

Isolation and Identification the Fungus *Trichophyton violaceum* from Human Skin Specimens in Iraq and Study Efficiency Antibacterial and some Plant Essential Oils

عزل وتشخيص الفطر *Trichophyton violaceum* من عينات الجلد وتقييم فعالية المضادات البكتيرية والزيوت الاساسية النباتية

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Abstract

This study was aimed to isolate and identify the fungus *Trichophyton violaceum* from human skin specimens and evaluate the activity of antibacterial and plant essential oils. The results showed of direct microscopy by using KOH examination and morphological identification, nine samples from skin were positive in KOH test. Microscopical examination appeared that colonies of *T. violaceum* were coarse with abundant aerial mycelium on SDA, growth rates 6-8cm/14 days. The color changes with age from white in the middle to brownish yellow at the edges which was more intense on the reverse side. Susceptibility test to antibacterial showed that 100% of *T. Violaceum* T2 isolate was resistant to TE (Tetracycline), AK (Amikacin), CTX (Cefotaxime), CFM (Cefixime), CIP (Ciprofloxacin), ERY (Erythromycin) and STR (Streptomycin). Also results showed that all plant essential oils at different concentrations significantly inhibit growth of *T. violaceum* T2. However, the peppermint oil, Myrrh oil, Cardamom oil, Chamomile oil and Castor oil at highest concentration (25mg / ml) caused highest reduction of mycelia growth (100%) followed by Olive oil (67.3%) and Clove oil (64%) at the same concentration, compared with the control treatment.

Key words: *Trichophyton violaceum*, skin, antibacterial, essential oils.

المخلص

هدفت الدراسة الى عزل وتشخيص الفطر *Trichophyton violaceum* من عينات جلد الانسان وتقييم فعالية المضادات البكتيرية والزيوت النباتية الاساسية ضده، اظهرت نتائج الفحص المجهرى باستعمال اختبار KOH ان تسعة من العينات كانت موجبة لاختبار KOH، المستعمرات للفطر كانت خشنة مع وفرة للغزل الفطري على وسط SDA (معدل النمو بلغ 6-8 سم / 14 يوم)، واللون كان يتغير حسب العمر من الابيض الى الاصفر المائل للون البني وبخاصة في الحواف للنمو الفطر. اختبار حساسية الفطر للمضادات البكتيرية اظهرت ان 100% لعزلة الفطر *T. violaceum* T2 كانت مقاومة للمضادات (AK(Amikacin), CTX (Cefotaxime), CFM (Cefixime), (STR) TE (Tetracycline), ERY (Erythromycin), (CIP) Ciprofloxacin, Streptomycin). ايضا اظهرت نتائج استخدام الزيوت النباتية الاساسية (زيت النعناع، المر، الهيل، البابونج وزيت الخروع) وجود فعالية تثبيطية معنوية كاملة على الفطر *T. violaceum* في تركيز 21%. ادى زيت الزيتون في تركيز 21% الى خفض معنوي لنمو *T. violaceum* اذ سجل 7.9 ملليمتر. زيت القرنفل خفض النمو للفطر في التركيزات (14,21)% ولم يؤثر في تركيز 7% وبلغ 3.5 ملليمتر. زيوت الهيل والبابونج تثبتت نمو الفطر في جميع التركيزات الثلاثة مقارنة مع معاملة السيطرة. اوضحت النتائج ان جميع الزيوت النباتية وبالتركيزات المختلفة تثبتت نمو الفطر *T. violaceum* T2، زيت النعناع وزيت المر وزيت الهيل وزيت البابونج وزيت الخروع في تركيز 25 ملغم/مل سببت خفض مرتفع لنمو الغزل الفطري بلغ 100% تلتها وبنفس التركيزات زيوت الزيتون وزيت القرنفل، اذ بلغ التثبيط (64، 67.3) % على التوالي مقارنة مع معاملة السيطرة.

الكلمات الدالة: *Trichophyton violaceum*، الجلد، المضادات البكتيرية، الزيوت النباتية الاساسية

Introduction

The dermatophytes that cause human infection comprise more 40 species of fungi which belong to three genera of imperfect fungi namely *Trichophyton*, *Microsporum* and *Epidermaphyton*. These infections are of public health importance because of their transmissibility from human to human or from animal to human [1]. *Trichophyton* spp. is one of the most commonly encountered dermatophytes that infect human keratinized tissue such as skin, nail and hair [2]. Members of the genus *Trichophyton* are the commonest agents of dermatophytoses. They are especially significant in onychomycosis but also invade skin and hair, causing infection associated with substantial morbidity [3-5]. The genus *Trichophyton* is characterized by the development of both smooth-walled macroconidia and

microconidia. Macroconidia are mostly borne laterally directly on the hyphae or on short pedicels, and are thin- or thick-walled, clavate to fusiform, and range from 4-8 x 8-50 mm in size [6].

Genus *Trichophyton* included 24 species. The colonies on agar media are powdery, velvety or waxy. The predominant spores are microconidia with sparse macroconidia [7]. Reverseside pigmentation is characteristic of the species and is used for the identification of the species within genus, the macroconidia are thin-walled with smooth surface and variable shape [8]. *Trichophyton* spp cause tinea capitis, tinea capitis is the medical term for an infection of the scalp (also known as scalp ringworm) involving the skin and the hair. Tinea capitis is a fungal infection of the scalp caused by two main genera called *Trichophyton* and *Microsporum*. It is common in children [9]. Dermatophytosis of scalp (tinea capitis) due to *T. violaceum*, *T. tonsurans* and *T. schoenleinii* [10]. Tinea capitis (TC) is a common childhood fungal infection, can cause long-term scarring [11]. *T. violaceum* is one of the important fungi [12]. *T. violaceum* colonies (SDA) are very slow growing, glabrous or waxy, heaped and folded and a deep violet in colour. *T. violaceum* is causing skin, nails, beard and scalp [6]. The azole antifungal agents block the synthesis of ergosterol, a major component of fungal cytoplasmic membranes [13]. Prevalence of tinea capitis among school children at primary school and the dermatophytes isolated were *T. violaceum* and *T. mentagrophytes* [14]. Nystatin was discovered in 1950 from the fermentation broth of *Streptomyces noursei*, and is still used as a topical antifungal agent. It is nonabsorbable after oral administration but is effective topically in the treatment of oropharyngeal candidiasis [15]. In this study indicates that bicyclic caryophyllene and caryophyllene oxide detected in *Bidens pilosa* essential oils may play an important role in antifungal activities *F. oxysporum*, *F. solani*. On the other hand, the fungi toxic activities of the flower essential oils were higher than those of the leaves [16]. In study on onion (*Allium cepa*) extract was effective in inhibiting the growth of the fungus *Aspergillus flavus* by 73% and reduce the production of aflatoxin and use an antifungal abstract [17]. Deabes Showed [18] in a study to assess the inhibition of growth and production of aflatoxin fungus *A. flavus* strain (ATCC16872) by essential oils extracted from various plants, including basil, fennel, coriander, caraway, pepper mint and rosemary oil has led to the inhibition of the growth of the mycelium and reduce the production of aflatoxin and complete inhibition in concentration 1000 Ppm to pepper mint oils and rosemary, coriander and caraway [19]. Essential oils been used as antifungal, anti-infectious and antimicrobial agents. Inhalation of vapours of the essential oils kill invaders attached to the inner respiratory lining and work synergistically with the body defences [20]. Reported the antimicrobial activity of zimmu (*Allium sativum* and *Allium cepa*) extract against many fungi and bacterial pathogens. Therefore, this study was aimed to isolate and identify the fungus *Trichophyton violaceum* from human skin specimens and evaluate the activity of antibacterial agents and plant essential oils against it.

Materials and Methods

Sample Collection

Eleven (11) of human skin specimens (the hands) were examined and then samples of fungus was collected under the supervising of doctor from dispensary in Baghdad, only nine (9) of them were symptomatic (suspected dermatophytosis). Skin of patients was scraping. Samples were transferred immediately to the laboratory and culture at the same day.

Isolation of *Trichophyton violaceum* from skin specimens

Trichophyton violaceum was isolated from skin specimens by the method in [21]. Using Sabourauds Dextrose agar (SDA: 40 g dextrose, 10g peptone, 20 g agar, in 1 L distilled water), then chloramphenicol 250 mg/1L and cycloheximid 0.4g were added, and incubated at 28 °C for 14 - 21 days and purified *T. violaceum* colony were subsequently subcultured on SDA medium.

Morphological and microscopical identification of *Trichophyton violaceum*

Trichophyton violaceum isolates were grown on 9 cm plates on SDA, for 14 days at 28 °C. When colonies were formed, microscopic examination was performed to identify them. Microscopic examination was achieved by putting the fungus on a microscope slide with lactophenol cotton blue, by use of a small piece of sticky tape. The slide was then examined under a light microscope. Species was identified based on specific keys described by [6]. Identification of *T. violaceum* is depended on (Colony observation, Colony reverse, Macroconidia and microconidia shape, size, hyphal structures, chlamydospores) [21, 22].

Antibacterial susceptibility test

This test was performed to know activity of antibacterial on pathogenic fungi. Seven antibacterial drugs were used in this study, their name illustrated in Table (1). Sensitivity of fungi to Antimicrobial agents, determined by Disk method [23].

Using a pair of flamed sterilized forceps apply the disks containing the antibacterial agent to be tested onto the surface of the inoculated agar plates and were press lightly to insure complete contact with the agar, inoculum plates that contain *T. violaceum* were incubated at 28°C for 10-14 days, results were recorded for susceptibility. Inhibition zone to the nearest whole millimeter was measured [24].

Table (1): Antimicrobial agents and their concentration.

Antimicrobial drug	Concentration	Origin
Tetracycline (TE)	10mg / disc	India
Amikacin (AK)	15mg / disc	India
Cefotaxime(CTX)	15mg / disc	India
Cefixime(CFM)	20mg / disc	India
Ciprofloxacin (CIP)	10mg / disc	India
Erythromycin (ERY)	20mg / disc	India
Streptomycin (STR)	20mg / disc	India

Plant essential oils

Seven types of plant essential oils, Peppermint oil, Myrrh oil, Olive oil, Clove oil, Cardamom oil, Chamomile oil and Castor oil were used in this study and produced by AL-Emad company - Iraq. The oils were 100% pure according to the manufacturers and were obtained from Pharmacies, use 100 ml of each oil. Oils sterilized according [25]. Oils were kept in dark bottle till it used.

Determination of Essential oils activity

Essential oils of Peppermint oil, Myrrh oil, Olive oil, Clove oil, Cardamom oil, Chamomile oil and Castor oil, were mixed separately with Tween 80 (0.05%). Dilute oils with ethanol 70% to give concentrations of 15, 20% and 25% mg/ml. 1 ml of each concentration was spread on 20ml of Sabourauds Dextrose agar plates. Then plates were inoculated by disc with diameter of 0.5 cm of the fungus *Trichophyton violaceum* at a rate of one disc in the center of each plate and concentration. The plates were incubated at 28°C for two week. The calculated inhibition through taking the colony diameter in cm by taking the radial growth rate of two diagonals perpendicular to the colonies growth upon the arrival of each fungus growth to the edge of the dish in the control treatment and A ruler used to measure the radial growth, calculated inhibition by the equation. The two readings were used to calculate percentage inhibition of radial growth using the formula developed [26].

$$\text{Inhibition \%} = \frac{R1 - R2}{R1} \times 100$$

Where

R1: maximum radial growth of the pathogenic fungus colony of control treatment.

R2: maximum radial growth of the pathogenic fungus colony in plates containing inoculate plant oils .

Statistical Analysis

Each treatment had three replications. A completely randomized design (CRD). Data were analysed using the ANOVA procedure of the (SPSS) statistical software. LSD test was performed for mean comparisons at the 5% probability level

Results and Discussion

Morphological identification

Four *Trichophyton violaceum* isolates (T1,T2,T3 andT4) Table (2), were identified based on cultural and microscopic characteristics. Following the identification keys used by another auther [6,21,22]. *T. violaceum* isolates showed slow growth rates (6-8cm/14 days). Colonies of *T. violaceum* on Sabourauds Dextrose agar slow growing, waxy and a deep violet in colour. Hyphae are relatively broad, tortuous, much branched and distorted, microconidia and chlamydo spores are present.

Table (2): Isolated of Fungus *Trichophyton violaceum* isolated from the skin samples

Isolate number	Fungal isolates	Isolates
4	<i>T. violaceum</i>	T1, T2, T3, T4

The results of direct microscopy by using KOH examination are mostly comparable with the results of [27]. Direct microscopic examination of samples using potassium hydroxide (KOH) is the most widely-used laboratory method [28]. *Trichophyton* genus is characterised by the development of both smooth-walled macroconidia and microconidia. Microconidia are spherical, pyriform to clavate or of irregular shape, [29]. Many studies have reported a significant burden of skin diseases in school children [30]. In study from India was incidence of tinea infections and their dermatophytes. Out of 83 samples with various infections 85.5 % were infected with dermatophytes. Among them, Tinea corporis was the highest, followed by tinea capitis [31]. Tinea capitis is of public health importance because of its transmissibility [32]. *T. violaceum* colonies on SDA are glabrous or waxy, heaped and folded and a deep violet in colour, *T. violaceum* is causing skin, nails, beard and scalp [6].

Antibacterial susceptibility test

Selected isolation T2 of *T. violaceum* was selected because of its growth rapid, therefore, used in subsequent experiments. Results of Table (3) showed that 100% of *T. violaceum* (T2) isolate was resistant to TE (Tetracycline), AK (Amikacin), CTX (Cefotaxime), CFM (Cefixime), CIP (Ciprofloxacin), ERY (Erythromycin) and STR (Streptomycin). A number of antifungal drugs with proven efficacy are available for the treatment of tinea capitis. However, varying dosage schedules, changes in epidemiology, and rising drug resistance are factors that hamper treatment in some cases [11].

The resistance of different types of fungi against ordinary antifungal drugs has been widely reported. The continuous search for a new compound with effective antifungal properties is therefore important and continuous [33].

Grover [11] showed that effective inhibition of growth of the fungi, especially at higher concentrations of the HCX compound. (3 µg/mL of HCX concentration) gave 100% inhibition. The new compound, formed from the reaction between cefotaxime and hydrocortisone (HCX), has the ability to inhibit the growth of five types of dermatophytes in low doses, comparable to well-known antifungal drugs. In study of three commonly used drugs (Terbinafine, Griseofulvin, and Fluconazole) was undertaken in children aged ≤ 12 years, *T. violaceum* was the most commonly isolated fungus and was found in 68% of patients. A total of 75 patients cure rates of 96%, 88%, and 84% were achieved with Griseofulvin, Terbinafine and Fluconazole, respectively.

Antifungal shampoos, such as ketoconazole 2%, selenium sulfide 2.5%, zinc pyrithione, and povidone iodine 4%, decreases spore load and the shedding of anthroconidia and the non-sharing of headgear and grooming equipment also helps in decreasing fomite-borne transmission. Not advise the shaving of hair because it can lead to peer group ridicule and ostracism [34].

Clinical isolates of yeast were cultivated with sublethal concentrations of Tetracycline and yeast cell counts, hyphal formation, drug efflux pump activity, biofilm production, and hemolysin production were determined by previously reported methods, Tetracycline concentrations above 150 g/ml inhibited *Candida albicans* [35].

Table (3): Degree of sensitivity (DS): S = Susceptible, R= Resistant of *Trichophyton violaceum* T2 isolates to different Antibacterial agents.

Antibacterial drug	Degree of sensitivity (I	susceptibility
Tetracycline (TE)	S	-
	R	+
Amikacin (AK)	S	-
	R	+
Cefotaxime(CTX)	S	-
	R	+
Cefixime(CFM)	S	-
	R	+
Ciprofloxacin (CIP)	S	-
	R	+
Erythromycin (ERY)	S	-
	R	+
Streptomycin (STR)	S	-
	R	+

Determination of Essential oils activity

Results demonstrated in Table (4) revealed that seven plant essential oils at different concentrations significantly inhibit growth of *T. violaceum* T2. However, the peppermint oil, Myrrh oil, Cardamom oil, Chamomile oil and Castor oil at highest concentration (25mg/ml) caused highest reduction of mycelial growth (100%) followed by Olive oil (67.3%) and Clove oil (64%) at the same concentration, compared with the control treatment.

Table (4): Percent inhibition of radial mycelial growth of *Trichophyton violaceum* T2 colonies by plant essential oils after 12days.

*Con. mg/ml)	% I. F.							
	Peppermint	Myrrh	Olive	Clove	Cardamom	Chamomile	Castor	Control
15	51.3	44.7	22.3	33.0	47.7	55.3	47.7	
20	75.3	66.6	37.5	45.5	65.0	75.3	81.3	100
25	100	100	67.3	64	100	100	100	
LSD	5.8	9.3	11.9	17.7	8.6	4.9	5.6	

* Con.: Concentration of plant essential oil I. F.: Inhibition of fungus

Rasooli [36] Showed that plant oils led to changes in plasma membranes and mitochondria of fungus *Aspergillus niger*. Another research [37], found that garlic oil effect upon Aflatoxin B1- induced DNA damage in cultured primary rat hepatocytes through the use of diallyl disulfide (DADS) and diallyl sulfide (DAS). DADS significantly decreased the DNA damage induced by AFB1 as compared with the AFB1 control. Moreover, [38]. found that cinnamon and clove oil were non-significant inhibit the growth of *Aspergillus niger*, *Alternaria alternata* and *Phomo psisviticola*, while significantly reduced growth of *Colletotrichum gloeosporioide*, and *Rhizopus stolonifer*, while [39] showed the effectiveness against molds of clove and cinnamon oil is affected by their chemical compositions, Eugenol is the main component of clove oil. Velluti [40] Mention that antimicrobial activity of this oil can form hydrogen bonds with-SH groups in the active sites of target enzymes, resulting in the deactivation of enzymes in fungi. Thirty three compounds identified in the essential oil of the peppermint plant were 96.25%. Main components in *Mentha piperita* oil were menthol, limonene, 1,8-cineole, sabinene, menthylacetate and menthone, showed strong antibacterial and antifungal activities [41, 42]. Suprapta and Khalimi [43] Showed that five plant species namely *Albizia saman*, *Piper betle*, *Syzygium aromaticum*, *Sphaeranthus indicus* and *Alpinia galangal* exhibited strong antifungal activities. The minimum inhibitory concentration (MIC) of the tested plants varied from 2 mg/ml to 13 mg/ml, however, the extract of *Albizia saman* showed the lowest MIC (2 mg/ml) against all tested isolates (LS05, LS14) of *F. oxysporum*.

Conclusion

The present study concluded the ineffectiveness of the antibacterial against *Trichophyton violaceum* fungus, and efficiency of Peppermint, Myrrh, Olive, Clove, Cardamom, Chamomile, Castor oil to inhibit growth of *Trichophyton violaceum* T2 colonies at highest concentration (25mg/ml)

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