A Comparative Study Between Specific and Non-specific Antifungal Agents to Treat the *Rhodotorula mucilaginosa* Athletes Foot

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**ABSTRACT**

The results of this study were detect the action of common anti-fungal used against *Rhodotorula mucilaginosa*, these agents were divided into two groups, first group was named specific antifungal agent which included Ketoconazole, Clotrimazole, Itraconazole, Fluconazole and Nystatin, while second group was named specific antifungal agent which included 1% Iodine (I), 3% Sodium bicarbonate (NaHCO₃), 3% Hydrogen peroxide (H₂O₂), 2% Acetic acid (CH₃COOH) and 10% Potassium permanganate (KMnO₄). The parameters were used in this study included isolation and identification of *Rhodotorula mucilaginosa*, antifungal susceptibility testing and minimum inhibitory concentration. All *Rhodotorula mucilaginosa* isolates were isolated from the fingers of the feet of athletes. Antifungal susceptibility testing showed *Rhodotorula mucilaginosa* sensitive to Ketoconazole, Nystatin, Clotrimazole, Acetic acid and H₂O₂, and resistance to Itraconazole, Ketoconazole, Iodine, Sodium bicarbonate and Potassium permanganate. Acetic acid was recorded lowest MIC value in comparison with other non-specific antifungal agents, while Ketoconazole was recorded lowest MIC value in comparison with other specific antifungal agents.

**Keywords:** Specific, non-specific antifungal, *Rhodotorula mucilaginosa*, athletes foot.

**INTRODUCTION**

An antifungal drug is a pharmaceutical (fungicide or fungistatic) used to treat and prevent fungal infection as athlete's foot and thrush (Baginski and Czub 2009).

Athlete's foot, is a skin disease of the feet caused by fungus. Signs and symptoms often include itching, rough skin, and congestion. In chronic cases the skin may blister. Athlete's foot disease may infect any part of the foot, but most often grows between the toes (Bell-Syer et al. 2012). *Rhodotorula* is an environmental yeast that is found in air, soil, lakes, water, milk, and fruit juice. *Rhodotorula* species, part of the Basidiomycota phylum, colonies plants, humans, and other mammals (Larone 1995). *Rhodotorula* produces pink to red colonies and blastoconidia that are single cell lacking pseudohyphae and hyphae. Several studies have isolated *Rhodotorula* in different ecosystems and environments as well as described infections in
Mammals. *Rhodotorula* spp. have been recognized as emerging yeast pathogens in humans in the last two decades (Hagan *et al*. 1995).

Among the few sources to the pathogenicity of *Rhodotorula* spp. in animals, there are several studies of an outbreak of skin infections in birds and sea animals and pulmonary infections and otomycosis in ruminants (Fernanda, 2012).

This study aimed to select best antifungal agent to treat *Rhodotorula mucilaginosa* athletes foot.

**MATERIALS AND METHODS**

**Antifungal agents**

In this study used two types of antifungal agents include Specific and non-specific antifungal agents. Specific antifungal agents were used to treat skin mycological infection between foot toes include Ketoconazole, Clotrimazole, Itraconazole, Fluconazole and Nystatin, While non-specific antifungal agents are using to treat skin mycological infection between foot toes include 1% Iodine, 3% Sodium bicarbonate, 3% H$_2$O$_2$, 2% Acetic acid and 10% Potassium permanganate. All non-specific antifungal agents were prepared from chemical agents stock solution (10% Iodine, 50% Sodium bicarbonate, 50% H$_2$O$_2$, 50% Potassium permanganate and 98% Acetic acid and use distilled water as a dilute of these substances). The required concentration has been prepared by V1C1=V2C2 equation (Mary, 2005).

**Microbe’s isolates**

*Rhodotorula mucilaginosa* were isolate from toes skin (Hagan *et al*. 1995; Galan-Sanchez *et al.*, 1999) of sport man by cut the small piece of infected skin between their toes figure (1) and kept in the cool container till trans to lab of Microbiology/the Technical institute of Babylon than this small piece cultivated in the Sabouraud Dextrose Agar (SDA) media for 48 hours at 27°C (Pawe³ and Anna, 2010).

**Macroscopic appearance**

*Rhodotorula* spp. are pigmented basidiomycetous yeasts in the family Sporidiobolaceae (Fell *et al*. 2000) *Rhodotorula* spp. produce colonies that are pink to red in color but can also be orange to red on Sabouraud Dextrose agar due to the presence of carotenoid colours. Colony shape has been appeared as thin, smooth, and sometimes mucoid. *Rhodotorula* spp. are nutritionally non-fastidious, grow easily on common media, and are characterized by a rapid growth rate (Larone, 2002).

**Biochemical test**

*Rhodotorula mucilaginosa* production of urease; and inability to assimilate inositol or to ferment sugars (Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts 2008).

**Rhodotorula mucilaginosa** cellular counts

In this study the method of *Rhodotorula mucilaginosa* cells count same calculation method of *Candida albicans* cell count include:–

*Rhodotorula mucilaginosa* cells suspension was prepared by adding five ml of distilled water to fresh SDA media contains *Rhodotorula mucilaginosa* (colonies aged 48 hour at 27°C). Its turbidity was adjusted accordance to the absorbance of 0.08-0.10 at 625nm corresponding to 5 x 10$^6$ CFU/ml (Ricardo and Edeltrudes, 2013).

**Determination of minimum inhibitory concentration (MIC)**

MIC of the effective specific and non-specific antifungal agents were determined by tube
dilution Method (Cruickshank, 1975). Ten test tubes with 8 ml of Sabouraud Dextrose Broth (SDB) in each were taken and autoclaved. To the first tube, 2 ml of the each concentration (50% H₂O₂, 10% Iodine and 98% Acetic acid) was added and serial double fold dilution was done up to the 10 tube and from the 10 tube, 2 ml of the mixture was discarded. To each tube 100μl of inoculums Rhodotorula mucilaginosa suspension (5 x 10⁶ CFU/ml) were added and mixed well. The tubes were incubated for 48 hours at 27°C. The least concentration of each one specific and non-specific antifungal agents capable of inhibiting the Rhodotorula mucilaginosa growth was considered MIC.

**Antifungal Susceptibility Testing**

**Specific antifungal Susceptibility Testing**

A standard antifungal disc diffusion susceptibility testing method (Clinical Laboratory Standards Institute 2009) were used by commercially available discs preloaded with Ketoconazole (10 μg), Clotrimazole (50 μg), Nystatin (100 I.U.), Itraconazole (25 μg) and Fluconazole (50 μg) were using to determine the inhibition zone against Rhodotorula mucilaginosa in SDA media.

**Non-Specific antifungal Susceptibility Testing**

Ager well diffusion method (Magaldi et al. 2004) were used to Non-Specific antifungal Susceptibility Testing.
One hundred µl of *Rhodotorula mucilaginosa* suspension were spread uniformly over SDA medium by using the class spreader, then left for one hour to dry of yeast cells on the media surface. By using cork borer, one central well (digs) was worked on the SDA media. One hundred microliter was taken from each non-specific antifungal agents (1% Iodine, 3% Sodium bicarbonate, 3% H$_2$O$_2$, 2% Acetic acid and 10% Potassium permanganate) that has been prepared previously and put in these wells. Same previous steps were used again for distilled water which considered as control group. Number of petri-dish for each agent repeated five times.

Inhibition activities of the non-specific antifungal agents were determined by measuring the zones inhibition formed around the well in millimeter. The plates were observed for presence of zones of inhibition around the well after 48 hours at 27°C (Mohit 2013).

### Statistical analysis

Data are presented as M±SE. For the statistical analysis, it was used one-way analysis of variance (ANOVA) using SPSS 13.0. Variances were considered significant if p < 0.05 (Joda 2008).

### RESULTS AND DISCUSSION

The results of the present study showed a difference in the effectiveness of antifungal agents

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**Table 1: Zone inhibition diameter of different specific antifungal agents against *Rhodotorula mucilaginosa* in the SDA media in comparison with distilled water. The age of colonies 48 hours at 27°C**

<table>
<thead>
<tr>
<th>Antifungal µg or I.U./Disc</th>
<th>Inhibition Zone M±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole (10 µg)</td>
<td>2.07±0.04</td>
</tr>
<tr>
<td>Clotrimazole (50 µg)</td>
<td>1.32±0.04</td>
</tr>
<tr>
<td>Nystatine (100 I.U.)</td>
<td>1.80±0.05</td>
</tr>
<tr>
<td>Itraconazole(25 µg)</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Fluconazole (50 µg)</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Control</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

M±SE =Mean ±Stranded error

Variant capital letters refer to significant values (P<0.05) between groups

**Table 2: MIC value of different specific antifungal agents against *Rhodotorula mucilaginosa* in the SDA broth for 48 hour at 27°C**

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>MIC value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>0.190 µg/ml</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>39.06 µg/ml</td>
</tr>
<tr>
<td>Nystatine</td>
<td>79.65 I.U./ml</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1.6 mg/ml</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>2.4 mg/ml</td>
</tr>
</tbody>
</table>

**Table 3: Zone inhibition diameter of different non-specific antifungal agents against *Rhodotorula mucilaginosa* in the SDA media in comparison with distilled water. The age of colonies 48 hours at 27°C**

<table>
<thead>
<tr>
<th>Antifungal Inhibition Zone M±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid (2%) 2.83±0.11</td>
</tr>
<tr>
<td>H$_2$O$_2$ (3%) 0.71±0.03</td>
</tr>
<tr>
<td>Sodium bicarbonate (3%) 0.00±0.00</td>
</tr>
<tr>
<td>Iodine (1%) 0.00±0.00</td>
</tr>
<tr>
<td>Potassium permanganate (10%) 0.00±0.00</td>
</tr>
<tr>
<td>Control 0.00±0.00</td>
</tr>
</tbody>
</table>

M±SE =Mean ±Stranded error

Variant capital letters refer to significant values (P<0.05) between groups

**Table 4: MIC value of different non-specific antifungal agents against *Rhodotorula mucilaginosa* in the SDA broth for 48 hour at 27°C**

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>MIC value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>0.62 mg/ml</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>20 mg/ml</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>120 mg/ml</td>
</tr>
<tr>
<td>Iodine</td>
<td>8 mg/ml</td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>40 mg/ml</td>
</tr>
</tbody>
</table>
(Specific and Non-specific) against *Rhodotorula mucilaginosa*.

**Specific antifungal agents**

In this study *Rhodotorula mucilaginosa* isolates appeared more susceptible (sensitive) to Ketoconazole, Nystatin and Clotrimazole respectively whereas other azole antifungal drugs (Itraconazole and Fluconazole) did not show any effectiveness (Resistance), as figure (3) and table (1).

The lowest MIC value (mg/ml) of different specific antifungal agents used in this study against *Rhodotorula mucilaginosa* showed in Ketoconazole in comparison with other agents used in this study, table (2).

The difference of antifungal drugs activity dependent on type and mechanism of action.

The Mechanism of Action of azole antifungal group (Ketoconazole, Clotrimazole, Itraconazole and Fluconazole) act by inhibit CYP P450 14 á- demethylase in mould and yeast. CYP P450 14 á- demethylase enzyme is necessary to convert of lanosterol to ergosterol. While mechanism of action of Nystatin act by Linked to ergosterol in mycological membrane causing membrane to become leaky (Myers 2006).

The use of antifungal drugs in the therapy of fungal diseases can lead to the development of antifungal resistance.

The resistance of *Rhodotorula mucilaginosa* to Ketoconazole and Clotrimazole may be result from low intracellular antifungal concentration by stimulation of efflux pathway or decreased of antifungal penetration, modification of the specific active sites, up regulation of the specific enzyme and development of bypass pathways (Pemán 2009).

**Non-specific antifungal agents**

In this study *Rhodotorula mucilaginosa* isolates appeared more susceptible (Inhibition zone) to Acetic acid and \( \text{H}_2\text{O}_2 \) respectively whereas other non-specific antifungal agents (Iodine, Sodium bicarbonate and Potassium permanganate) did not show any effectiveness (Resistance), as figure (4) and table (3).

The lowest MIC value (mg/ml) of different non-specific antifungal agents used in this study against *Rhodotorula mucilaginosa* showed in acetic acid in comparison with other agents, table (4).

Some non-specific agents used in this study have antifungal effect but variety in cellular yeast damage depended on type, concentration and mechanism of action of antifungal agent used.

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**Fig. 4:** Inhibition Zone diameter (Agar well diffusion method) of specific antifungal agents against *Rhodotorula mucilaginosa* in the SDA media for 48 hours at 27 °C. A=Acetic acid (2%), N=Sodium bicarbonate (3%), H=Hydrogen peroxide (3%), P=Potassium permanganate (10%), I=Iodine (1%) C=Control.
CH3COOH has been mostly used in medical fields for more than 6000 years for the disinfection of wounds infections and especially as an antiseptic agent in the treatment and prophylaxis of deferent microorganisms. The antimicrobial effect of acetic acid, even at concentrations as low as 5%, has been attributed to its ability to decrease pH both in intra- and extracellular conditions and therefore to altering the cell membrane’s transportation and integrity, as well as enzymatic activity, and even precipitating cytoplasmic proteins (Ryssel 2009). Whereas antimicrobial activity of H2O2 acts as an oxidant by producing •OH which linked with macromolecules of cell example lipids, proteins, and DNA. It has been proposed that exposed sulphydryl groups and double bonds are particularly targeted (Block. and Peroxygen 1991).

NaHCO3 is used as an alkalinizing material for glutaraldehyde sterilization of medical apparatus. In watery solution, it ionizes to form sodium ions and bicarbonate ions ions. The dissociation of HCO3- ions from NaHCO3 increases the pH of a solution (Enfors and Molin 1975).

I- ions acts by lowering the O2 concentration in aerobic microbes cell. I- ions interacts with the respiratory chain of the microbes by blocking the transport of electrons through electrophilic reactions with the respiratory chain enzymes. I- also interacts high affinity with the proteins of the cytoplasm membrane in a form with a positive (H2O +I- or neutral (I2 or HOI) charge (Maris, 1995).

KMnO4 is a mild antiseptic with astringent properties. It is used in dermatology to treat weeping skin conditions (Anderson 2003). KMnO4 oxidation of organic pollution also may induce biodegradability and/or toxicity to microbes (Bowers 1992). Also KMnO4 act on the cell membrane phospholipids, containing unsaturated fatty acids, may be sensitive to MnO4- oxidation at C=C bonds that led to defect of cellular components (Bui and Cotton 2002).

All non-specific antifungal agents which resistance to Rhodotorula mucilaginosa may be resulted from:

First cause:- Fungal resistance to chemical biocides is very limited. One commonly accepted theory about the mechanism of fungal resistance to biocides involves natural (intrinsic) resistance. A fungal cell may have an innate ability to present a permeability barrier to one or more biocides, or to inactivate a biocide due to the presence of existing enzymes (McDonnell and Russell 1999).

Second cause:- The defiance mechanism of Rhodotorula mucilaginosa to Potassium permanganate, Iodine and Sodium bicarbonate may be resulted from lowest concentrations were used to treatment of Rhodotorula mucilaginosa for that may be Increasing the concentration of these substances may increase their effectiveness against this yeast.

CONCLUSION

Specific antifungal drugs
Rhodotorula mucilaginosa were highly sensitive to Clotrimazole and Ketoconazole and Nystatin and resistance to Fluconazole and Itraconazole.

Non-Specific antifungal drugs
Rhodotorula mucilaginosa were sensitive to Acetic acid and H2O2 and resistance to Sodium bicarbonate, Iodine and Potassium permanganate.

Recommendation
1- Molecular study of cellular defect of Rhodotorula mucilaginosa after specific and non-specific antifungal agents treated.
2- Studying Rhodotorula mucilaginosa resistance to specific and non-specific antifungal agents in this study.
3- Studying the direct use of antifungal agents (Clotrimazole, Fluconazole, Nystatin, 2% Acetic acid and 3% H2O2) in a sample of patients infected by Rhodotorula mucilaginosa athletes’ foot.

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