparation of ethanolic propolis extract:

The first step to prepare stock solution by weighting 0.375 gm from the dry extract and dissolving in 15 ml of 70% ethanol to obtain the final concentration, 25 mg/ml in stock solution. Then make the following concentration as given:

Stock solution ml	Ethanol 70% ml	Final concentrate mg/ml
2	-	25
1.2	0.4	20
1.6	0.8	15
0.8	1.6	10
0.4	1.2	5
-	2	0

Antifungal activity of ethanolic Propolis extract:

Fungal suspensions were prepared by picking up four or five *Candida albicans* colonies from a 24 hr Sabarodes dextrose agar culture plate and suspending them in 5 mL of sterile distilled water. Fungal suspensions were standardized to a 0.5 McFarland turbidity standard, then diluted 1 in 1000 with sterile distilled water to yield an initial inoculum of approximately $1x10^3$ to 5×10^3 cfu /ml,

25 ul were swabbed over the surface of Müller- Hinton agar plate. Swab in three directions to ensure complete plate coverage. Let the plate stand for 5 minutes. Cut 5-mm squares on agar.

Pour the ethanolic propolis extract on wells. Incubate the plate inverted at 37°C for 24 to 48 hrs. The diameters of the zones of inhibition were measured in millimeters, using a ruler on the underside of the plate. Record the zone size (Kirby-Bauer Disk Diffusion Susceptibility Test Protocol 2016)

Determination of fungistatic concentrations

The value of fungistatic concentrations is determined as the zero intercept of a linear regression of the squared size of these inhibition zones, x, plotted against the natural logarithm of the antibiotic concentration, c:

$$\ln(\text{fungistatic concentrations}) = \ln(c) - \frac{x^2}{4Dt}$$

Here, D is the diffusion coefficient, presumed to be independent of concentration and t the time of antibiotic diffusion (Bonev et al., 2008).

Determination of minimum fungistatic concentrations (MFCs)

The MFCs are determined by sub-culturing the test dilution (used in fungistatic concentrations) on to a fresh solid medium and incubated further for 24 hours. The concentration of the ethanolic propolis extract that completely killed the fungi has been taken as MFCs (Antara and Amla , 2012) .

RESULTS

Results in Table 1 revealed that out of 30urine samples were taken from hospitalized patients. (41.7%) were positive for *Candida* spp, including *Candida albicans* (46%) and *Non-Candida albicans* (54%)

Results in Figure 1 was shown *Non Candida albicans* was the most Percentage of isolates from urine samples

Photo1 and photo 2 showed Candida SPP isolated from urine samples, and Photo 3 showed Germ Tube of Candida albicans.

Table 1: Number of *Candida albicans* and *Non-Candida albicans* isolates from urine samples

Isolates	Number	Percentage %
Candida albicans	14	46%
Non-Candida albicans	16	(54%)

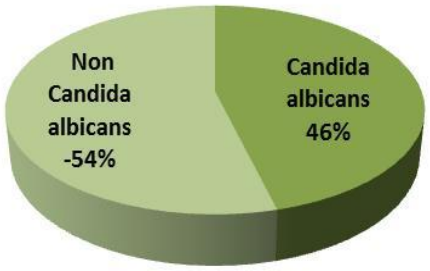


Figure 1: Percentage of *Candida albicans* and *Non-Candida albicans* isolated from urine samples

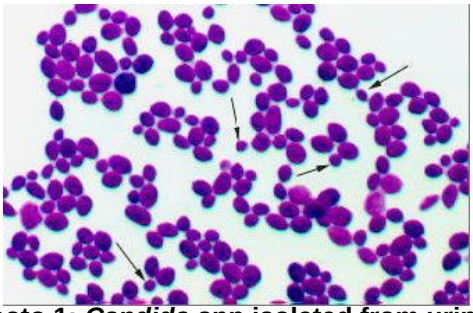
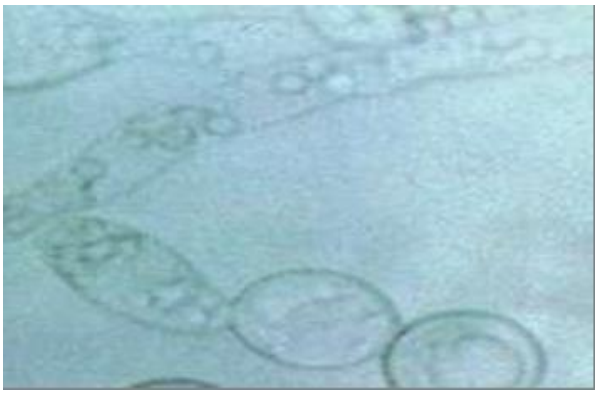


Photo 1: *Candida* spp isolated from urine samples

Photo 2: *Candida* spp on Sabouraud's



Dextrose Agar

Photo 3: Germ Tube of *Candida albicans* isolated from urine samples

Table 2: Inhibition zone (mm) of ethanolic propolis extract against *Candida albicans*

Concentration of ethanolic propolis extract (mg/ml)	0	5	10	15	20	25
Inhibition zone (mm)	0	3	6	11	13	15

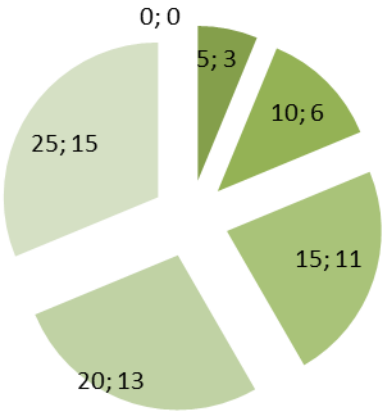


Figure 2: Inhibition zone (mm) of ethanolic propolis extract against *Candida albicans*

Photo 4, 5 and 6: Inhibition zone (mm) of ethanolic propolis extract

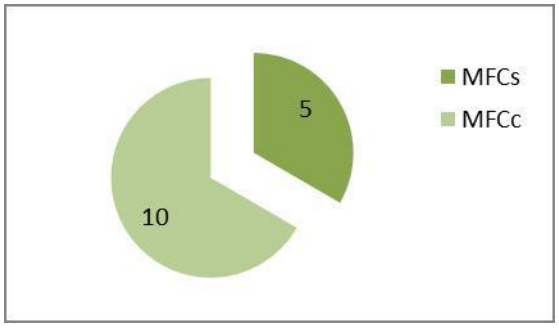


Figure 3: Minimum fungistatic concentrations (MFCs) and Minimum fungicidal concentration (MFCc) of ethanolic propolis extract against *Candida albicans*

The results in Table 2 and Figure 2 showed that the ethanolic propolis extract concentration increased with increased inhibition zone compared with the control on the one hand and with concentrations on the other hand. The inhibition zones were 0, 3, 6, 11, 13 and 15 mm of ethanolic propolis extract concentrations 0, 5, 10, 15, 20, 25 mg/ml, respectively Photos 4, 5 and 6 showed Inhibition zone of ethanolic propolis extract against Candida albicans

Table 3: Minimum fungistatic concentrations (MFCs) and Minimum fungicidal concentration (MFCc) of ethanolic propolis extract against *Candida albicans*.

MFCs	MFCc
5mg/ml	10mg/ml

Our Result in Table 3 and Figure 3 revealed that minimum fungistatic concentrations (MFCs) and minimum fungicidal concentration (MFCc) ethanolic propolis extract against Candida albicans were ranged 5mg/ml and 10mg/ml respectively.

DISCUSSION

The last decade has seen *Candida* spp are continuously medical importance, they could be opportunistic can causes life to threaten systemic infections and chronic mucocutaneous infection in immune compromised patients (Özer et al., 2016).

The presence of *Candida albicans* and *Non-Candida albicans* species in urine is known as candiduria, which may occur in both asymptomatic and symptomatic UTIs (Behzadi and Behzadi, 2011) .

Candida spp. are of medical importance because they are the most common opportunistic mycosis worldwide, a common cause of nosocomial urinary tract infections (UTIs) (Karwan et al., 2018).

In present study revealed that out of 30 urine samples were taken from patients hospitalized. 41.7% were positive for *Candida spp* result agree with previous study carried out by Karwan (Karwan et al., 2018) who reported that The sample urine cultured on the SDA (40%) was positive *Candida* spp and (60%) sample urine cultured on the SDA was negative.

The present study explained that the fungal isolates include *Candida albicans* and *Non-Candida albicans. Candida* with the variable percentage also some urine sample isolated more than one species

Candida albicans: 14 out of 30 (46%) and *Non-Candida albicans* 16 out of 30 (54%) this agree with Ali (Ali et al.,2015) who record that *Candida albicans* was the most common recovered agent (44%) and dis agree with Laura (Wiebusch et al .2017) who found that all yeasts isolated from urine were identified as *Candida albicans*.

Recently recognized worldwide an increase in the number of *Candida* spp that are resistant to antifungal drugs (Jamil et al., 2017). These facts make the management of patients urinary tract infections difficult and put into evidence the need of searching for new, effective, safe, low-cost antifungal alternatives against this pathology. Resistance is an important problem for clinicians during therapy and prophylaxis (Seifi et al., 2013).

Propolis is characterized by a broad range of biological activities. Propolis has been

documented to possess antibacterial, antifungal, antiviral, anti-parasitic, antioxidative, anticancer, anti-inflammatory, antiulcer, and antidiabetic effects besides the bioactivities and pharmacological properties of propolis, a number of studies indicate that propolis has no toxicity and no side effects in animal models or humans (Demir et al., 2016).

The results of this study confirm that Minimum fungistatic concentrations (MFCs) and Minimum fungicidal concentration (MFCc) ethanolic propolis extract against *Candida albicans* were ranged 5mg/ml and 10mg/ml respectively. Our results agree with those of Pobiega (Pobiegaet al., 2019)

who found that ethanolic propolis extract had MfCs/MFCc for fungi ranged from 2 to 32 mg/mL and the growth of *Candida albicans* was subject to stronger inhibition by ethanol extracts of propolis.

The deleterious effect of the propolis on the cell wall of the *Candida albicans* may be the main reason for the decrease in the rate of yeast budding, because the integrity of the cell wall is necessary for cell division (Sisti et al., 2003)

CONCLUSION

The most common pathogen in Urinary tract system was *Candida* spp both *albicans* and *Non-Candida -albicans,* this study has shown non-albicans a major cause of candiduria. Ethanolic propolis extract have fungi static and fungicidal activity against *Candida albicans*

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

NMME suggested the point of this manuscript. FHA developed the idea .MSFA, NMMA, and NMME conceived and designed the experiments. FHA follow up the results of the different experiments. MSFA prepared and wrote the introduction and methodology sections. NMME performed the practical part. NMMA and NMME prepared the study tools and collected data, executed the program and reviewed data analysis. MSFA, NMMA, wrote the results and discussion section. All authors read and approved the final version.

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