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Detection Of Some Virulence Enzymes Of *Malassezia* spp. Isolated From Patients Of Pityriasis Versicolor And Their Sensitivity To Some Antifungal Agents

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Detection of some virulence enzymes of *Malassezia* spp. isolated from patients of Pityriasis versicolor and their sensitivity to some antifungal agents

Authors Names	ABSTRACT
<p>a. Abeer T NOOR b. Ali A Alsudani</p> <p>Article History Received on: 6/11/2020 Revised on: 16/11/2020 Accepted on: 24/12/2020</p> <p>Keywords: virulence enzymes, <i>Malassezia</i> spp., Pityriasis versicolor, antifungal agents.</p> <p>DOI: https://doi.org/10.29350/jops.2021.26.1.1239</p>	<p>This study included isolation and identification of <i>Malassezia</i> spp. from patients of Pityriasis versicolor (PV) diagnosis by the dermatological consultant at Al-Diwaniyah Teaching Hospital for the period from 1/10/2019 to 5/3/2020, and detection of the virulence enzymes of this species and their sensitivity to some antifungal agents. Eighty seven specimens were collected (38 females 43.7%, and 49 males 56.3%). Seventy three specimens (84%) were positive while 14 specimens (16%) were negative to direct and indirect examination. The results of isolation and identification of <i>Malassezia</i> spp. based on direct examination of specimens with KOH10%, indirect methods of growing specimens on SDA medium, and biochemical tests showed that the most occurrence isolates is <i>M. furfur</i> with 30 isolates (34.4%) followed by <i>M. globosa</i> with 22 isolates (25.2%), then <i>M. slooffiae</i> with 16 isolates (18.3%), and <i>M. pachydermatis</i> with 5 isolates (5.7%), while 14 specimens (16.0%) that did not give positive growth. The results also showed the ability of some species to produce protease enzyme, the rates of transparent halo diameters around the colonies on the skimmed milk medium ranged from (9.66-11.66) mm, and <i>M. pachydermatis</i> showed the highest ability to produce lipase enzyme with a precipitation diameter (24 mm), followed by <i>M. furfur</i> (20.33 mm), <i>M. globosa</i> (15 mm), then <i>M. slooffiae</i> (13.3 mm), also <i>M. pachydermatis</i> showed the highest ability to produce hemolysin enzyme with a degradation zone diameter. (18.33 mm), followed by <i>M. furfur</i> (17.33 mm), <i>M. slooffiae</i> (13 mm), and <i>M. globosa</i> (11 mm). The antifungal sensitivity of <i>Malassezia</i> spp. was tested using eight tablet, the results showed that the Ketoconazole is the most effective in inhibiting the growth of the fungal species compared to other antifungals agents, as the average diameter of the inhibition zone was (46.66 mm) for <i>M. globosa</i>, which is the highest rate of inhibition compared with other species, followed by <i>M. furfur</i> (45.33 mm), <i>M. slooffiae</i> (43.66 mm), <i>M. pachydermatis</i> (38.66 mm), followed by the antifungal Fluconazole with an inhibition diameter (35.66 mm) for <i>M. furfur</i>, followed by <i>M. slooffiae</i> (33.33 mm), <i>M. pachydermatis</i> (33 mm) and <i>M. globosa</i> (20 mm), while Nystatin showed no effect on the growth of <i>Malassezia</i> spp.</p>

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1. Introduction

The fungus *Malassezia* spp. one of the lipophilic yeasts known for more than a century as symbionts with the skin may be pathogenic under certain conditions [17]. It is part of the normal flora of the skin of human vertebrates and other warm-blooded vertebrates [23]. Being lipid-dependent, it is usually found in areas rich in sebaceous glands, and current evidence indicates higher rates of infection in healthy adults, in contrast to the low incidence in children before puberty [15]. The species of *Malassezia* spp. are associated with some diseases affecting the skin of humans such as Pityriasis versicolor, folliculitis, seborrheic dermatitis, dandruff, steroid acne, atopic dermatitis, and psoriasis [3,32].

Pityriasis versicolor most often affects young adults of both gender and can also affect children, adolescents, and the elderly, initially, these spots or pigment usually appear in the area rich in the sebaceous secretory gland (neck, chest, arm scalp, trunk, face and shoulders) and the most rife species related with (PV) are *M. globosa*, *M. sympodialis*, and *M. furfur* [11].

Malassezia spp. fat dependence due to the absence of cellular fatty acids and the multifunctional enzyme required for the formation of fatty acids in a multi-step process usually produces palmitic acid which acts as a precursor for long-chain fatty acids [13]. The pathogenic potential of these fungal species is related to several factors including the ability to produce enzymes such as Esterase, Lipase, and Protease, these enzymes that enable the growth of these yeasts on the host skin lead to changes in sebum composition, for example, the release of synthetic fatty acids can lead to (FAs) from triglycerides to inflammation, irritation, and desquamation in susceptible subjects [28].

Microscopic observations showed that these fungi have a thick, multi-layered cell wall with buds that form successively from one position on the parent cell, and these results in the formation of a prominent scar that gives the typical bottle shape of the mother cell and buds, *Malassezia* spp. it is spherical, oval, or elongated in shape and at one end has the shape of a bottleneck, the cell size is about (1.5-4.5 mm) in width and (2-6 mm) in length, and that all species have a fixed morphological characteristic except for *M. furfur* the bottleneck shape of *M. globosa* and sometimes *M. furfur*, elliptical shape of *M. furfur* and *M. slooffiae*, *M. restricta*, *M. sympodialis*, *M. pachydermatis*, and a cylindrical shape of *M. obtosa* and *M. furfur*, and the types of *Malassezia* spp. is an absolute fat requirement in the SDA (Sabouraud dextrose agar) except *M. pachydermatis* [12].

Because of the increasing occurrence of Pityriasis versicolor, the present study aimed to identify the most important species responsible for these infections in Al-Diwaniyah city/Iraq (Latitude: 31.9868 Longitude: 44.9215), and detect the ability of these species to produce some virulence enzymes and their sensitivity to some antifungal agents.

2. Material and methods

Direct examination

Eighty seven specimens were collected from patients of (PV) diagnosis by the dermatological consultant at Al-Diwaniyah Teaching Hospital for the period from 1/10/2019 to 5/3/2020, and the samples used were skin scrapers. Forceps, a surgical blade, and sterile slides were used to collect the

skin specimens, then they were transferred in sterile containers to the fungal laboratory in the Department of Biology/ College of Science, Scraping specimens were prepared for direct examination by placing them on a clean glass slide and mixing them with KOH10% covered with the slide cover, and then gently heated and examined under the 40 X microscope [22].

Indirect examination

All collected specimens were cultured in sterile Petri dishes containing SDA medium with 0.04 ml Penicillin, 2 ml Streptomycin, 0.05 g Chloramphenicol, and 0.5 g Cycloheximide added to olive oil or without olive oil. Scraping specimens were cultured by scattering them on the SDA medium, then incubated at (30-32 °C) for two weeks, and after the results of the culture appeared, the colonies were examined morphologically and microscopically [4].

Identification of fungal isolates

The identification was based on the phenotypic characteristics of the colonies, such as the colony's shape, color, size, texture, enzymes that produce, and its growth with oil or without oil in the medium. Microscopic characteristics, included the shape of the yeast, size, and arrangement, this was done by taking part of the fungal colony by using a sterilized loop on a glass slide using a drop of the stain of lactophenol and examined under a microscope [24]. The microscopic examination also included staining with Gram stain, according to the method presented in [14].

Biochemical tests

Catalase test

The test was performed by adding a drop of 3% hydrogen peroxide to a swab on a glass slide of the fungal isolates under study and the positive reaction is the production of gas bubbles [14].

Tween assimilation tests

A part of the pure colonies aged 4-5 days were taken and mixed in 3 ml of sterile distilled water, this inoculum was added to 18 ml of sterile SDA medium and the mixture was poured into Petri dishes 9 cm and after complete solidification 4 pits were made in the center, using a 6 mm Cork Borer, and filled the holes with about 15 µl of each of 20, 40, 60, and 80 Tweens, the plates were incubated for 7-10 days at 32-34 ° C the assimilation of the tween was evaluated by a reaction (precipitation) around the pits [10].

Growth in the mDA medium at 32, 37, and 41 ° C

This test performed by prepared Petri dishes containing mDA medium with 0.04 ml Penicillin, 2 ml Streptomycin, 0.05 g Chloramphenicol, and 0.5 g Cycloheximide then inoculated the medium with 0.1 ml fungal inoculum 10^6 cell/ml, the Petri dishes were incubated at 32, 37 and 41 ° C, respectively, for 4-7 days with three replications [9].

Production of some virulence enzymes

The medium for protease analysis was prepared using skimmed milk powder according to the method of Rùchel *et al* (1982) [29], lipase medium prepared using Tween 80 according to the method of Lee *et al* (2013) [18]. Hemolysin medium prepared using sheep blood according to the method of Luo *et al* (2001) [19]. The culture media were inoculated with a 5 mm diameter disc of the pure colony using a cork borer with three replicates. The plates were incubated in the incubator at 30-32 ° C. for 4-5 days.

Antifungal sensitivity of *Malassezia* spp.

The test was carried out by following the method of Casals (1979) [5] by using SDA medium prepared for this purpose and inoculated with 0.1 ml fungal inoculum 10^6 cell/ml, and using sterile forceps, the antifungal tablets were placed on the surface of medium (one tablet for each antifungal) with three replicates for each isolation. The dishes were incubated at 30-32 °C for 3 days, after which they were measured the area of inhibition of growth (inhibition zone) represented by the area free of fungal growth around each disc was measured by using a standard ruler.

Statistical Analysis

The results of the study were analyzed statistically using the statistical program known as the Statistical Package for Social Sciences (SPSS, version 25). The significant differences were identified at 5% and 1% probability levels [21].

3. Results and Discussion

Isolation and identification of *Malassezia* spp.

Direct examination

Direct examination of skin scrapings using KOH% 10 showed short, thick strands, and the spores appeared in the form of grape-like clusters (spaghetti and meatballs), and there was a variation in the number of spores and strings from one lesion to another for different patients, and in patients who used some preparations, they were very few and difficult to detect.

Macroscopic features

The colonies growing on the culture media showed different characteristics, as the colonies were high and smooth at the beginning and dried and wrinkled after some time, and the color of *Malassezia* spp. white to cream color, different texture, and distinct edge, there was no growth on the SDA medium without the addition of oil, which excludes the presence of all *Malassezia* spp. Except for *M. pachydermatis*, the only species that can grow in culture media without adding oil [16] (Fig.1).

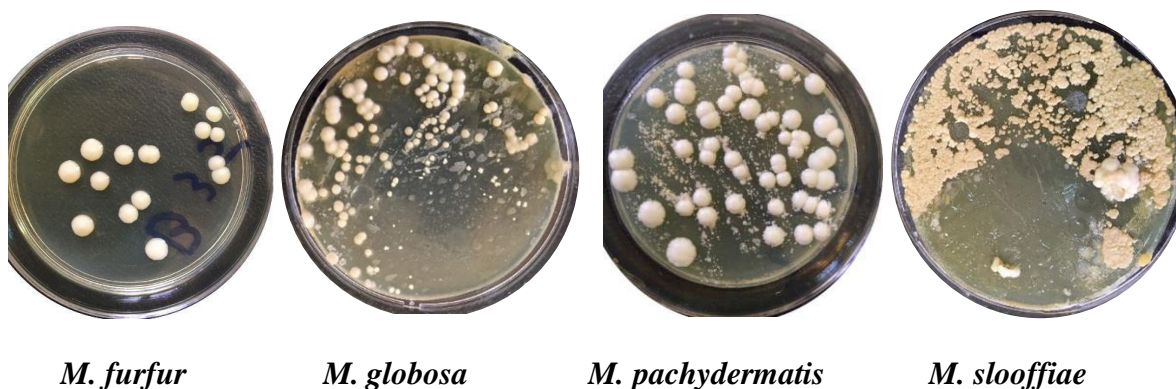


Figure (1) Macroscopic features of *Malassezia* spp.

Microscopic features

After cultivating the species on SDA medium at 32° C, *Malassezia* spp. appeared elliptic to cylindrical short 2-2.5x 4-5 mm with a unipolar bud on a wide base and there is a variety according to *Malassezia* spp. (Fig.2).

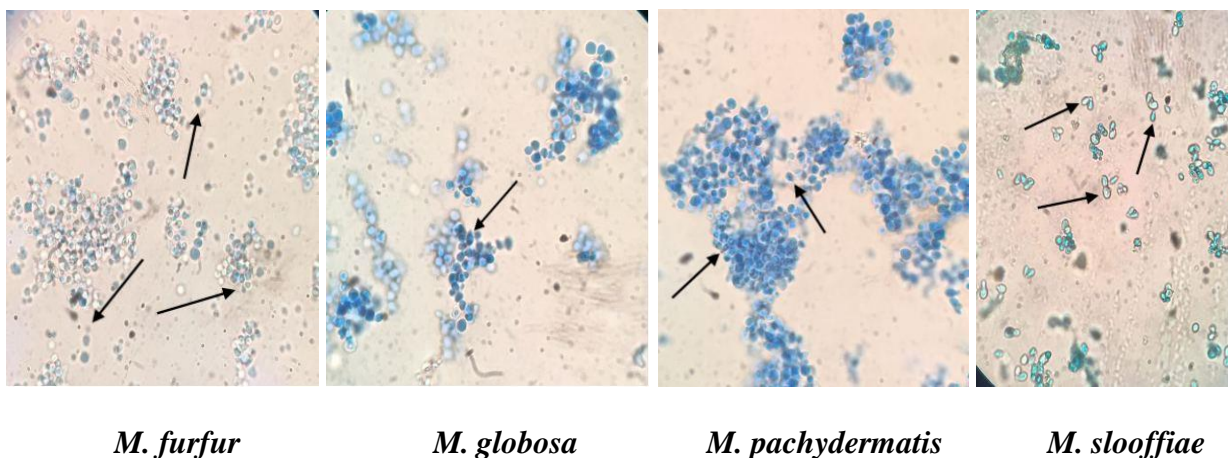


Figure (2) Microscopic features of *Malassezia* spp.

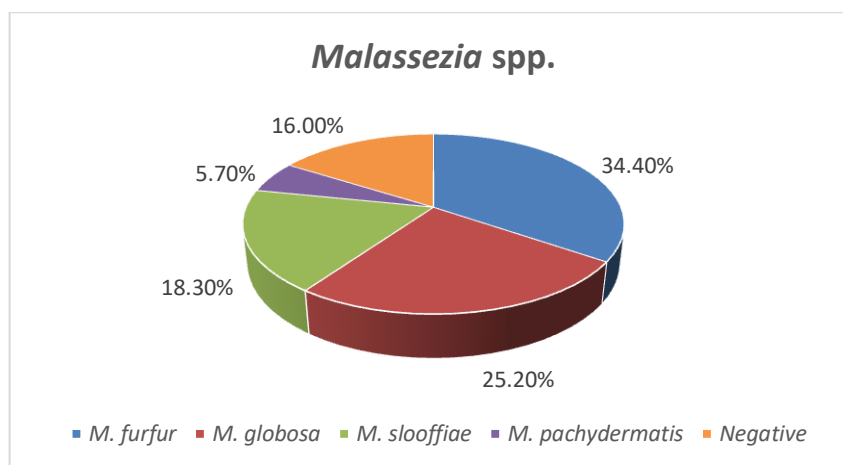
Biochemical tests

The biochemical tests (Table 1) showed a clear contrast between the studied species of *Malassezia* spp. in the assimilation of the tween 20,40,60,80 and the growth on mDA medium at 32, 37, and 41 ° C. All species were positive for the catalase enzyme test, as for the growth in SDA culture media without adding oil, all species were negative (no growth) except for *M. pachydermatis* [16].

Table (1) Biochemical tests for *Malassezia* spp.

<i>Malassezia</i> spp.	Tw 20	Tw 40	Tw 60	Tw 80	mDA 32°C	mDA 37°C	mDA 41°C	Cat	SDA Without oil
<i>M. furfur</i>	+	+	+	+	+	+	+	+	+
<i>M. pachydermatis</i>	-	-	-	-	+	-	+	+	-
<i>M. globosa</i>	-	-	-	-	+	+	-	+	+
<i>M. slooffiae</i>	+	+	+	-	+	+	+	+	+

The results of isolation and identification of *Malassezia* spp. based on direct examination of specimens with KOH10%, indirect methods of growing specimens on SDA medium, and biochemical tests showed that the most occurrence isolates is *M. furfur* with 30 isolates (34.4%) followed by *M. globosa* with 22 isolates (25.2%), then *M. slooffiae* with 16 isolates (18.3%), and *M. pachydermatis* with 5 isolates (5.7%), while 14 specimens (16.0%) that did not give positive growth (Fig. 3).

**Figure (3): *Malassezia* spp. among patients with (PV)**

Detection of some virulence enzymes of *Malassezia* spp.

Ability to produce protease enzyme

The results showed the ability of *Malassezia* spp. on the production of the protease enzyme (Fig. 4), the rates of transparent halo diameters around the colonies on the skimmed milk medium ranged from (9.66-11.66) mm with no significant differences between the enzyme producing species at a probability level of 0.05 (Table 2). The fungi produce proteolytic enzymes within a wide range of

activity in different environmental conditions, such as temperature and pH, and their effectiveness increases with the availability of casein protein, gelatin, peptone, or aspartic acid [27].

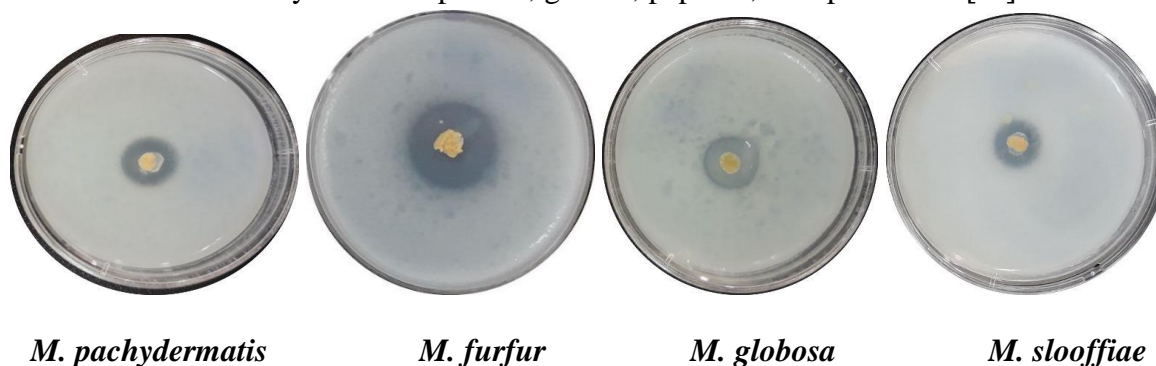


Figure (4) Ability of *Malassezia* spp. on the production of protease enzyme

The protease produced by *Malassezia* spp. acting as a mediator for itchy nerve free endings in the skin, Seborrheic dermatitis is categorized by irritation and flaking in parts rich in sebaceous glands such as the face, scalp, and trunk, while dandruff is a non-inflammatory, scaly state of the scalp [6,7].

It is interesting to note that the protein activity of *M. furfur* isolates is consistent with chronic (PV), which is like to that of *Trichophyton rubrum* from chronic dermatophytes fungi cases, the potential role of the protease enzyme in inducing the immune response in vivo may not be excluded, and the common activity of lipase and protease enzymes may be responsible for the medical indicators and the pathogenic potential of *Malassezia* spp. [8].

Table (2) Ability of *Malassezia* spp. on the production of protease enzyme

<i>Malassezia</i> spp.	Protease
<i>M. furfur</i>	11.66±1.2
<i>M. pachydermatis</i>	11.66±1.76
<i>M. globosa</i>	10±1.15
<i>M. slooffiae</i>	9.66±0.33
<i>LSD</i> _{0.05}	3.994

The values represent the rates (mm) of three replicates ± the standard error.

Ability to produce lipase enzyme

M. pachydermatis showed the highest ability to produce the lipase enzyme, with significant differences at a probability level of 0.05, as the average diameter of precipitation around the colony was 24 mm, followed by *M. furfur* with a precipitation diameter of 20 mm, then *M. globosa* with a precipitation diameter of 15 mm and *M. slooffiae* with a precipitation diameter of 13.3 mm (Fig. 5 and Table 3).

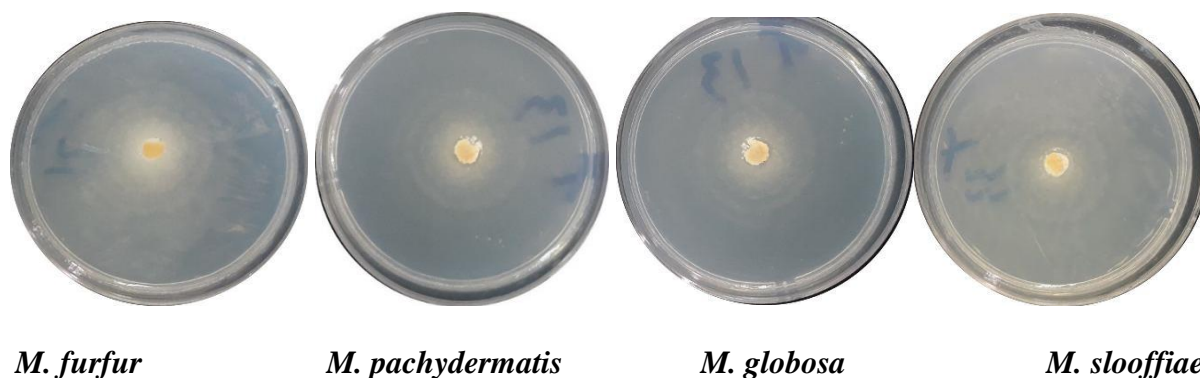


Figure (5) Ability of *Malassezia* spp. on the production of lipase enzyme

Fungi differ in their lipolytic activity, and these differences are related to the rate of decomposition of triglycerides to produce free fatty acids and glycerol [2]. Also, the lipolytic activity of fungi may be the result of individuals showing specificity and priority using fatty acids as a source of carbon [26].

The lipase enzyme alters sebum production and produces free fatty acids on the external of the skin. *M. pachydermatis* strains are known to produce the lipase associated with a parasitic lifestyle. However, few studies have been conducted to investigate the role of the lipase enzyme in the pathogenesis of the pathological variations related to dermatitis [20].

Table (3) Ability of *Malassezia* spp. on the production of lipase enzyme

<i>Malassezia</i> spp.	Lipase
<i>M. furfur</i>	20.33±3.71
<i>M. pachydermatis</i>	24±2.3
<i>M. globosa</i>	15±2.64
<i>M. slooffiae</i>	13.33±0.88
<i>LSD</i> _{0.05}	8.46

The values represent the rates (mm) of three replicates ± the standard error.

Ability to produce hemolysin enzyme

The results showed that the effectiveness of *M. pachydermatis* fungus was higher compared with the other species, with significant differences at a probability level of 0.05, as the average diameter of the area of decomposition was 18.33 mm, followed by *M. furfur* with a value of 17.33 mm, then *M. slooffiae* with a diameter of the area of decomposition of 13 mm and the fungus *M. globosa* with a diameter of 11 mm lysis area (Fig. 6 and Table 4).



Figure (6) Ability of *Malassezia* spp. on the production of Hemolysin enzyme

Pathogenic microorganisms have a blood-analyzing factor, the enzyme hemolysin, which is one of the enzymes of potential virulence due to its important part in the occurrence of infection [1]. Because of its toxic effect on the membranes of red blood cells and macrophages, these effects lead to the formation of pores and decomposition in other eukaryotic cells and cell structures, which leads to the release of iron, which is an important factor for the growth of fungi, especially during infection, metabolism and as a catalyst for various biochemical processes [25].

Table (4) Ability of *Malassezia* spp. on the production of Hemolysin enzyme

<i>Malassezia</i> spp.	Hemolysin
<i>M. furfur</i>	17.33±1.76
<i>M. pachydermatis</i>	18.33±2.02
<i>M. globosa</i>	11±0.57
<i>M. slooffiae</i>	13±1.15
<i>LSD</i> _{0.05}	4.861

The values represent the rates (mm) of three replicates ± the standard error.

Antifungal sensitivity test of *Malassezia* spp.

The results showed (Table 5) that Ketoconazole was the most effective in inhibiting the growth of the *Malassezia* spp, as the rates of inhibition areas ranged between (38.66-46.66) mm, with significant differences from the other antifungal agents at a probability level of 0.05, followed by Fluconazole, as the rates of inhibition zones ranged between (20-35.66) mm, then Econazole and Caspofungin with rates of inhibition diameters between (13-20.66) mm and (12.33-21) mm respectively, while Nystatin was not effective in inhibiting the growth of *Malassezia* spp. (Fig. 7). Pityriasis versicolor diseases are frequently treated topically with antifungals, while in cases of widespread disease a systemic antifungal treatment may be essential, the test for sensitivity to different species shows that ketoconazole has high activity against all species tested and the activity varies with different species and occasionally among different isolates of the similar species [30]. ketoconazole is available as a

shampoo which is often used in this case and some studies indicated that shorter periods of using ketoconazole shampoo were very effective [31].

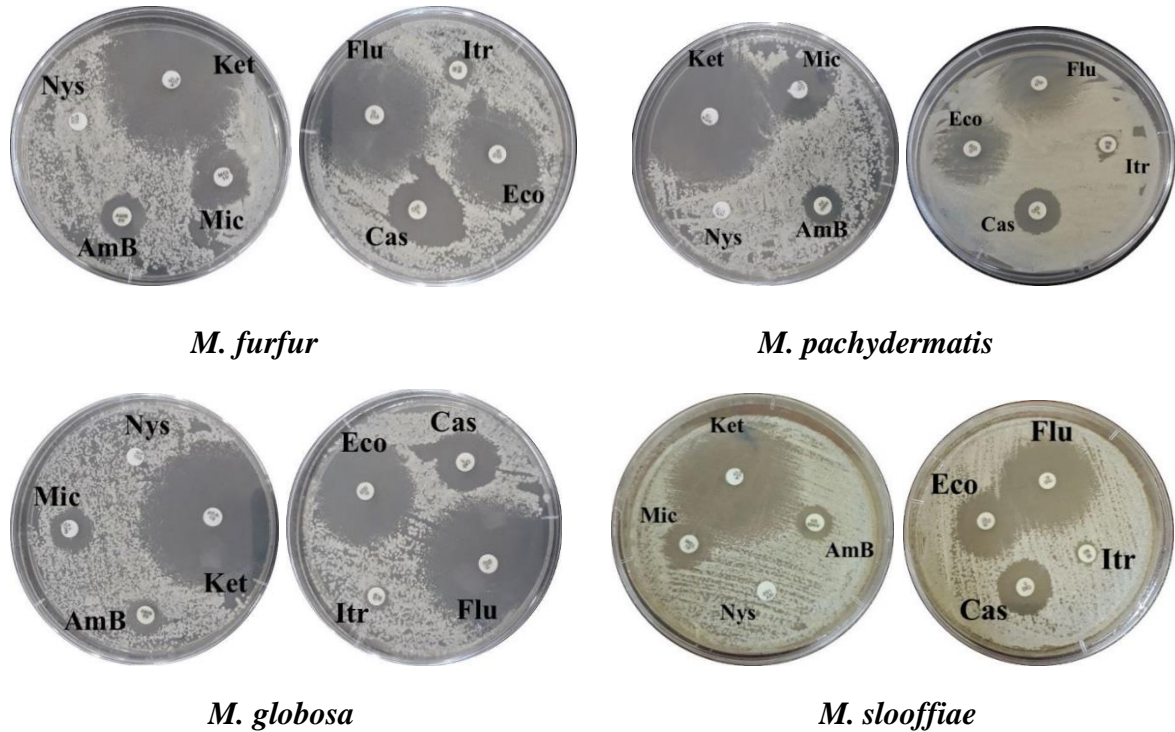


Figure (7) Antifungal sensitivity test of *Malassezia* spp.

Table (5) Antifungal sensitivity test of *Malassezia* spp.

<i>Malassezia</i> spp.	Ket	Flu	Eco	Cas	Mic	Amph B	Itr	Nys
<i>M. furfur</i>	45.33±2.6	35.66±2.33	20.66±5.36	21±2.08	13.66±2.02	12±1.15	10.66±1.2	0±0
<i>M.pachydermatis</i>	38.66±2.02	33±1.15	17.33±1.45	18.66±0.88	20.33±0.88	11.33±0.88	9.66±0.33	0±0
<i>M. globosa</i>	46.66±2.02	20±1.15	13±1.15	12.33±1.15	13.66±2.72	11.33±1.2	10.33±0.33	0±0
<i>M. slooffiae</i>	43.66±3.17	33.33±0.88	18.66±4.7	18.33±1.2	19.33±2.96	13.66±0.33	8±1.15	0±0
<i>LSD_{0.05}</i>	5.579							

The values represent the rates (mm) of three replicates ± the standard error.

4. Conclusions

Virulence enzymes of *Malassezia* spp. such as protease, lipase, and hemolysin enzymes, which qualify them to cause infection and at different rates according to the species. The Antifungal agent Ketoconazole achieved the highest growth inhibition of *Malassezia* spp. among the other, the antifungal Nystatin showed no effect on the growth of *Malassezia* spp.

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