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Antifungal effect of propolis and *Oreganum vulgaris L.* extract

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By

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بسم الله الرحمن الرحيم

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Supervisors Certification

We certify that this thesis entitle (**Antifungal effect of propolis and *Oreganum vulgaris L.* extract**) was prepared under our supervision at the department of Biology, College of Science for Women, University of Babylon as a partial requirement for the degree of Master in Biology.

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Dedication

To the first teacher of mankind .. Messenger of humanity Muhammad bin Abdullah (PBUH).
To those who carry his name proudly to the spirit pure My father, may God have mercy on him .

To the inexhaustible fountain ... to the meaning of love and affection ... To whose supplication was the secret of my success ... to the most beloved.

My husband beats my heart (Hagg Amer)
To the smile that added to my life all the meanings.

My family

To those who bore the burdens of my preoccupation with studies and stood by my dear family.

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List of Abbreviations

Abbreviations	Meaning
PES	Propolis extract solution
GC MS	Gas chromatography mass spectrometry
DC	Direct current
MIC	Minimum inhibitory concentration
BLAST	Basic local alignment search tool
bp	base pair
EO	Essential oil
ITS	Internal transcriptional spacer
PCR	Polymerase chain reaction
SDA	Sabouraud's dextrose agar
EDTA	Ethylene damien tetra acetic acid
PDA	Potato dextrose agar
UV	Ultraviolet
ITOL	Interactive Tree of life
ICUs	Intensive care units
LSD	Least significant difference
UTI	Urinary tract infection
CF	Cystic fibrosis

Summary:-

The results of (115) isolation fungi showed six Genera of fungi in different clinical cases from different hospital and out patant clinics in Babil governorate, they are :- *Candida spp*. (34.78%), *Penicillum* spp. (17.4%), *Aspergillus* spp. (33.9%), *Cladosporium* spp. (6.96%), *Trichophyton* spp. (4.35%), *Curvularia* spp. (2.6%). 4 species of *Candida* genus were isolated by Agricullture method on differential medium Chromo agar candida based on colony color, as well as by molecular methods, the presence of two types of *Candida albicans, Candida glabrata* were recorded. as well as isolating two species of *Aspergillus (Aspergillus flavus, A.niger*), where most of the fungi obtained were diagnosed by DNA sequencing of PCR methods. The study proved that Oregano plant was the most effective in inhibiting the isolated fungal species compared to Propolis. The alcoholic extract induced antifungal activity in the laboratory using the food poisoning method against isolated fungi species by making three concentrations (5, 10 and 15) mg / ml and comparing the positive control represented by the antifungal ketoconazole and the negative control represented by 10% dimethyl sulfoixide.

The results showed that the extract Alcoholic (A. Propolis) had an inhibitory effect of 100% on *A.niger* and *T.mentagarophytes*, compared with control and antifungals with a significant level of 0.05, and their inhibition rate was (0.00 for negative control and 100% for positive control). The sensitivity of other fungi to the alcohol extract concentrations under study varied. Also, the effect of alcoholic extract of Oregano leaves was observed to have 100% inhibitory effect on *A.flavus, A.niger and C.albicans, T.menTagraophyTes* compared with negative and positive control, respectively.

The effectiveness of anti-origano activity is due to the presence of active compounds, most of which have been investigated with GC MS, technology. , including flavonoids, alkaloids, phenols and fatty acids, had a clear effect on the growth of the fungi within the current study.

Chapter One

Introduction

1.1 Introduction

Fungi represent the second largest group of eukaryotic organisms on earth, with estimating ranges from 1.5 to 5.1 million species (O'Brien *et al*., 2005; Blackwell, 2011; Hawksworth, 1991). Members of the fungal kingdom play important roles in human life and can flourish in a number of natural and man-made environments (Stajich *et al*., 2009). In scientific research, species-level identification of fungi is critical in both fundamental (ecology, taxonomy) and applied (genomics, bioprospecting) applications (Schmitt and Barker, 2009).

Fungi are important decomposers, mutualists, and pathogens in ecosystems, but the role of individual fungi in nature is often unknown (Mueller and Schmit, 2007). Fungi-borne infectious diseases are becoming more virulent, posing a global threat to food security (Hyde *et al*., 2018).

One of the causes of increasing antifungal resistance is the widespread use of antifungal agents in agriculture; medical and surgical devices, such as catheters and artificial heart valves, are known to lead to biofilm formation and thus drug resistance. There are two types of microbial resistance: primary and secondary. Primary resistance occurs when a pathogen is born resistant to an antifungal drug, while secondary resistance occurs when a pathogen develops resistance during antifungal treatment. As a result, drug penetration to the infection site can lead to antifungal resistance (subclinical reservoirs of fungal spores/cell not reached/killed by drug seed new infection). As a fungistatic drug must function synergistically to regulate and clear the fungal abundance, the host immune status is one of the most significant factors in antifungal resistance (Cowen *et al*., 2014).

Natural products derived from plants have been known to have a wide range of health benefits for thousands of years through traditional uses, such as instructions for the preparation and administration of medicinal herbs in Babylon around 2000 BC and a list of 500 plants with medicinal properties in a Greek herbalist. By introducing over 200 plants to Britain, the Romans contributed to the growing body of knowledge about medicinal plants. In reality, modern scientific research is raising awareness of the benefits of using natural products for health care. Scientists have introduced an intriguing trend in pharmaceutical production at the turn of the twenty-first century: a return to nature as a source of new medicines (Georgiev, 2012; Lanzotti, 2014).

Various phytochemicals, like Flavonoids, alkaloids, phenolic acids, anthocyanins, lignans, stilbenes, polysaccharides, carotenoids, essential oils, and some vitamins (A, C, and E) have all received a lot of attention as a result of this effort because of their important role in preventing and managing diseases like cancer, diabetes, Alzheimer's disease, and cardiovascular disease (Andrae-Marobela *et al*., 2013; Shao and Xiao, 2013; Xiao, 2015; Xiao and Jiang, 2015; Xiao *et al*., 2014).

Medicinal plants have been used in folk medicine in Asian and African populations for thousands of years, and many plants are consumed in developing countries for their health benefits. According to the World Health Organization (WHO), some countries still rely on plant-based medicine as their primary source of medicine, while developing countries are taking advantage of the therapeutic benefits of naturally sourced compounds (Rajeswara Rao *et al*., 2007). Polyphenols, brassinosteroids, and taxols are examples of anticancer compounds that have been identified and extracted from terrestrial plants (Greenwell and Rahman, 2015).

Propolis can be used to treat recurrent valvo vaginal candidiasis (RVVC) and can be an alternative to antibiotics for patients who are unable to take antibiotics due to another medical condition. The efficacy of propolis against antifungal nystatin has been shown to be satisfactory. In human cells, propolis extract solution (PES) has low toxicity and may be used as an alternative treatment for chronic vaginitis. PES also has antifungal properties and can be used as an antibiofilm material for RVVC to fight C. *albicans* biofilm growth and antifungal drug resistance (Capoci *et al*., 2015).

1.2 Aims of Study

One of the causes of increasing antifungal resistance is the widespread use of antifungal agents in agriculture; medical and surgical devices, such as catheters and artificial heart valves, are known to lead to biofilm formation and thus drug resistance

1.3 objective of Study

1. Isolation and diagnosis of pathogenic fungi from different clinicle cases
2. Finding natural, plant-based alternatives, such as extracts, in the treatment of fungal diseases
3. Test the effectiveness of these extracts in inhibiting the growth of isolated fungi
4. Analysis of the active compounds in these extracts with GC-MS technique.

Chapter Two

Literature Review

2. Literature review:

2.1 Important of Fungal infections:

The advent of fungi capable of infecting humans is becoming a major public health problem. Fungi with clinical importance can be classified as either primary or opportunistic. Primary infection occurs in healthy people who haven't been exposed to fungi, while opportunistic infection occurs in immunosuppressed people (Dixon *et al*., 1996). Modern medical devices and services increase the number of people who depend on healthcare, which raises the number of vulnerable hosts. Fungal infections like *Candidiasis, Mucormycosis (Zygomycosis), Aspergillosis, Cryptococcosis, and Pneumocystosis* often appear and disappear. *Candidemia*, on the other hand, is one of the most common causes of blood-stream infections, with a mortality rate of more than 30% (Zarger and Stepp, 2004), while *Aspergillus* can infect more than 45 % of susceptible hosts (Maschmeyer *et al*., 2007).

Furthermore, despite the expanded spectrum of antifungal agents now available, the incidence of invasive fungal infections is increasing, and invasive fungal infections continue to cause significant morbidity and mortality (Low and Rotstein, 2011). External pressures from antibiotic use make populations vulnerable to invasive fungal infections, which explains why the burden of invasive fungal infections is sadly increasing in lockstep with medical advances (Bansod and Rai, 2008).

Fungal infections kill about 1,350,000 people per year, and more than 300 million people are infected (Brown *et al*., 2012).

Aspergillus, along with many fungi has been reported as an allergen which can cause an allergic reaction in the human population, with the allergenicity of *Aspergillus fumigatus* specifically being often reported (Kespohl and Raulf, 2014). In the 1980s many allergens were identified with the

development of molecular biological techniques of the time, and proteins identified as allergens were classified into databases containing details of allergenic gene sequences and features (Onami *et al*., 2019).

Aspergillus fumigatus is the most common disease causing species of *Aspergillus* in CF, and is most frequently isolated in adolescents and adults. However, this may be due to children being less able to give effective and repeated sputum samples (Burgel *et al*., 2016).

Dermatophytosis is a fungal infection caused by the skin fungus *Dermatophytes*, also known as ringworm. *Dermatophytosis*, also known as Tinea, is a highly contagious skin disease that affects both humans and animals (Pal, 2017). *Dermatophytosis* is one of the most common infections in the world, with a prevalence of 20 to 25% (Sahoo and Mahajan, 2016). *Dermatophytes* are classified into three groups based on their preferred environment: Zoophilic, Geophilic,Anthrpophlic (Ziółkowska *et al*., 2015). *Microsporum, Trichophyton*, and *Epidermophyton* are three of these groups. These fungi can infect the skin as well as other body parts including hair and nails (Reddy, 2017).

In several intensive care units, *Candida* is the fourth most common pathogen in bloodstream infections, with *C. albicans* the most frequently isolated species (Mukherjee *et al*., 2003). *Candida* is also a common cause of infective endocarditis in children (Marom *et al*., 2011) and has been linked to ventilator-acquired pneumonia (Delle Rose *et al*., 2016).

2.2 Fungi species:

2.2.1 *Candida* spp*:*

Candida species are found in the mucous and can cause a variety of infections in humans. This genus contains at least 30 clinically significant

species (Pfüller *et al*., 2011; Silva *et al*., 2012).Infections caused by the *Candida* genus have risen dramatically in recent decades (Sobel, 2007; Pfüller *et al*., 2011).

More than 30 different *Candida* species are linked to human infection with *C. albicans* considered the most pathogenic species despite an apparent increase in the prevalence of the non-*C. albicans* (McManus and Coleman, 2014).

2.2.1.1 Characterization and Classification of *Candida* spp

Taxonomical of *Candida* species according to (Walsh and Groll, 1999; Johnson, 2008), which classified as below;

Kingdom: *Fungi*

Division: *Ascomycota*

Class: *Saccharomycetes*

Order: *Saccharomycetales*

Family: *Saccharomycetaceae*

Genus: *Candida*

The genus *Candida* accommodates a heterogeneous collection of asporogenous (imperfect fungi) yeast like fungi. The yeasts belonging to genus *Candida* are oval, elliptical, or cylindrical unicellular or bicellular measuring 3 to 5 µm, with double-layer cell walls. They are capable of forming true or pseudohyphae except *C. glabrata* and *C. parapsilosis*. *C. glabrata* is incapable of forming both true hyphae and pseudohyphae whereas; *C. parapsilosis* does not produce true hyphae, but can generate pseudohyphae (Walsh and Groll, 1999).

Pseudohyphae are formed by budding yeast cells or hyphae, but the bud remains attached to the parent cell. The new growth continues to elongate and form filaments at cell-cell junctions that are constricted (Silva *et al*., 2012).

Internal cross-walls are absent in pseudohyphae (septa). True hyphae form from yeast cells or from the branches of established hyphae. Its development is triggered by the creation of a ‘germ tube.' The hyphae are divided into separate fungal units by this germ tube, which elongates and then branches with established septa (Silva *et al*., 2012).

Candida species include *C.albicans, C.krusei, C.parapsilosis, C.tropicalis,* and *C. glabrata*, which cause numerous diseases in animals and humans. The five most common Candida species are *C.albicans, C.krusei, C.parapsilosis, C.tropicalis,* and *C. glabrata*, which cause human candidemia). The other non-*Candida albicans* species include *C.dubliniensis*, *C.guilliermondii*, *C.hisitaniae*, *C.kefyr*, *C.lipolytic and C.pelliculos* also cause *candidiasis* infection (Khan *et al*., 2004; Diaz-Fuentes *et al*., 2007).

Candida's ability to form biofilms is a crucial factor in its virulence. Describe biofilms as organized microbial communities attached to a surface and encased in an exopolymeric substance (EPS) matrix (Ramage *et al*., 2006). *Candida* biofilms shield it from host immune systems, improve antifungal tolerance, and can withstand competition from exogenous microorganisms (Sardi *et al*., 2013).

To cause oral *candidasis*, *Candida* must adhere to and persist on oral surfaces, so adherence is critical for effective host colonization and pathogenicity. *Candida*'s initial adhesion to an epithelial cell or a prosthetic surface is aided by hydrophobic and electrostatic interactions. *Candida* adhesions must interact with unique host receptor molecules for long-term adhesion (Höfs *et al*., 2016). The agglutinin-like sequence (ALS) proteins,

hyphal wall protein (Hwp1), and enhanced adherence to polystyrene (EAP1) proteins are the most studied adhesions. Als3 and Ssa1 (an invasin and a heat shock protein HSP70 expressed on hyphal surfaces) bind to host epithelial surface receptors, allowing host cells to attach and invade. The epidermal growth factor (EGF) receptor on oral epithelial cells is one of the host ligands (da Silva Dantas *et al*., 2016)

Because of their ability to form hyphae and/or pseudohyphae, *C.albicans* and *C.dubliniensis* are considered truly polymeric species of the genus. When inoculated with human serum or other substances, they can develop germ tubes in as little as 2 hours. As a result, a positive germ tube test or Reynolds Braude's phenomenon can be used to distinguish these two species (Silva *et al*., 2012; Deorukhkar *et al*., 2012).

2.2.1.2 *Candidiasis:*

Candida infections have seemed to have risen in recent decades, and this has been due to a variety of factors, including the AIDS outbreak, population aging, and the increased use of immunosuppressive medications (Silva *et al*., 2012).

Candidiasis is a fungus infection caused by the *Candida* yeast (a form of fungus). *C.albicans* is the most common *Candida* species that can cause infection in humans. *Candida* can live on the skin and within the body, in places like the mouth, throat, stomach, and vaginal canal, without causing problems. *Candida* can cause infections if it grows out of control or reaches the body deeply (for example, the bloodstream or internal organs such as the kidney, heart, or brain) (Centers for Disease Control and Prevention, 2020).

Systemic *Candiaosis*, which is linked to deep organ infection, is much less common than superficial mucous membrane and skin infection (Naglik *et al*., 2014). The occurrence of solid organ infection in transplant patients is

estimated to be less than 2%, highlighting this lower prevalence (Silveira *et al*., 2013).

Candida usually colonizes 35-55 percent of healthy mouths (De-la-Torre *et al*., 2016). Oral *Candida* carriage can be as high as 90% in immunocompromised patients, such as those with acquired immunodeficiency syndrome (Ribeiro *et al*., 2015).The underlying cause of the transition from commensal candidal carriage to infection has sparked a lot of discussion and study (Patil *et al*., 2015).

Chronic hyperplastic *candidosis* lesions may appear anywhere on the oral mucosa, but are most commonly seen in the commissure regions of the buccal mucosa, the dorsum of the tongue, and the hard palate (López *et al*., 2012). Hyperplasia and parakeratosis of the surface epithelium, which is invaded by *C. albicans* hyphae, are histopathological characteristics. In the epithelium beneath the hyphae, polymorphonuclear micro abscesses form, and a poorly defined chronic inflammatory infiltrate is visible in the lamina propria. The condition is significant because it has the ability to progress to squamous cell carcinoma (SCC) (López *et al*., 2012). Up to 15% of cases have been confirmed to progress to SCC (Sitheeque and Samaranayake, 2003).

2.2.2 *Aspergillus Spp:*

There are numerous published studies describing the function of *Aspergillus,* which includes over 340 taxonomically species, such as *A. fumigatus*, *A. flavus*, *A. niger*, *A. parasiticus*, *A. nidulans,* and *A. terreus,* recognized as cell factories for human, agricultural, and biotechnological applications (Meyer *et al*., 2015; Park *et al*., 2017).

Aspergillus species are ubiquitous saprotrophic filamentous moulds that can be found in air, soil and decaying vegetation (Van De Veerdonk *et al*., 2017). Upon examining the fungal composition of air in hospitals, *A. fumigatus*

is the most prevalent *Aspergillus* spp (Pini *et al*., 2008; Mahieu *et al*., 2000), and *A. fumigatus* accounts for 65% of all invasive *Aspergillus* infections in humans while 185 other species are known (Warris, 2014). *Aspergillus* can exist in different forms, the one observed in air is hydrophobic conidia. These conidia have a diameter of 2-3 μm and because of this small size can penetrate deep into the lungs of humans. It is estimated that 100-1000 *A. fumigatus* conidia are inhaled by humans each day, with the highest exposures occurring in those who work with soil or vegetation such as farmers (Latgé, 1999).

A. flavus is more virulent than *A. fumigatus* and is the second most common cause of invasive and non-invasive *Aspergillosis* (Hedayati *et al*., 2007). Fungal sinusitis and cutaneous infections are caused by the fungus *A. flavus*. *A. flavus* has only been linked to a few chronic disorders, such as chronic cavitary pulmonary *Aspergillosis* and sinus fungal balls. *A. fumigatus*, on the other hand, is the most common species in the genus (Guinea *et al*., 2005). Aflatoxins, the most toxic and active carcinogenic natural compounds ever identified, are among the secondary metabolites formed by *A. flavus* (Hedayati *et al*., 2007).

A. fumigatus is a ubiquitous spore-forming fungus present in the environment, found mostly in soil and decaying organic matter, where it plays a role in decomposition and nutrient recycling (Zacharias and Sheppard 2019).

2.2.2.1 Characterization and Classification of *Aspergillus Spp:*

Aspergillus spp. are filamentous fungi that are usually spread in seeds, grains, soil and decaying vegetation where they grow as saprophytes, they are sometimes can be harmful to humans, most *Aspergillus* species are spread in a wide range of environments and substrates on the earth everywhere considered as important opportunistic pathogens (Kwon-Chung and Sugui, 2013; Seyedmousavi *et al*., 2015).

It has the potential to survive at temperatures ranging from 12°C to 48°C but the optimum growth temperature ranges from 24°C to 17°C, its ability to develop at relatively high temperatures leads to its pathogenicity against humans and other warm blooded animals, for most of its lifecycle, the fungus exists in the form of mycelium or asexual spores known as conidia (Akar and Tunali, 2006).

The appearance of *Aspergillus* spp. depends on many factors like the availability of substrates, microclimate, in addition to activity of water and complex ecological interactions, living in geographical habitats and different environments can be involved to a high ability of reproductive, metabolic variation and competitive abilities of *Aspergillus* strains in nature (Mehl and Cotty, 2013)

Based on National Centre for Biotechnology Information (NCBI) classification system *Aspergillus* is classes as below (Geiser, 2009):

Kingdom: *Fungi*

Subkingdom: *Dikarya*

Phylum: *Ascomycota*

Subphylum: *Pezizomycotina*

Class: *Eurotiomycetes*

Subclass: *Eurotiomycetidae*

Order: *Eurotiales*

Family: *Aspergillaceae*

Genus: *Aspergillus*

2.2.2.2.3 *Aspergillosis:*

Aspergillosis is a fungal infection caused by *Aspergillus*, a common mold (fungus) that can be found both indoors and out. Most people will inhale *Aspergillus* spores without being ill. People with compromised immune systems or lung disorders, are more likely to develop health problems as a result of *Aspergillus*. Allergies, lung infections, and infections in other organs are among the health problems caused by *Aspergillus* (Centers for Disease Control and Prevention, 2020).

2.2.2.2.1 Classification of *Aspergillus* Disease:

1.Allergic bronchopulmonary *aspergillosis* (ABPA): *Aspergillus* causes pulmonary inflammation and allergy symptoms such as coughing and wheezing, but not infection (Agarwal *et al*., 2013).

2. Allergic *Aspergillus* sinusitis: *Aspergillus* causes sinus inflammation and symptoms of a sinus infection (drainage, stuffiness, headache), but not infection (Glass and Amedee, 2011).

3. *Aspergilloma*: also known as fungus ball, it is an *Aspergillus* ball that grows in the lungs or sinuses but does not normally spread to other areas of the body (Lee *et al*., 2004).

4. Chronic pulmonary *aspergillosis*: a long-term (3 months or longer) disease in which *Aspergillus* causes cavities in the lungs, one or more fungal balls, and other symptoms. The presence of *aspergillomas* in the lungs is also possible (Denning *et al*., 2003).

5. Invasive *aspergillosis*: a severe infection that most often affects people with compromised immune systems, such as those that have undergone an organ transplant or a stem cell transplant. It most often affects the lungs, but it may also spread to other parts of the body (Bao *et al*., 2017).

6. Cutaneous (skin) *aspergillosis*: *Aspergillus* enters the body through a crack in the skin (for example, after surgery or a burn wound) and causes infection, most often in people with compromised immune systems. Cutaneous *aspergillosis* may also occur if invasive *aspergillosis* spreads to the skin from elsewhere in the body, such as the lungs (Neki *et al*., 2016).

2.2.3 *Dermatophytes*

Dermatophytes are a group of fungi that belong to three genera: (*Tichophyton, Epidermophyton and Microsporum*). The genus *Epidermophyton* has only one pathogenic species (*Epidermophyton floccosum*), while the genera *Trichophyton and Microsporum* are more complex, with many known species (Baldo *et al*., 2012). *Dermatophytes* are keratinophilic fungi that attack keratinized tissue including skin, nails, and hair. They are responsible for cutaneous mycoses. *Dermatophytosis* (tinea or ringworm) is the medical term for this condition (Sahai and Mishra, 2012).

The infection of *Dermatomycosis* may also be caused by the members of the genus *Candida* and *non-dermatophytes* moulds such as *Aspergillus*, *Fusarium and Scopulariopsis* (Naveed and Naeem, 2009).

2.2.3.1 *Trichophyton mentagrophytes*:

Trichophyton mentagrophytes is a fungal species belonging to the *Trichophyton* genus (de Hoog *et al*., 2017). Ringworm in companion animals is caused by one of three common fungi. It's also the second-most frequently isolated fungus in humans that causes tinea infections, and it's the most common (or one of the most common) fungus that causes zoonotic skin disease (i.e., transmission of mycotic skin disease from species to species). *T. mentagrophytes* is commonly isolated from dogs, cats, rabbits, guinea pigs, and other rodents (Cafarchia *et al*., 2012; Mesquita *et al*., 2016; Bartosch *et al*., 2019), though some genetic variants, such as Type VII (Gallo *et al*., 2017) and

Type VIII (Larionov *et al*., 2017). The geographic ranges of different genetic variants of the fungus differ (Taghipour *et al*., 2019).

T.mentagrophytes and its clonal offshoot *T. interdigitale* are unique among *Trichophyton* species in that they have a large number of genotypes in the internal transcribed spacer (ITS) region, accounting for 34% of the genus' diversity (Pchelin *et al*., 2019). As a result, the same DNA sequences can be used for molecular strain typing and species identification. The basis for species delineation is differences in epidemiological sources and clinical pictures between different genotypes (Pchelin *et al*., 2019; Heidemann *et al*., 2010). *T. interdigitale* is the name given to exclusively anthropophilic isolates, which are often present in tinea unguium and tinea pedis cases, as opposed to zoophilic, *T. mentagrophytes* isolates, which can be found in clinical cases other than nail and foot infections (de Hoog *et al*., 2017).

2.2.4 *Penicillium spp.*

Many species of *Penicillium* are common contaminant on various substrates and commonly found in house dust. Some species grow in-door on dry wall, decaying fabrics, moist chip boards and paint. It is also found in dried foodstuff, cheese fresh herbs, dry cereals, nuts, decaying vegetation among others. It can cause pneumonitis, asthma and allergic alveolitis in susceptible individuals. Type I allergies (hay, fever, asthma) and Type III hypersensitivity has been associated with *Penicillium* spp. (Sigei, 2013).

Penicillium chrysogenum, P. citrinum, P. janthinellum, P. marneffei, and P. purpurogenum are the most common species in the *Penicillium* genus. Patients who do not receive the proper antifungal treatment have a poor prognosis; however, primary Amphotericin B treatment and secondary itraconazole prophylaxis are effective (Supparatpinyo *et al*., 1998).

2.2.4.*1 Penicillium chrysogenum*:

Penicillium. chrysogenum is a flamentous fungus that produces -lactam antibiotics like penicillins and cephalosporins in industrial quantities (van den Berg, 2011). Three genes [acvA (pcbAB), ipnA (pcbC), and aatA (pende)] are involved in penicillin biosynthesis (Terfehr *et al*., 2017).

Since Alexander Fleming (1944)discovered penicillin, which is produced by the filamentous fungus *P. notatum*, the genus *Penicillium* has been extensively studied for its ability to produce a wide range of natural products (NPs), many of which have biotechnological and pharmaceutical applications. *P. rubens* recently renamed *P. chrysogenum* is the most important of the 354 Penicillium species that make up the genus (Nielsen *et al*., 2017). *Penicillium* is a fungus that thrives in enclosed spaces and is linked to food spoilage. It is a major producer of B-lactam antibiotics, particularly penicillin, and its current production strains are the result of decades of classical strain improvement (CSI) (Gombert *et al*., 2011; Houbraken *et al*., 2011).

2.2.5 *Cladosporium Spp* :

Cladosporium species are one of the most common fungi on the planet, surviving in almost any climate and geographical location (Bensch *et al*., 2012). The genus is distinguished by its conidiophores, which are erect, straight, or geniculate, produce abundant branched acropetal chains of smooth to roughened dry conidia, and have a distinct darkened coronate hilum, i.e., a conidial scar with a thick rim covering a central convex dome (David, 1997; Crous *et al*., 2007). *Cladosporium* is one of the most commonly isolated airborne fungi (David, 1997). The relatively small conidia are easily detached and disseminated by the wind (Ellis *et al*., 2007).

The *Cladosporium* genus has been shown to be morphologically and phylogenetically diverse (De Hoog *et al*., 1995). The true human-pathogenic

species *C. bantiana, C. carrionii, and C. devriesii*, which are distinguished by thermotolerance and the absence of conidiophores with pigmented conidial scars, were transferred to *Cladophialophora* based on molecular evidence (De Hoog *et al.,* 1995;Bensch *et al.,* 2012;.) More recently, *Cladosporium* underwent extensive revisions based on polyphasic approaches (Schubert et al., 2007; Zalar et al., 2007;Bensch *et al*., Bensh et al., 2010 Bensh et al.(2012;) , which resulted in the delimitation of 169 species currently accepted in *Cladosporium sensu stricto* (*Cladosporiaceae*, *Capnodiales*). On the other hand, a large number of taxa

2.2.6 *Curvularia Spp:*

Curvularia is a hyphomycete (mold) fungus that is found in soil and is a facultative pathogen, or beneficial partner, of many plant species (Priyadharsini and Muthukumar, 2017).

Curvularia lunata, C. clavata, C. pallescens, C. intermedia, C. senegalensis, C. inaequalis, C. australiensis, and C. tuberculate are only a few of the *Curvularia* species that can infect maize. *C. lunata* is the most destructive of these pathogens in China (Jie *et al*., 2000; Dou and Jin, 2006; Liu *et al*., 2008) .

Curvularia is a type of phaeoid (black) fungus that can cause opportunistic infections in people and animals. *Curvularia hawaiiensis*, also known as *Cochliobolus hawaiiensis* (formerly *Bipolaris hawaiiensis*), is primarily a plant pathogen that is isolated from soil and vegetative matter (Gunasekaran *et al*., 2017)

2.3 Antifungal activities:

As fungi are eukaryotic organisms therefore selection of antifungal drug for controlling human pathogenic fungi is a great challenge. The most

commonly used azole drugs are also have static effect on human pathogenic fungi like *Candida albicans.* Development of fungal resistance against frequently used antifungal agents is also creating life threatening problems (Baddley *et al*., 2001; Whaley *et al*., 2017). In addition to the production of antibacterial compounds, some endophytic fungal strains were also found effective to produce diverse metabolites which have very good antifungal potential against both pathogenic molds and yeasts.

Although most of the organisms showed activities against pathogenic bacterial strains, number of reports regarding antifungal potential is comparatively lower. Enfumafungin, a hemiacetal triterpene glycoside produced by endophytic *Hormonema* sp. showed specific inhibition to the glucan synthesis required for fungal cell wall development and it was found effective against *C.albicans* and *A. fumigatus* (Aly *et al*., 2011).This compound as the first oral glucan synthase inhibitor has entered pahse I trials (Motyl *et al*., 2010) . Cryptocandin produced by endophytic *Cryptosporiopsis sp.* showed antifungal potential against human pathogenic fungi causing skin and nail Mycoses (Strobel and Daisy, 2003).

Ketoconazole is abroad antifungal agent active against systemic and superficial mycoses. As it is readily but incompletely absorbed after oral administration, its topical preparation is good for its activity (Hardman and Gilman's, 2001).

Ketoconazole is effective against *Candida, Histoplasma, Coccidioides*, and *Blastomyces* (but not *Aspergillus*), as well as *chromomycosis* and *paracoccidioidomycosis* (Finkel *et al*., 2009; Food and Drug Administration, 2013). The first orally active azole antifungal drug was ketoconazole (Finkel *et al*., 2009). Because of ketoconazole's higher toxicity, lower absorption, and more limited spectrum of activity, other azole antifungal agents, such as

itraconazole, have largely replaced it as a first-line systemic antifungal drug (Finkel *et al*., 2009; Kauffman, 2004).

2.4 Antifungal Activity to some plant extracts:

Many ethnic groups use medicinal plants as a source of medicine to treat a variety of illnesses in humans and domestic animals. These plants produce antimicrobial,plant extrad so screening medicinal plants provides another option for developing chemical fungicides that are relatively non-toxic and cost-effective (Mahlo *et al*., 2016).

Hassan (2011) isolated *C.albicans* in 35.7 percent of 70 samples of infected women with vaginal and Thrush candidiacis in Iraq, relative to other infected agents. The antifungal activities of ethanolic and aqueous extracts of *Quercus infectoria galls* with hot and cold water treatments were tested in vitro against the growth of *C. albicans* and *C. glabrata* at various concentrations. The results demonstrated that ethanolic extracts of *Quercus* infectoria were more effective against *C. albicans* at 700 mg/ml concentrations, while aqueous (hot and cold distilled water) extracts were more effective against *C.glabrata*, as compared to *C. albicans* growth at the same extract concentrations (Hassan, 2011).

A study by Itlal *et al*., 2020) was conducted in Babylon governorate about Molecular diagnosis of toxigenic *Fusarium spp*. associated with corn seeds and detection the effect of some plant extracts against it, which found that the chemical compound extracts were shown by two medicinal plants (*Curcuma longa L.*)the local name(Curcum) and (*Boswellia carteri Birdwood*)the local name(Luban Dakar) are able to limit the growth of *Fusarium* species. While Experience showed that (Curcum) plant is more effective in controlling *Fusarium* species compared to (Luban Dakar), especially methanol extract

Conducted a study about the antifungal activity of some plant extracts and essential oils against fungus-infested organic archaeological artifacts. The antifungal activities of nine powdered plant extracts and five essential oils were tested *in vitro* against four common fungal species (*A. flavus, A. versicolor, Penicillium sp., and P. purpurogenum*), which were isolated from various organic materials from archaeological artifacts such as papyrus and linen. Essential oils were found to be more effective than powdered plant extracts, with thyme and lemongrass oils being the most effective. In addition, the minimum inhibitory and fungicidal concentrations of these oils were determined. Moreover, gas chromatographymass spectrometry (GCMS) analysis was used to identify their chemical components. *In vivo* experiments were conducted using biodegraded samples for treatment, and microbiological tests showed that both oils would completely kill both tested fungi after exposure time, which ranged from two weeks for papyrus samples to fourteen weeks for linen samples (Othman *et al*., 2020).

The aqueous extract of anise fruits and their volatile oils is characterized by inhibiting activity against four yeast`s species *C. albicans, C.parapsilosis, C. tropicalis, C. pseudotropicalis.*and four species of skin infected fungi *Trichophyton rubrum, T. mentagrophytes*, *Microsporum canis and M. gypseum* (Kosalec *et al*., 2005).

Moghimipour *et al*. (2014) tested the antifungal activity of *Glycyrrhiza glabra* saponin against Candida species (*Candida albicans, Candida tropicalic and Candida glabrata*). Quillaja saponaria complete saponin (QST) was also tested for antifungal activity. In a soxhlet apparatus, the plant's roots were dried, powdered, and prepared with petroleum ether. Methanol, n-butanol, and diethyl ether were used to remove the air-dried powder in order. The saponins' antifungal activity was tested using the well diffusion process, and the value of the Minimum Inhibitory Concentrations (MIC) was calculated. Positive controls

such as clotrimazole were used to determine the species' sensitivity. The saponins of *G. glabra* and *Q. saponaria* were found to be sensitive to *C. albicans* and *C. tropicalic,* while saponin isolated from *G. glabra* only inhibited the growth of *C. glabrata*. Saponins isolated from *G. glabra* and Q. saponaria have been shown to be potential candidates for the development of new antifungal agents in vitro (Moghimipour *et al*., 2014)

Verastegui *et al*. (1996) examined the antifungal activity of a number of widely distributed plants found in northern Mexico and the southern United States. *C. albicans, C.krusei, C.rugosa, Cryptococcus neoformans, C. laurentis, C. albidus, M. canis, M. gypseum, T. tonsurans, E.flocosum, and Sporotrix schenckii* were tested against yeasts and moulds. The extracts analysed demonstrated good antifungal activity against more than one organism (Verástegui *et al*., 1996).

Anti-*Candida albicans* activity was also tested in ethanol extracts from the leaves and/or roots of 35 medicinal plants widely used in Brazil. Extracts from thirteen plants were found to be active (Duarte *et al*., 2005).

Queiroz *et al.,* (2005) found that a dichloromethane extract of the aerial component of *Blumea gariepina D.C.* was active against the phytopathogenic fungus *Cladosporium cucumerinum*, (Queiroz *et al*., 2005), and that aqueous and petroleum ether extracts of *Spilanthes calva D.C.* were active against *Fusarium oxysporum and T.mentagrophytes* (Rai *et al*., 2004).

Devi and Chhetry (2013) tested the antifungal activity of aqueous extracts of locally available medicinal plants in vitro against Drechslera oryzae, the organism that causes brown leaf spot in rice. By using the poisoned food technique, plant extracts at concentrations of 5%, 10%, 15%, and 20% were tested against D. oryzae mycelial growth. Among the plant extracts, Acorus calamus extract at a concentration of 20% inhibited mycelial growth by 80.0

percent, while the other plants tested had a lower inhibitory effect. When compared to a control map, the aqueous extract of Acorus calamus showed the highest percentage of disease control and decreased disease incidence by 45.29 percent in a field trial (Devi and Chhetry, 2013).

Kumar *et al*. (2010) investigated the antimicrobial activity of ethanolic extracts of the medicinal plant *Acorus calamus* against three bacterial (*Pseudomonas sp., Bacillus sp., Staphylococcus aureus*) and three fungal *Aspergillus niger, Aspergillus flavus, and Trichoderma sp.* species in vitro. On *Pseudomonas sp., Staphylococcus aureus, and Aspergillus flavus*, the ethanolic extract of Acorus calamus had moderate antimicrobial activity. Gas Chromatography and Mass Spectroscopy (GCMS) are used to screen the phytocompounds found in the rhizomes of *Acorus calamus.* The GC-MS analysis showed the presence of approximately ten active phytocompounds in the rhizomes, including alkaloids, aromatic, palmitic, and linoleic acid

Mathur *et al.* (2011) tested the antifungal activity and minimum inhibitory concentration (MIC) of various plant extracts in different solvents such as hydroalcohol (50 percent v/v), hexane, and hexane of plants traditionally used as medicines such as *Valeriana jatamansi (Sugandhbala), Coleus barbatus (Pathar choor), Berberis aristata (Kingore), Asparagus racemos* In contrast to hexane extracts, hydroalcoholic extracts of all plants had the highest antifungal activity. Hydroalcoholic extracts of *Andrographis paniculata and Achyranthes aspera* had the highest MIC values of 0.5 and 0.3 mg/ml against *Aspergillus niger* and *Candida albicans*, respectively. *Andrographis paniculata* hexane extracts had the maximum MIC value of 0.7 mg/ml against *Aspergillus niger* (Mathur *et al*., 2011).

Lalitha *et al*. (2013) used a standard procedure to test the antifungal activity of an aqueous extract of *Centella asiatica* L. leaf against seven test fungi and four test bacterial species. The extract was tested at concentrations ranging

from 10% to 100%. All of the test fungi showed substantial activity at all concentrations, with Minimum Inhibitory Concentration (MIC) values ranging from 80% to 90%. The findings were compared to synthetic fungicides in every case. All four bacterial species tested showed substantial activity, with inhibition ranging from 29mm to 31mm (Lalitha *et al*., 2013).

2.5 The Medical Plants:

2.5.1 *Oreganum vulgare L:*

Oregano the object of our investigations is an aromatic perennial herb that belongs to the Lamiaceae family. Approximately 60 species are known as oregano in the world (Leyva-Lopez et al., 2017) It grows in different areas at a wide range of ecological conditions in Armenia: populations are located mostly the Southern regions(Abrahamyan et al., 2014) .Oregano *O.vulgare* Oregano. OEO and carvacrol have been investigated for their antioxidant, antibacterial, antifungal, anticancer, and anti-inflammatory effects, among other biological and pharmacological properties (Suntres *et al*., 2015). *O. vulgare* has been gathered for its essential oils since ancient times to flavor traditional dishes and to treat a variety of illnesses such as convulsive coughs, colds, skin diseases, and digestive disorders (Pieroni, 2008;Ibadullayeva *et al*., 2012; Polat and Satıl, 2012). *O. vulgare* is now one of the most traded and consumed spice plants due to its importance as a source of oregano.(Lukas *et al*., 2015).

Oregano is a valuable source of natural secondary metabolites that are high in essential oil (EO) and have a wide range of applications in medicine, pharmaceutics, food, and cosmetics. Oregano essential oil is a complex mixture of various compounds, with terpenes as the main constituent (mono- and sesquiterpenes). The presence of thymol and carvacrol as the key components in oregano oil gives its flavor (Baranska *et al*., 2005). However, EO composition varies, and the oregano chemotype is determined by the proportion of

compounds within the same genus identified many chemotypes, each with its own distinct flavor (De Martino *et al*., 2009; Lukas *et al*., 2015). In 502 plants from 51 populations from 17 countries, three chemotypes of *O. vulgare* were discovered, which were categorized based on the content ratios of cymyl, sabinyl, and linalool/linalyl acetate. There are four major chemotypes of oregano, according to other literature sources, based on the proportion of phenolic substances: thymol/carvacrol; carvacrol; thymol; and the chemotype with low phenol and high alcohol content (Werker *et al*., 1985).

2.5.2. Scientific Classification of Origano:

According to United States Department of Agriculture (United States Department of Agriculture, 2021)

Kingdom: *Plantae* – Plants

Subkingdom: *Tracheobionta* – Vascular plants

Superdivision: *Spermatophyta* – Seed plants

Division: *Magnoliophyta* – Flowering plants

Class: *Magnoliopsida* – Dicotyledons

Subclass: *Asteridae*

Order: *Lamiales*

Family: *Lamiaceae* – Mint family

Genus: *Origanum L. – origanum P*

Species: *Origanum vulgare L.* (oregano)

2.6.2 Propolis

Honeybees contain propolis, a natural resinous mixture made up of substances collected from plant parts, buds, and exudates. The word propolis comes from the Greek pro, which means "at the entrance to," and polis, which means "community" or "city," meaning that this natural product is used in hive

protection. Propolis is also used as bee glue. Bees use propolis in the construction and repair of their hives for sealing holes and cracks and smoothing out the internal walls (Burdock, 1998; Bankova *et al*., 2000) and as a defensive shield against external invaders such as snakes, lizards, and so on, as well as wind and rain. Propolis is obtained by bees from a variety of plants in temperate climate zones (Wagh, 2013).

Honey and propolis both have a positive impact on human wellbeing. Propolis has been used by man since ancient times, especially in folk medicine to treat a variety of ailments. Egyptians embalmed their cadavers with bee glue because they were aware of its putrefactive properties. Propolis was used by the Incas as an antipyretic. It was prescribed for topical therapy of cutaneous and mucosal wounds by Greek and Roman physicians as a mouth disinfectant, antiseptic, and healing product in wound care (Bankova *et al*., 2000).

Propolis is a lipophilic, stiff, and brittle substance that when heated becomes flexible, pliable, gummy, and extremely sticky (Hausen et al., 1987). It has a distinct and pleasant aromatic odor, and its color ranges from yellow green to red and dark brown, depending on the source and age (Bankova et al., 2000; Monti et al., 1983; Wollenweber et al., 1990; Hausen et al., 1987; Marcucci, 1995).

Propolis is a complex mixture of plant-derived and bee-released compounds. In general, raw propolis is made up of 50% resins, 30% waxes, 10% essential oils, 5% pollen, and 5% organic compounds (Burdock, 1998; Park *et al*., 2002; Pietta *et al*., 2002). More than 300 constituents have been found in various samples (Marcucci, 1995; Park *et al*., 2002; Pietta *et al*., 2002; Castro, 2001), with new ones being discovered during chemical characterization of new forms of propolis (Bankova *et al*., 2000; Banskota *et al*., 1998; Alencar *et al*., 2007).

2.7. Moleculer Detection of fungi

PCR is a molecular biology technique for amplifying a single or a few copies of a piece of DNA and producing thousands to millions of copies of a specific DNA sequence. It was invented in 1985 by Kary Mullis, an American biochemist who won the Nobel Prize and the Japan Prize for developing PCR in 1993. It is also one of the most commonly used molecular biology techniques due to its speed, simplicity, and low cost (Bartlett and Stirling, 2003).

PCR with fungal-specific primers targeted the sequences of 5.8S and 28S rDNAs, as well as those of 18S and 28S rDNAs, resulting in the amplification of the species-specific internal transcribed spacer ITS1 and ITS2 region, which change in amplicon length and sequence approval to species, to achieve the specific identification of fungal species and determined the lengths of PCR fragments by using agarose gel electrophoresis (Hui *et al*., 2000).

2.8 Gas chromatography mass:

Two-dimensional analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) can be used to assess the presence of metabolites or their derivative products in the extract (LC-MS). The most versatile instrument for detecting and distinguishing secondary metabolites is liquid chromatographymass spectrometry (Visagie *et al*., 2014). It plays an important role in determining the precise masses of ions of known and unknown compounds, as well as estimating their molecular composition. Its high sensitivity and specificity enable it to differentiate between isobaric compounds with different elemental compositions at microgram concentrations (Altemimi *et al*., 2017). Electrospray ionization (ESI) combined with tandem mass spectrometry (MS/MS) could be used to perform structural analysis of various bio-active metabolites (Cuyckens and Claeys, 2004), allowing for direct screening of a wide range of natural products

without the need for time-consuming isolation procedures. The volatile and semi-volatile secondary metabolites present in the extract are usually analyzed using GC-MS (Dart *et al*., 1976; Lopes da Silva *et al*., 1998).

Chapter three

Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Equipment's and Instruments

The following equipment's and instruments were used for proceeding of study listed in table (3-1).

Table (3.1): Equipment's and instruments used in the study

No.	Equipment's and Instruments	Manufacturing company
1.	Autoclave	HIR Yama -Hve-50 /Japan
2.	Compound Microscope	Miji Japan
3	Sensitive Balance	Denver Swizer land
4	Incubator	Memmert Germany
5	Water Bath	GFL Germany
6	Centrifuge	Centrifuge Series /Germany
7.	Loop	Hana Romania
8	Micropipette	Eppendorf /Germany
9	Vortex Mixture	LAB-MX-F /Germany
10	Hood	Lab Tech Germany
11	Camera	Camera Alptek /China
12	Cork porer	Janetzki/Germany
13	Refrigerator	Vestel Europe
14	Oven	Memmert /Germany
15	Water Distiller	GFL/Germany
16	Eppendorf	Sigma /England
17	UV- light Transilluminaton	Germany

18	Burner	Amal /Turkey
19	Microscope Slides	Sail Brand /China
20	Thermocyclr	Trios/Germany
21	GC-MAS	USA

3.1.2. Chemicals and Biological Materials

Mentions Chemicals and Biological Materials used in This study in table(3.2)

Table (3.2)Chemicals and Biological Materials used in this study.

NO.	**Biological and Chemical materials**	**Manufacturing company/Origin**
1.	Ladder 100-1000 bp	Intron /korea
2	Agarose	Intron /korea
3	Ethidium bromide	Promega /USA
4	TBE buffer	Promega /USA
5	TE buffer	Promega /USA
6	Primers	Boneer, korea
7	Ketoconiozol	India
8	Chloramphenicol	Samarra Pharmaceutical Factory/Iraq
9	Lactophenol-cotton blue stain	Fluka/Switzerland
10	Formaline	BDH/England
11	EDTA	Promega USA
12	Ethanol 70%, 99%	Switzerland
13	MG-CL	BDH/England

Culture media listed in table (3.1.3) are prepared according to the manufacturer instructions.

Table (3.1.3) culture media used in this study.

NO.	culture media	Manufacturing company
1.	Potato Dextrose agar	Himedia (India)
2	Chromagar candida	Liofilchem/Italy
3	Sabouraud dextrose Agar	HI-Media(India)
4	Potato Dextrose broth	Himedia (India)

3.1.4 The DNA Extraction kit:

The contents of the DNA Extraction KIT from Favorgen used in the laboratory in table(3.4).

Table (3.4) The contents of the DNA extraction compound

No.	Material	Volume
1.	FA buffer	60ml
2.	FB Buffer	32ml
3.	TG 1Buffer	22ml
4.	TG 2 Buffer	15ml
5.	Wash buffer	10 ml
٦	Elution buffer	7ml
7.	Lyriacas sloution	550ml x 10
8.	Proteinase K	11Mg
9.	Collection Tube	100pcs
10.	Elution Tube	50pcs

3.1.5. Primers Used in DNA Amplification:

Primers used in targeted DNA amplification are shown in table (3-5).

Table (3.5)The primer pairs which were used in the study

Primer	Primer sequence `5- --`3	Gene Targeted	Refrenses gene
ITSF	`5GGAAGKARAGTCGTAACAAG`3	ITS	(Cheng *et al*., 2016)
ITSR	`5RGTTTCTTTTCCTCCGCTTA`3		

* : R = (AG)

3.2. Methods

3.2.1. Samples Collection:

Samples were collected from patients with different clinical cases (wounds burns ,urinary tract infection. UTI, sputum, mouth of new born, various skin injuries such as hair, nails, scalp, vaginal in).were obtained from hospitals and Outpatient clinics in Babylon province, from October 2020 to December 2020 a sample collected by using swab media from the infected area and cultured it in the culture media (Weitzman and Summera bell, 1995).

3. 2.2. Sterilization:

3.2.2.1. Sterilize by Autoclaving:

All the media and extraction tools such as Eppendorf tube tips, PCR tube used were sterilized by autoclaving at 121 °c and 15 lbs pressure for 15 minutes. All glass were sterilized by oven at 200°c for two hours.

3.2.2.2. Sterilization by Alcohol Sprayer and Heating:

Stitch, tweezers and other instruments were sterilized by heat and the Hood surface was sterilized by alcohol sprayer.

3.2.2.3.Sterilization by formalin

Sterilization of incubators and refrigerators were performed with formalin supplementation with 15 ml of formalin full into petri dish and left for (2-7) days for complete the sterilization.

3.2.3. Prepare of culture media :-

3.2.3.1. Potato Dextrose Agar(PDA)

This medium was prepared by following the instructions of the manufacturing by suspending 39 gm dissolved in 500ml and complete volume for 1000m| and add 250 mg of antibacterial chloramphenicol, then mixed and autoclaved at 121 °C \15 minutes .

3.2.3.2. Sabouraud dextrose agar (SDA)

This medium was proper with 65 gm of sabouraud's dextrose dissolved 500 ml of distilled water and complete to 1000 ml then placed in a hot plate and magnetized to a boiling point. that add 250 gm of chloramphenicol, then mixed and sterilized. This medium for culturing and maintaining the pathogenic fungi and yeast isolates .

3.2.3.3 Potato Dextrose broth

This medium was prepared by following the instructions of the manufacturing by suspending 50 gm dissolved in 200ml and complete volume for 1000m| and add 250 mg of antibacterial chloramphenicol, then mixed and autoclaved at 121 °C \15 minutes .

3.2.3.4.CHROMO Agar

This medium was used for primary isolation for rapid species identification and detection of mixed flora, especially from non-sterile sites. Depending on the brand of CHROMO agar medium presumptive identification of *C. albicans, C. tropicalis and P.kudriavzevii* is possible. It is especially useful for detection of mixed infections, The CHROM agar is prepared by suspending 47.7 gm of CHROM agar in 1000 ml of D.W. and heated to the point of effervescence (for yeast cultivation), as instructed by the manufacturers. It was then poured into a plastic 9 cm petri dishes. The media is used for researching and diagnosing the Candida spp. Their appearance is based on color (Nadeem *et al*., 2010).

3.2.4. Identification of fungal isolates :

3.2.5. Morphological Identification:

The shape, size, color, edge and appearance of fungal isolates were studied on SDA and PDA media after 24-48 hr of incubation for yeast isolates and 5-7 days for molds isolates. while chromagar test was used to aid in the diagnosis of the *Candida* species depending on color, single cell was picked up from the yeast growth on SDA and culture planning by the loop method incubated for 24-48 hr. at 28 C (Horvath *et al*., 2003).

3.2.6 .Microscopic identification

A direct slide preparation method was used A portion of colonies removed from the culture and placed on a slide with lactophenol cotton blue and tested under the light microscope (McGregor *et al*.,1992).

3.2.7 CHROM agar Test:

This test is performed by inoculating CHROM agar Candida medium which is prepared previously from *Candida* isolate culture grown on SDA for 24 h and then incubated at 28˚C for 24-48h (Paritpokee *et al*.,2005).

CHROM agar test is used for the presumptive identification of *Candida* species by production of different colors on this medium (C. *albicans*= green/ blue green, *C.parapsilosis=white* pale pink ,C. *tropicles*= cream white, C.*glabrata* =Dark pink) (Horvath et al.,2003).

3.2.8. Molecular Detection of Fungal Isolates

3.2.8.1. Preparing the primers

The primer was prepared by adding distilled water free of nucleace in the a different volume according to the manufactures instructions company to obtain a solution of with a concentration of 100 pico mol/ul, mixed by vortex, then centrifuged for 10sec rpm. Then 10 mol/ul of primer was taken and but in the micro centrifuge tube with 90 of deionized distill water prepared working solution.

3.2.8.2. : DNA Extraction from fungi

Use the factory card from (Microgen .Korea) to extract DNA samples of instinct insulation under study and according to the following working steps

1- Transferred 1-5x10 of fungi culture(48 h age) into micro a 1.5 ml microcentirfuge tube for each isolate .

2- 1 ml of FA buffer was added to the cells and resuspend the cells by pipetting.

3- The cells were descended by centrifuge at 5,000 rpm for 2 min and discarded the supernatant completely.

4- The cells were resuspend in 550 µl of FB buffer and 50 µl of lyticase solution were added , mixed well by vortexing . the samples were incubated at 37° C for 30 min.

5- About 8µl of 50 mg\ml RNase A was added and incubated for 2 min at room temperature .

6- The cells were descended by centrifuge at 5,000 rpm for 10 min . The supernatant were removed completely.

7- About 300 µl of TG1 buffer was added and mixed well by pipetting , the sample mixture was transferred to bead tube.

8- Mixed well by plus-vortexing for 5 min.

9- Total 20 µl was added of Proteinase K (10 mg/ml) and mixed well by vortexing. Incubated at 55 °C for 15 min, vortex 30 seconds for every5 minutes incubation.

10- The cells were descended by centrifuging at 5,000 rpm for 1 min and 200 µl of supernatant were transferred to a new 1.5 ml microcentrifugetube.

11-About 200 µl of TG2 Buffer was added and mixed well by pipetting.

12- About 200 µl of ethanol (96-100%) was added and mixed well by pulse-vortexing for 10 seconds.

13- A TG Mini Column was placed in Collection Tube. The sample mixture (including any precipitate) were transferred carefully to TG Mini Column. Centrifuged at 11,000 rpm for 30 second then the TG Mini Column was placed to a new Collection Tube.

14- A total 400 µl of W1 Buffer was added to the TG Mini Column. Centrifuged at 11,000 rpm for 30 seconds and discarded the flow-through. The TG Mini Column was placed back to the Collection Tube.

15- About 750 µl of Wash Buffer was added to the TG Mini Column. Centrifuged at 11,000 rpm for 30 seconds and discarded the flow-through. Placed the TG Mini Column back to the Collection Tube.

16- Centrifuged at full speed (12000 rpm) for an additional 3 min to dry the column.

17- Placed the TG Mini Column to a Elution Tube.18. Added 50 ~100 µl of Elution Buffer or ddH2O to the membrane center of the TG Mini Column. Stranded TG Mini Column for 3 min.

19- Centrifuged at full speed (12000 rpm) for 1 min to elute total DNA.

20- Total DNA was stored at 4°C or -20°C.

3.2.9. Amplification ITS region of rRNA gene

The PCR amplification for its gene was carried out using PCR master Mix. The PCR reaction components show in Table (3.4).

Table(3.6) PCR Reaction mixture

Component	Volumes
Master Mix	12 µ
DNA template	2 µ

Forward Primer	1 µ
Reveres Primer	1 µ
Deionized water	9 µl
Total	25

Table (3.7) PCR Thermo cycler Conditions

PCR step	Temp	Time	Cycle number
Initial Denaturation	94C °	5min	1
Denaturation	94C°	30sec	35
Annealing	58C°	30sec	
Extension	72C°	40sec	
Final extension	72C°	5min	1

3.2.10. DNA electrophoresis :

1- 100 ml of the T.B.E buffer is placed in a beaker.

2- Then 2 g of agarose is added to the buffer.

3- the buffer with the agarose is heated on a micro wave oven boiling point so that all of its components are diveloved.

4- The agarose mixture is cooled by leaving it between 50-60˚C.

5- Ethidium Bromide dye is added at 0.5 µl to the agarose before solidification of the liquid and mixed it well.

6- The comb is put into one of the ends of the agarose gel Tank.

7- Agarose was is poured into theTank to prevent the bubbles and left to cool at room temperature until solidified.

8- The migration electric basin is filled with the T.B.E buffer solution so that it rose from the gel surface.

9- The samples are placed in the wells with the addition of the agarose gel loading dye so that the dye was linked to the DNA.

10- The electrophoresis is performed in 70 V to 45 min.

11- The agarose gel is exposed to UV transilluminator for DNA bands visualized and documented.

3.2.11. Sequencing of Selected PCR Product of fungi and yeast Isolates:

The PCR products of fungi and Yeast isolates were sent to Macrogen Laboratory in Korea and the received the positive sequence data for different species. The sequencing results were subjected for alignment with reference strains those deposits in Genbank for sequence species

I dentification.

3.2.12. Comprehensive phylogenetic tree construction

A specific comprehensive tree was constructed in this study according to the cladogram construction described by Hashim *et al.* (2020). The observed variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (Zhang *et al.* 2000). Based on Clustal omega suit (Sievers and Higgins, 2014), multiple sequence alignments were made for the retrieved nucleic acid sequences. Subsequently, an inclusive tree was built by the neighbor-joining method and visualized using iTOL suit to generate a cladogram of clades construction (Letunic and Bork, 2019). The observed variants as well as

their corresponding reference sequences were incorporated in the constructed comprehensive cladogram. The sequences of each classified phylogenetic species in the comprehensive tree were annotated accordingly.

3.2.13. The plant Extract samples

Phytochemicals compounds were extracted by using digestion methods by using 50 gm of plants materials powder instant in 250 ml of Eethanol solvents separately for each plant, then shake it well for 1 hour, after that leave it for 72 hours in water path at 45C˚ to complete the process of extraction (Handa, 2008), and then dried. Stock solution of 100 mg/ml was prepared in 10% Dimethyl Sulfoxide (DMSO) sterilized by Millipore filter (0.22µm) and stored at (-20C˚) until use (Al-Jassani, 2017).

3.2.13.1. Antifungal activity assay of extract:

SDA medium was prepared and autoclaved after that a known volume (2ml) of the each plant extracts is placed in the center of the petri dishes and complete the volume to 20ml with SDA medium to obtain the required final concentrations (5,10, and 15mg/ml) of the medicinal plants after complete solidification of the medium, 5 mm disc of seven days old culture of the test fungus were placed aseptically in the center of the Petri plates and incubated at 25 - 28C˚ for seven days,and 24-48hr of Yeast simultaneously 0.02ml of antibiotic solution was added to each assay plate to check the bacterial contamination as suggested by Gupta and Banerjee (1970). Antifungle ketoconazole 5 mg/ml(Ganabi,2004).was used as positive control and as negative Dimethyl Sulfoxide control observations were recorded on 7-10 day. The colony diameter was recorded in terms of millimeters. For each treatment three replicates were maintained. The fungi toxicity of extracts was calculated in terms of percent inhibition of mycelia growth by using the formula (Singh and Tripathi,1999).

Percent Inhibition = (dc- dt/ dc)* 100

Where:

dc = Average increase in mycelia growth in control.

dt = Average increase in mycelia growth in treatment

3.2.14. :Method for analyzing chemical compounds with GC-MS technology

The active compounds were diagnosed and quantified by the GC-MS device type(Agilent Aechnologies,7820A GC system) American made. According to the following circumstances:

1- Separation column type HP-5ms ultra lnert By dimensions(30mX250MmX0.25Mm)which compound of which works in Electron Effect Mode 70-EV(Electron fixed Detector).

2-helium gas(99.99%) used as a carrier gas at a continuous flow rate of 1.2ml/min.

3- The volume of injected fluid is 1 Micro liter with a split ratio 1:20

4- Injector temperature is 250C

5- Oven temperature is automatically on 60C,withn an increase 10C/min until 280C ,than6C min to 300C,hold for 2min then increase to 300C by5C/min.Settle in300C.

6- The device pressure is10.7 Psi with avrege 1.2ml/min.

7- The total time to start and operation of the device for the sample is 36min.

3.2.15. Statistical analysis

All statistical analysis were performed by the using of SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). All the results were expressed as mean. A $p < 0.05$ was considered statistically significant.. Analysis of variance was employed to evaluate the presence of significant differences. LSD was carried out to find the significant difference using(Payne *et al.,* 2002).

Chapter four

Results and Discussion

4. Results and discussion

4.1 Isolation and identification

4.1.1 Collection of samples

The current study included 55 samples from hospitalized patients, various special clinics, and tuberculosis center in Babylon province who had various clinical cases that included 20 (36.36%) Vaginal swab, followed by 13 (23.6%) various skin injuries samples. While 9 (16.4%) of kids mouth, 7 (12.7%) of burns samples, only 3 (5.45%) of urinary tract infection samples, and finally 3 (5.45%) of Sputum.(Table 4.1).

Table (4.1):Numbers and percentages of samples collected from hospitalized patients with various clinical cases in Babylon province

Type of sample	No. of samples	Percentage
Various skin injuries	13	23.6%
Burns	7	12.7%
Kids mouth	9	16.4%
UTI	3	5.45%
Sputum	3	5.45%
Vaginal (Pregnant women and not pregnant)	20	36.36%
Total	55	100.0%

This study demonstrated that *Candida spp.* 13(37.14%), *Aspergillus spp.* 10 (28.57%), *Penicillium spp.* 8 (22.86%), and *Trichophyton spp.* 4 (11.4%) were obtained from various skin injuries. While *Aspergillus spp.* 9 (34.61%), *Candida spp.* 9 (34.61%), *Penicillium spp.* 5 (19.23%), *Curvalaria spp.* 2 (7.7%), and *Tricophyton spp.* 1 (3.85%) were isolated from burns. Also, *Aspergillus spp.* 10 (50%), *Cladosporium spp.* 4 (20%), *Penicillium spp.* 3 (15%) , and *candida spp.* 3 (15%) were isolated from UTI. As for other samples, the study found that 8(57.14%), 4(28.57%), and 2(14.3%) of *Aspergillus sp, Cladosporium sp,* and *Penicillium* sp were isolated from sputum respectively. Regarding Kid's mouth samples, the study revealed that *Candida* 8 (72.73%), *Aspergillus sp* 2(18.18%), and *Penicillium* 1 (9.1%). Finally, *Candida* 7 (77.78%), and 1 (11.1%) of *Penicillium*, and *Curvalaria* for each of them, were isolated from vaginal (pregnan Also, the study revealed six genera from different samples which involved Candida spp. 40 (34.78%), followed by *Aspergillus spp*. 39 (33.9%), *penicillium spp*. 20 (17.4%), *Cladosporium spp*. 8 (6.96%), *Trycophyton spp*. 5 (4.35%), and *Curvalaria spp*. 3 (2.6%). Table (4.3)t and not pregnant).(Table 4.2)

Table(4.2):Number and percentage of genera isolated from samples distributed according to various clinical cases

Samples	Genera	No.	Percentage
Various skin injuries	*Tricophyton*	4	11.4%
	Aspergillus	10	28.57%
	Candida	13	37.14%
	Penicillium	8	22.86%
	Total	**35**	**100%**
Burn	*Aspergillus sp*	9	34.61%
	Penicillium sp	5	19.23%
	Curvularia sp	2	7.7%
	Candida sp	9	34.61%
	Trichophyton sp	1	3.85%
	Total	**26**	**100%**
UTI	*Candida sp*	3	15%
	Penicillium sp	3	15%
	Aspergillus sp	10	50%
	Cladosporium sp	4	20%
	Total	**20**	**100%**
Sputum	*Aspergillus sp*	8	57.14%
	Penicillium sp	2	14.3%
	Cladosporium sp	4	28.57%
	Total	**14**	**100%**
Kids mouth (New born)	*Candida sp*	8	72.73%
	Aspergillus sp	2	18.18%
	Penicillium sp	1	9.1%
	Total	**11**	**100%**
Vaginal (pregnant and not pregnant)	*Candida sp*	7	77.78%
	Penicillium sp	1	11.1%
	Curvalaria sp	1	11.1%
	Total	**9**	**100%**

Table 4.3 Frequency percentage and number of fungal genera isolated from hospitalized patients with various clinical cases

Fungal genera	No. of isolates	Percentage
Candida sp	40	34.78%
Aspergillus sp	39	33.9%
Penicillium sp	20	17.4%
Cladosporium sp	8	6.96%
Trycophyton sp	5	4.35%
Curvalaria sp	3	2.6%
Total	115	100.0

The results of this study demonstrated that the highest percentage 74.35% of *A. niger*, while the recorded lowest percentage 25.64% of *A. flavus*. Table (4.3.1)

Table (4.4.):Frequency percentage and number of *Aspergillus* spp. isolated from hospitalized patients with various clinical cases

Aspergillus spp.	No. of isolate	Percentage
A. niger	29	74.35%
A. flavus	10	25.64%
Total	39	100.0

On the other hand, the study revealed the highest frequency 20 (50.0%) of *C. albicans,* while recorded the lowest frequency for 8 (20.0%) of *C. tropopical* , 6 (15.0%) of *C. parapsilosis*, finally 6 (15.0%) of *C. glabrata*. Table (4.3.2)

Table (4.5.2) :Frequency percentage and number of *Candida spp.* isolated from hospitalized patients with various clinical cases

Candida spp.	No. of samples	Percentage
C. albicans	20	50.0%
C. tropopical	8	20.0%
C. parapsilosis	6	15.0%
C. glabrata	6	15.0%
Total	40	100.0%

Higher prevalence of *Candida sp* in hospitalized patients may be due to increasing use of antibiotics and better adaptation to the hospital environment. Also prolonged ICU stay of patients may lead prevalence of Candida infections in immunocompromised and patients on prolonged indwelling medical devices (Pawar, 2019). Our findings may explain the increase in the risk of *Candida* infection in hospitalized patient as well as healthy individuals.

In a study conducted by Leinberger *et al*.,(2005), the study revealed that *C. albicans* isolated had first rank (14.5%) in skin injuries. This result is in agreement with the findings conclude in our study.

A study by (Pawar, 2019) about morphological and genotypic characterization of *C. albicans* isolated from clinical samples from a tertiary care hospital in PCMC region, revealed that *C. albicans* 26 (21.9%) were isolated of UTI.

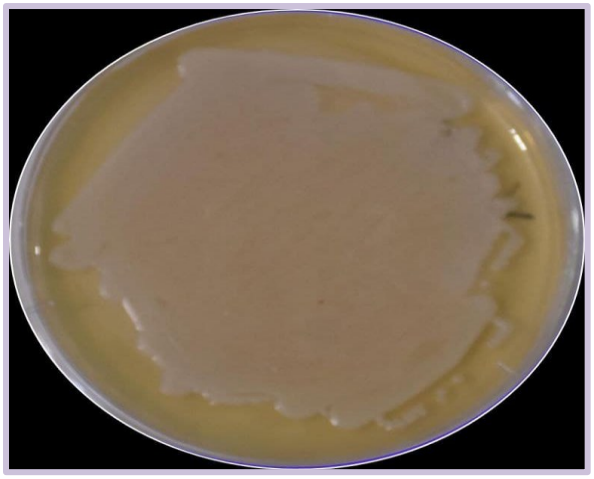
Also, study conducted in India by Nageswari *et al* (2013) and Alim A *et al* (2018) reported highest *C. albicans* 59.59% from sputum and 40.28% respiratory samples respectively (Alim *et al*., 2018; Gandham *et al*., 2013).

A study by Gujarat (2016) also reported the similar pattern and prevalence of species wise distribution of *Candida* isolates (Mehta and Date, 2016)

4.1.2 Morphological identification

4.1.2.1 Identification on SDA medium

All of the samples were cultured on SDA, and the colonies of *Candida spp*. ranged in color from cream to yellowish, grew quickly, and matured in 24 to 48 hours, with a smooth, glistening, or dry texture (Figure 4.1). Who findings the same results AL-Ameri, (2018).

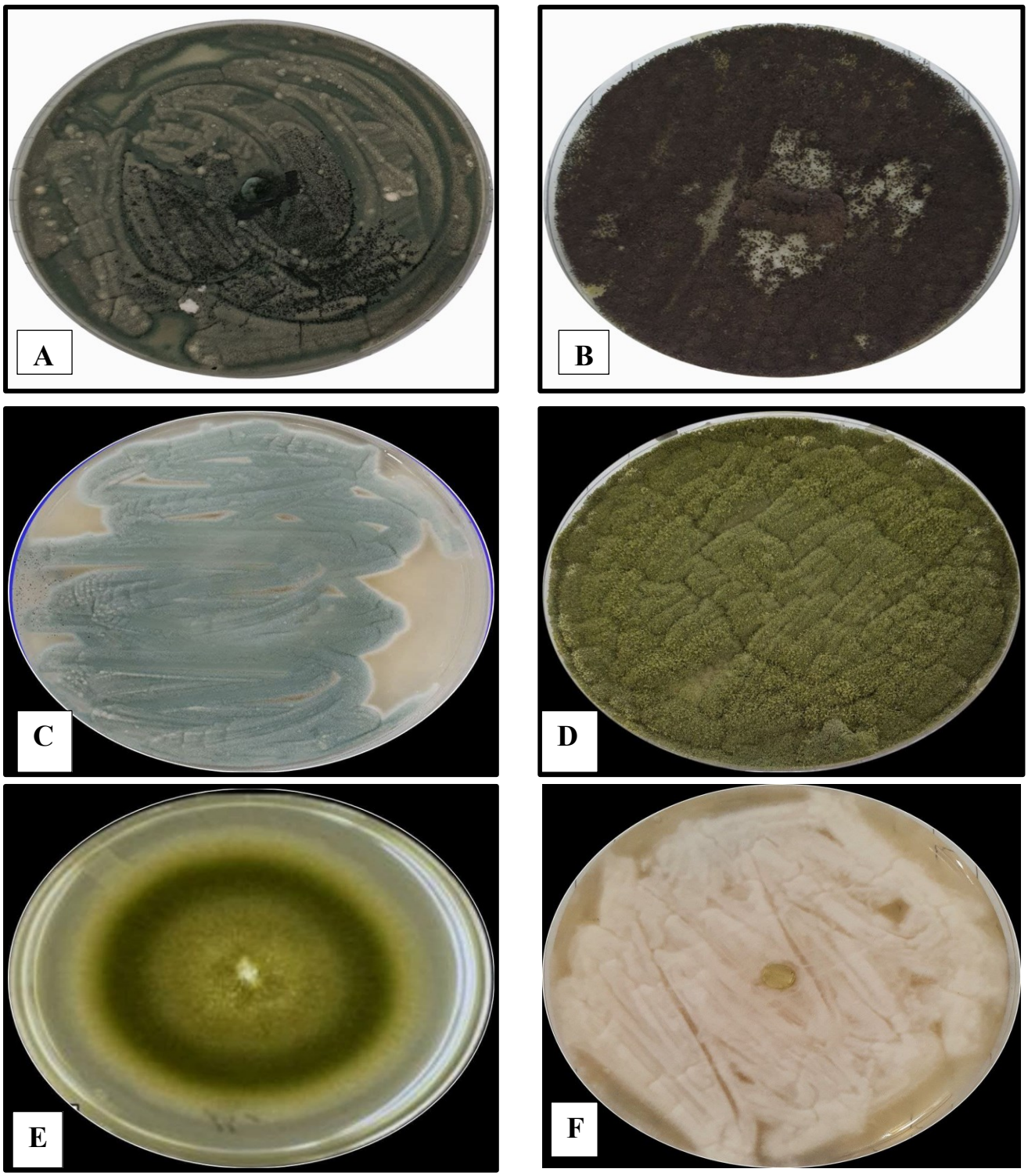


Figure(4.1):Macrograph showing *Candida spp.* colonies growing on SDA , 24-48 hrs.

On media, particularly SDA, macro and micro morphological characteristics of the genus *Aspergillus*, as well as available taxonomic keys, have been used (Klich, 2002). The macro-morphological characteristics that have been used to identify *Aspergillus spp.* included colony color and texture, presence of exudates and production of soluble pigments by the fungi in the media, and plate reverse color, and the micro-morphological characteristics that have been used to identify *Aspergillus spp.* included shape of conidia head, serration, vesicle shape and diameter,

stipe; length, width, texture and color, conidia size and color (Figure 4.2). Conidia heads may be radiate, globose, clavate, or columnar in shape (Samson et al., 2014).

Morphological identification is still widely used, although the characteristics observed cannot be used to accurately identify species at the species level, but are sufficient to give indication or evidence of complex divisions or species (Balajee et al., 2007). According to a survey conducted by the American Society for Microbiology, 89% of laboratories in the United States still use morphological methods because isolation and cultural identification are easy, quick, and less expensive than molecular methods (Zulkifli, 2015).



Figure(4.2):Macrographs showing fungal spp. colonies growing on SDA, 5-7 days 28 C°, A: *Penicillium spp.*, B: *A. niger*, C: *Cladosporium spp*., D):*Aspergillus spp*., E: *Curvularia* spp., F: *Trichophyton spp.*

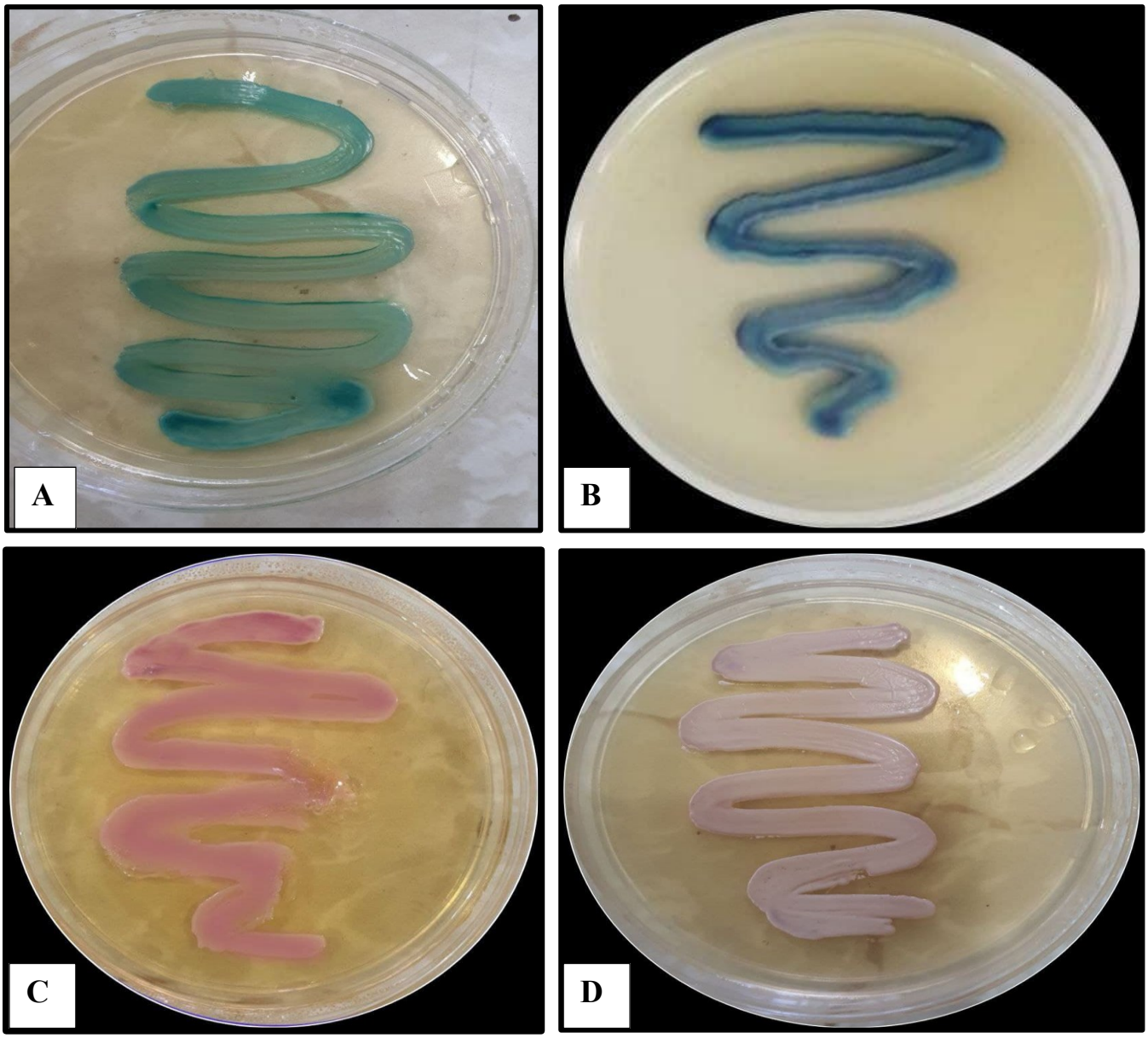
4.1.2.2 Identification of *Candida* spp. on chromagar medium

This is an effective and rapid testing in the diagnosis of certain species of yeasts on the output color level after inoculation and incubation, compared with tests and other culture medium from the traditional methods (Momani, 2000).

CHROM agar Candida contains enzymatic chromogenic substrates, which combine with certain enzymes secreted by the types of *Candida* when they grow on this medium, which leads to different colors depending on the *Candida* species, this test is useful in the laboratory diagnosis of yeast (Murray *et al*., 2005).

The current study found that using chromagar *Candida*, a differential agar, the colonies appear dark pink in *C.glabrata* and white pale pink in *C.parapsilosis* (Hospenthal *et al*., 2002), and light green color smooth colonies in *C.albicans* and dull greenish-blue in *C.tropicalis*, after incubation for 48 hours at 37°C, (Figure 4.3). These findings are in agreement with those of (AL-Ameri, 2018; Daef *et al*., 2014 and Safarali, 2020), who discovered the same characteristics of *Candida spp. colonies* on chromagar.

Also, Other studies in Iraq conducted by Al-Sendi, (2019) and Shaba'a, (2011), who revealed that *C. albicans* produce the light green color on CHROMagar medium. These results agreed with the study our findings.



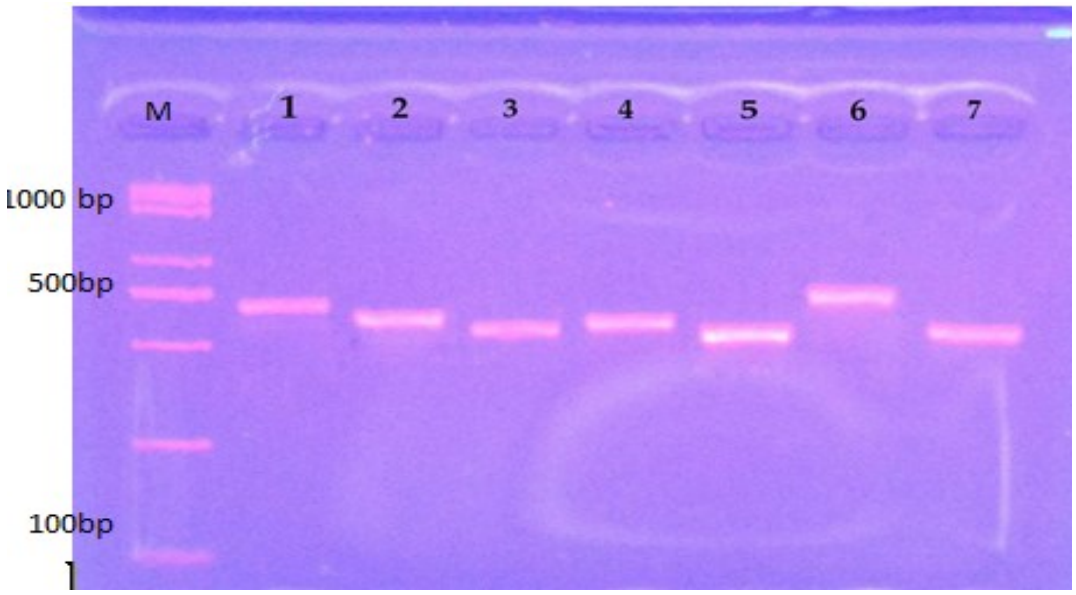
Figure(4.3):Macrographs showing *Candida* spp. colonies growing on chromagar 24-48 hr. 37 Cº, A) : *C.albicans*, B): *C.tropicalis*, C): *C.glabrata* , and D): *C.parapsilosis.*

4.2 Sequencing-Phylogenies Methods

4.2.1 DNA Sequencing of PCR amplicons

1-Fungal samples

The resolved PCR amplicons were commercially sequenced from forward, and reverse, termini ,according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of fungal samples with the retrieved reference sequences of the fungal database, the virtual positions, and other details of the retrieved PCR fragments were identified.



Figure(4.4): ITS regions PCR product electrophoresis on an agarose gel using pair primers ITS4-ITS5**from different Fungal and yeast species (1.3g agarose gel, 80 volts for 1 hour) (M: DNA ladder; lane 1-7: yeast spp.)**

1.GROUP-1; Sequencing of ribosomal amplicons of one (S1) *Trichophyton mentagrophytes*

Within this locus, one samples was detected in this study. These samples was screened to amplify internal transcribe spacer regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons (https://www.snapg ene.com). Concerning the supposed 707 bp amplicons, the NCBI BLASTn engine shown about high 100% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. MT371235.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure 4-5).

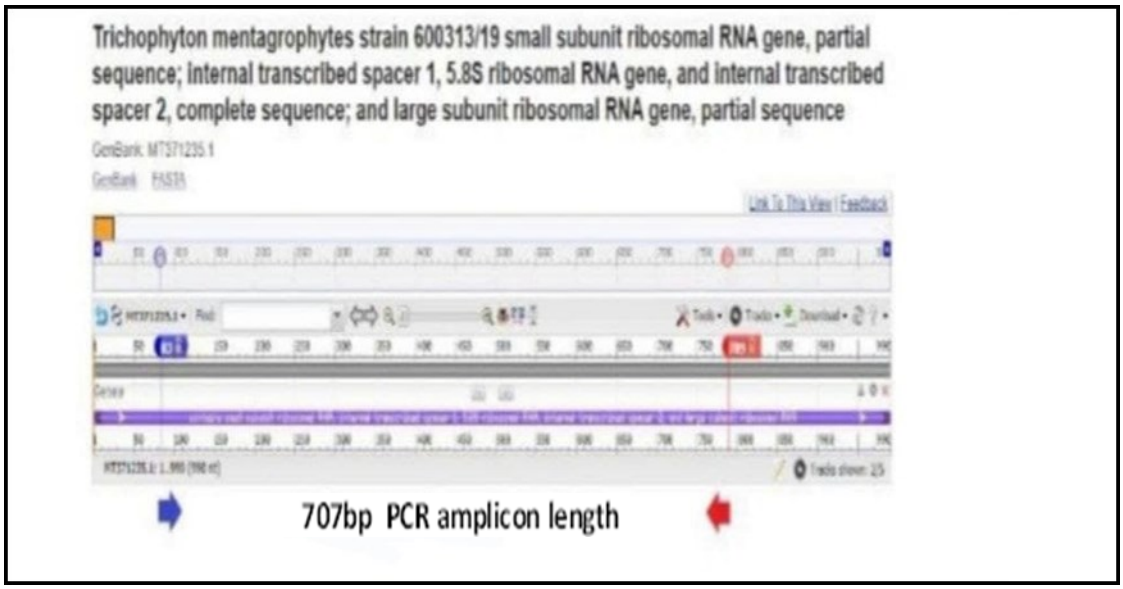


Fig (4-5): The exact position of the retrieved707pb amplicon that partially covered a portion of the rRNA gene within *T. mentagrophytes* (GenBank acc. MT371235.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint. The letter "S" refers to sample numbers

After positioning the 707 bp amplicons' sequences within the the details of its sequences we *Trichophyton mentagrophytesre* highlighted, in terms of the positioning of both forward and reverse primers of the 707 bp amplified amplicons (Table 4-4).

Amplicon	**Reference locus sequences (5′ - 3′**	**length**
DNA sequences within the internal transcribed region of the rRNA gene	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGA AGGATCATTAACGCGCAGGCCGGAGGCTGGCCCCCCACGATAGG GCCAAACGTCCGTCAGGGGTGAGCAGATGTGCGCCGGCCGTACC GCCCCATTCTTGTCTACCTTACTCGGTTGCCTCGGCGGGCCGCG CTCTTCCAGGAGAGCCGTTCGGCGAGCCTCTCTTTAGTGGCTAA ACGCTGGACCGCGCCCGCCGGAGGACAGACGCAAAAAAATTCTT TCAGAAGAGCTGTCAGTCTGAGCGTTAGCAAGCAAAAATCAGTT AAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTG AATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTCCG GGGGGCATGCCTGTTCGAGCGTCATTTCAGCCCCTCAAGCCCGG CTTGTGTGATGGACGACCGTCCGGCGCCCCCGTTTTTGGGGGTG CGGGACGCGCCCGAAAAGCAGTGGCCAGGCCGCGATTCCGGCTT CCTAGGCGAATGGGCAACAAACCAGCGCCTCCAGGACCGGCCGC CCTGGCCTCAAAATCTGTTTTATACTTATCAGGTTGACCTCGGA TCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGA GGA	707 bp

Table (4-4): The positions and length of the707pb PCR amplicons used to amplify a portion of the internal transcribed spacer region of the rRNA gene within *T. mentagrophytes* (GenBank acc. MT371235.1). The highlighted sequences refer to the positions of the forward and reverse primers respectively.

The alignment results of the 707bp samples revealed the Absence of any mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-6).

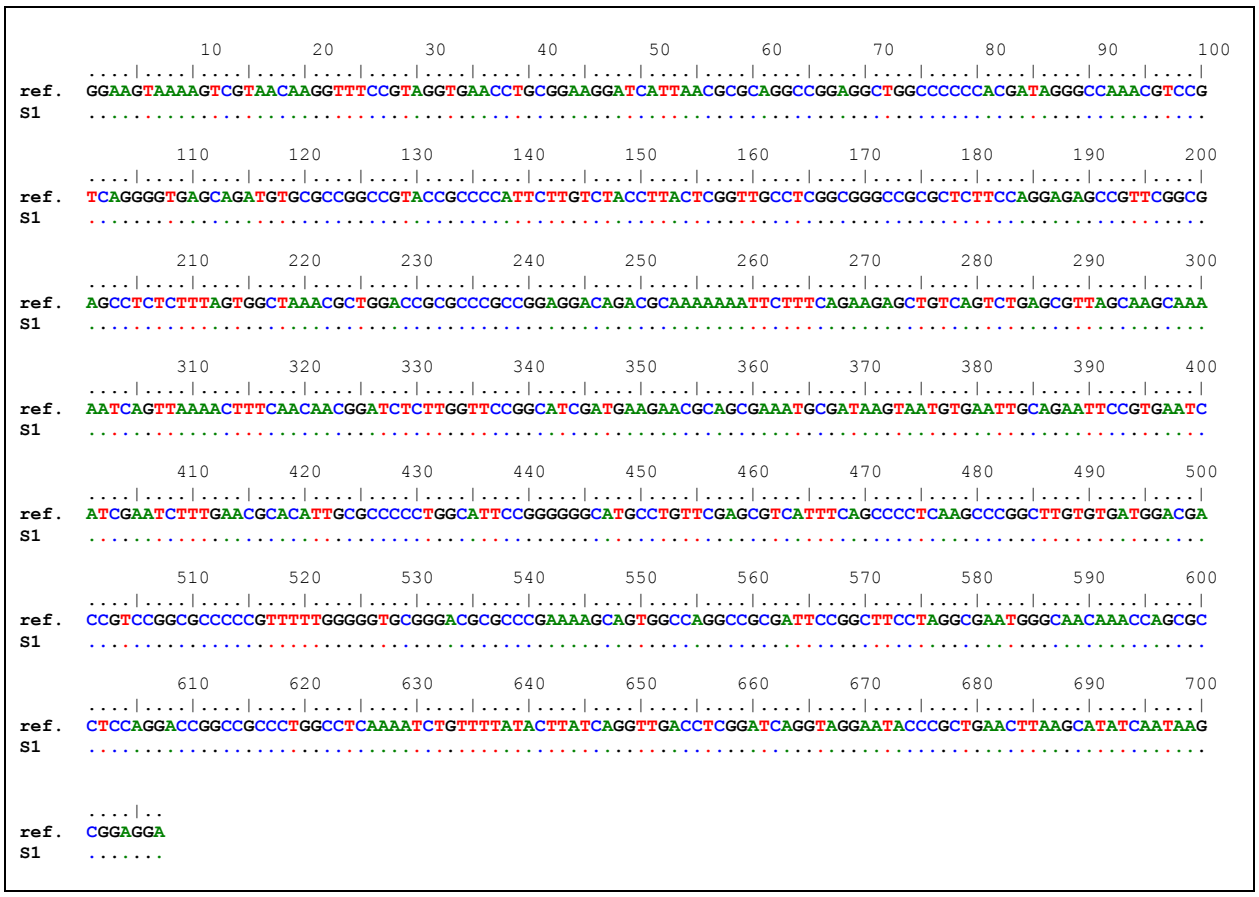


Figure (4-6).DNA sequences alignment of *T. mentagrophytes* with its corresponding reference sequences of the 707 bp amplicons of the internal transcribed region of the ITS4-ITS5rRNAgenetic DNA sequences. The symbol ―ref‖ refers to the NCBI referring sequence, ―S1,‖ refer to the samples no. 1.

2.GROUP-2; Sequencing of ribosomal amplicons of three (S2, S7, S9)

Penicillium chrysogenum

Within this locus, three samples was detected in this study. These samples was screened to amplify internal transcribe regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons .Concerning the supposed 609 bp amplicons, the NCBI BLASTn engine shown about high 99% sequences similarities between the sequenced samples and the intended reference target sequences. By

comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. MN839773.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure 4-7).

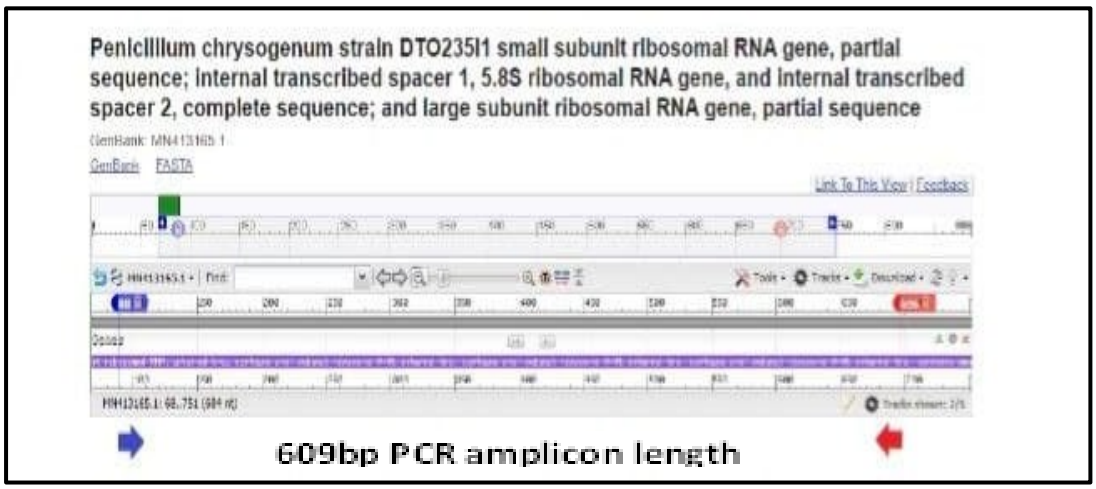


Figure (**4-7**): The exact position of the retrievedpb609 amplificon that partially covered a portion of the rRNA gene within *P. chrysogenum* (GenBank acc. MN839773.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint**.**

After positioning the 609 bp amplicons' sequences within the *Penicillium chrysogenum* the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 609 bp amplified amplicons (Table 4-5).

Amplicon	**Reference locus sequences (5′ - 3′**	length
DNA sequences within the internal transcribed region of the rRNA gene	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGG AAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCA CCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTAA CTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGA AGACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAA ATATAAATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCC GGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAA TTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCG CCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGC TGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGTCCTCCGATC CCGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGT CCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGGC CGGCGCTTGCCGATCAACCCAAATTTTTATCCAGGTTGACCTC GGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAG CGGAGGA	609 bp

Table (**4-5**): The positions and length of the609bp PCR amplicon used to amplify a portion of the internal transcribed region rRNA gene within *Penicillium chrysogenum* (GenBank acc. MN839773.1), the highlighted sequences refer to the positions of the forward and reverse primers respectively.

The alignment results of the 609bp samples revealed the percent of three mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-8).

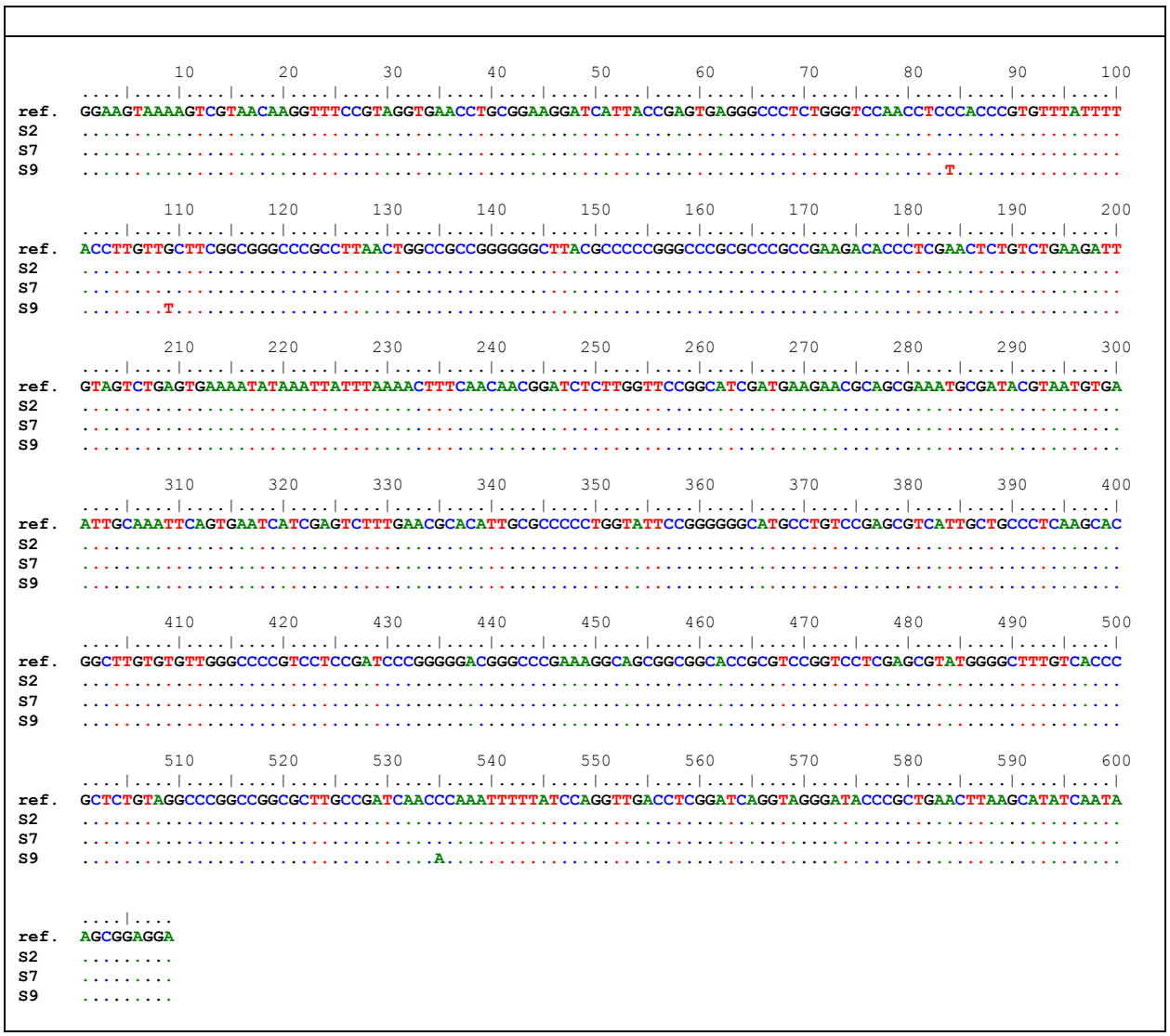


Figure (**4-8**).DNA sequences alignment of three varieties of *P. chrysogenum* with its corresponding reference sequences of the 609 bp amplicons of the internal transcribed region of the ITS1-ITS2rRNAgenetic DNA sequences. The symbol "ref " refers to the NCBI referring sequence, "S2, S7, S9" refer to the samples no.2,7,9.

Three nucleic acid substitutions (NCBI) was observed in all of the investigated specimens, were G84T.G109T,C535A,in S9 The sequencing chromatogram of the identified variation region, as well as their detailed annotations, were documented, and the chromatogram this sequence was chromatogram of the identified variation region, as well as their detailed

annotations, were documented, and the chromatogram this sequence was shown according to its position in the PCR amplicons (Figure 4-9).

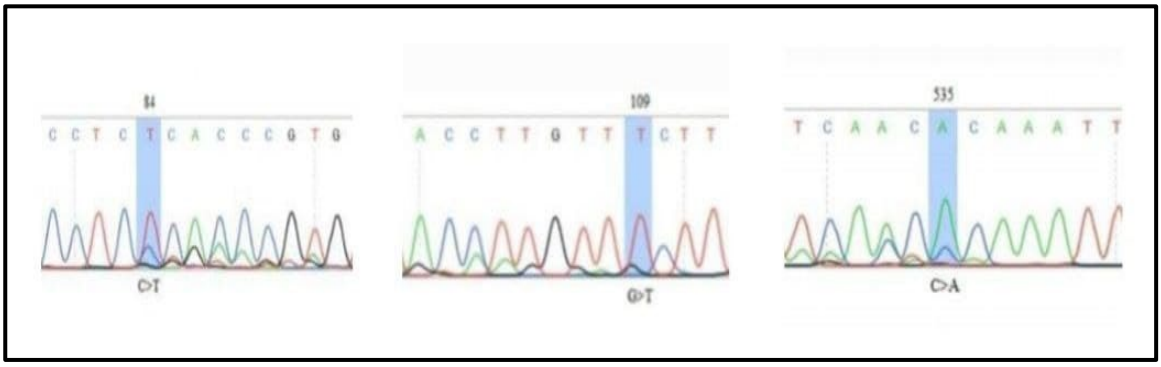


Figure (4-9): The pattern of the detected (NCBI) within the DNA chromatogram of the targeted 609 bp amplicons of the ITS4-ITS5 rRNA gene. The identified SNP were highlighted according to its position in the PCR amplicons. The symbol ”>”‖ refers to”substitution”‖ mutation.

3.GROUP-3; Sequencing of ribosomal amplicons of three (S3, S5, S6)

Cladosporium limoniforme

Within this locus, three samples was detected in this study. These samples was screened to amplify internal transcribe regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons. Concerning the supposed 576 bp amplicons, the NCBI BLASTn engine shown about high 99% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. MN413165.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure 4-10).

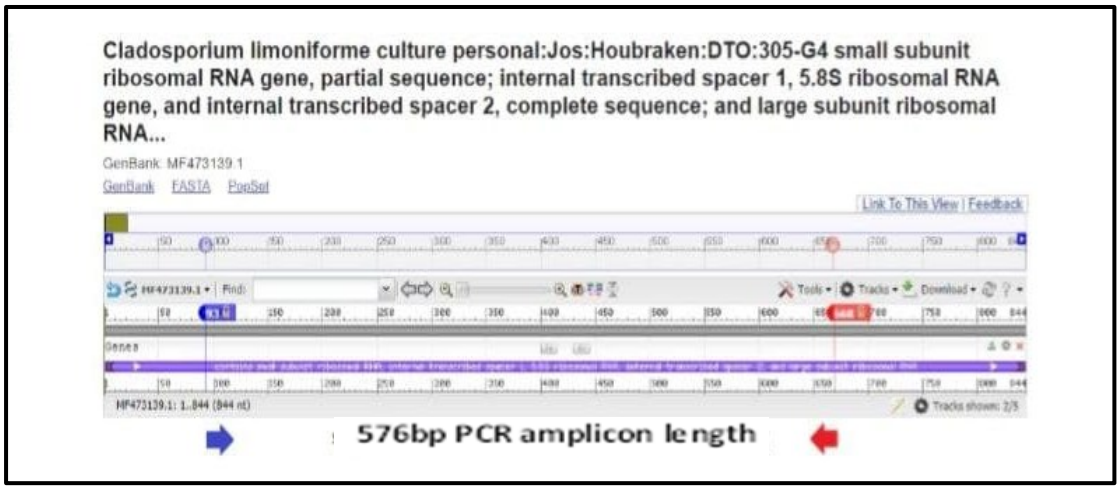


Figure (4-10): The exact position of the retrievedpb576 amplicon that partially covered a portion of the rRNA gene within *C. limoniforme* (GenBank acc. MN413165.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 576 bp amplicons' sequences within the *Cladosporium limoniforme* the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 576 bp amplified amplicons (Table 4-6).

Amplicon	**Reference locus sequences (5′ - 3′**	**length**
DNA sequences within the internal transcribed region of the rRNA gene	GGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGA GGGATCATTATAAGTTCACCCAGGCTTGTACAGCTGGGGACTGA CAACCCTTTGATTTCCGACTCTGTTGCCTCCGGGGCGACCCTGC CTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAA CTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAA CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT CTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC TGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGTATTGGG CAACGCGGTCCGCCGCGTGCCTCAAATCGTCCGGCTGGGTCTTC TGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGTTCGG GAGGCTACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGG ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGG AGGA	576 bp

Table (4-6): The positions and length of thebp576 PCR amplicon used to amplify a portion of the internal transcribe regions rRNA gene within *Cladosporium limoniforme* (GenBank acc. MN413165.1), the highlighted sequences refer to the positions of the forward and reverse primers respectively.

The alignment results of the 576bp samples revealed the Percent of three mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-11).

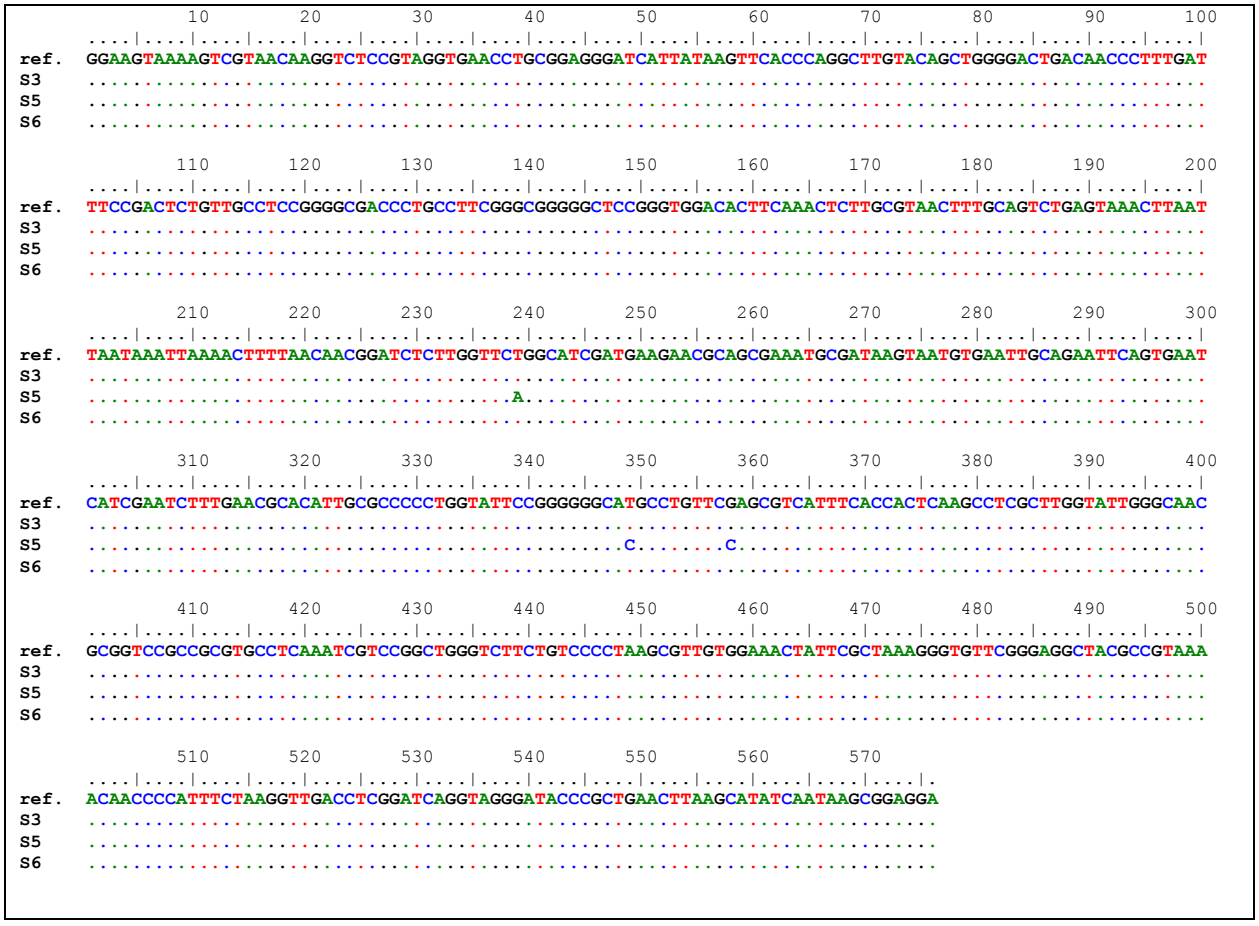
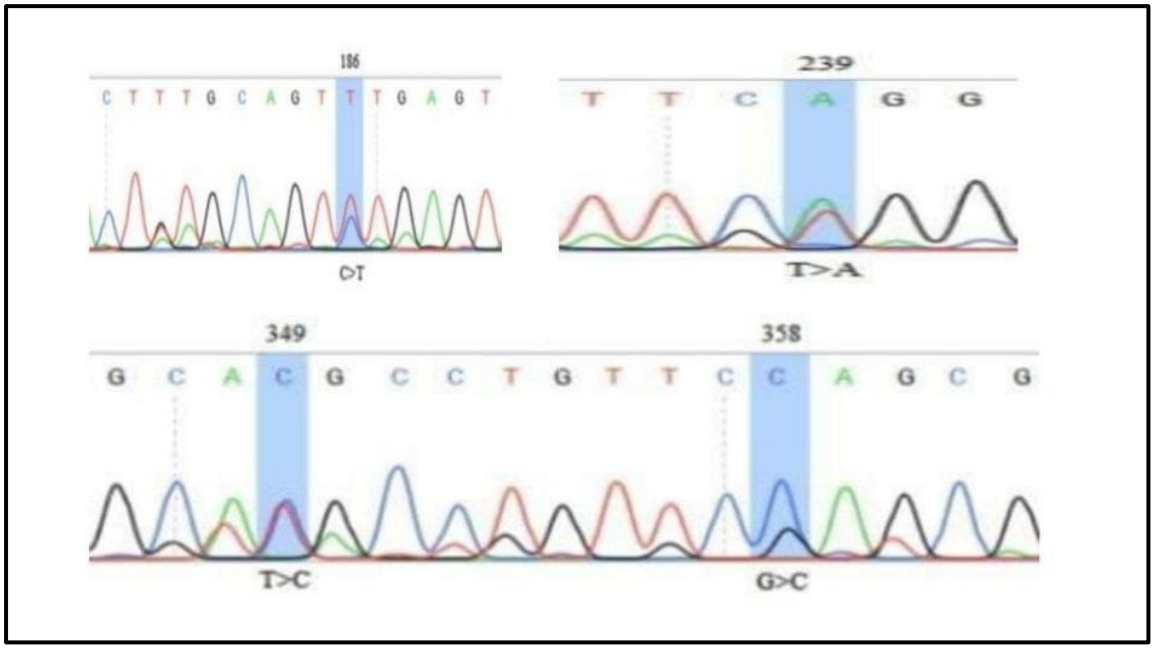


Figure (4-11): DNA sequences alignment of three varieties of *Cladosporium limoniforme* with its corresponding reference sequences of the 576 bp amplicons of the internal transcribed region of the ITS1-ITS2rRNAgenetic DNA sequences. The symbol "ref " refers to the NCBI referring sequence, "S3, S5, S6" refer to the samples no.3,5,6.

Four nucleic acid substitutions (NCBI) was observed in all of the investigated specimens, were C>T 186, T>A 239, T>C 349, and G>C 358) in S5 The sequencing chromatogram of the identified variation region, as well as their detailed annotations, were documented, and the chromatogram this sequence was shown according to its position in the PCR amplicons (Figure 4-12).



Figure(4-12): The pattern of the detected four (NCBI) within the DNA chromatogram of the targeted 576 bp amplicons of the ITS4-ITS5 rRNA gene. The identified NCBI were highlighted according to its position in the PCR amplicons. The symbol ”>”‖ refers to”substitution”‖ mutation.

4.GROUP-4; Sequencing of ribosomal amplicons of one (S4) *Aspergillus flavus*

Within this locus, one samples was detected in this study. These samples was screened to amplify internal transcribe regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons .Concerning the supposed 585 bp amplicons, the NCBI

BLASTn engine shown about high 99% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. MW522554.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure4-13).

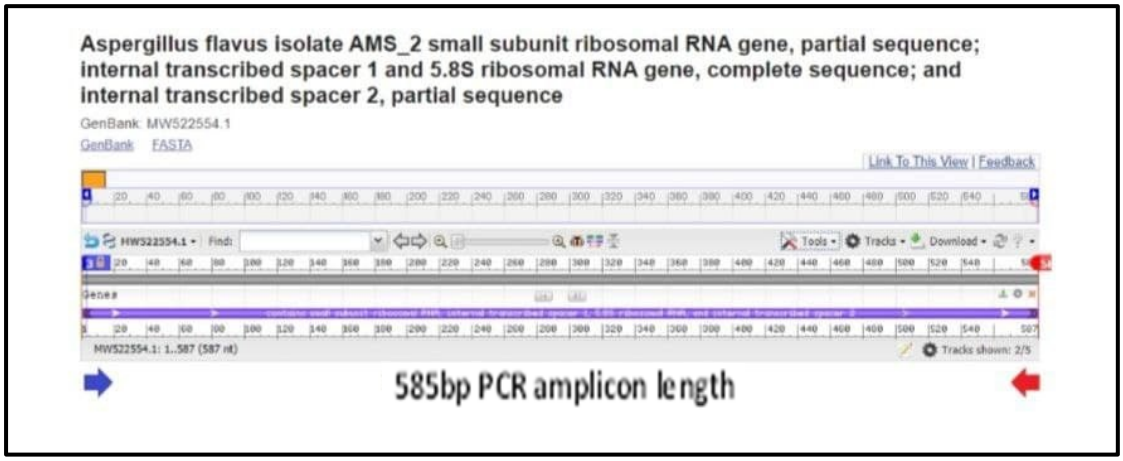


Figure (4-13): The exact position of the retrieved585pb amplicon that partilly covered a portion of the rRNA gene within *Aspergillus flavus* (GenBank acc. MW522554.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint. The letter "S" refers to sample numbers.

After positioning the 585 bp amplicons' sequences within the *Aspergillus flavus* the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 585 bp amplified amplicons (Table 4-7).

Amplicon	Reference locus sequences (5′ - 3′	length
DNA sequences within the internal transcribed region of the rRNA gene	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCG GAAGGATCATTACCGAGTGTAGGGTTCCTAGCGAGCCCAACC TCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCCC GCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGC CCGCCGGAGACACCACGAACTCTGTCTGATCTAGTGAAGTCT GAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGA TCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGA TAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTT TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCT GTCCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGTT GGGTCGTCGTCCCCTCTCCGGGGGGGGACGGGCCCCAAAGGCA GCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGT CACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAAT CAATCTTTTCCAGGTTGACCTCGATCAGTACGGATTCCT	585 bp

Table (4-7): The positions and length of the585bp PCR amplicon used to amplify a portion of the internal transcribe spacer regions rRNA gene within *Aspergillus flavus* (GenBank acc. MW522554.1), the highlighted sequences refer to the positions of the forward and reverse primers respectively.

The alignment results of the 585bp samples revealed the presence of one mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-15).

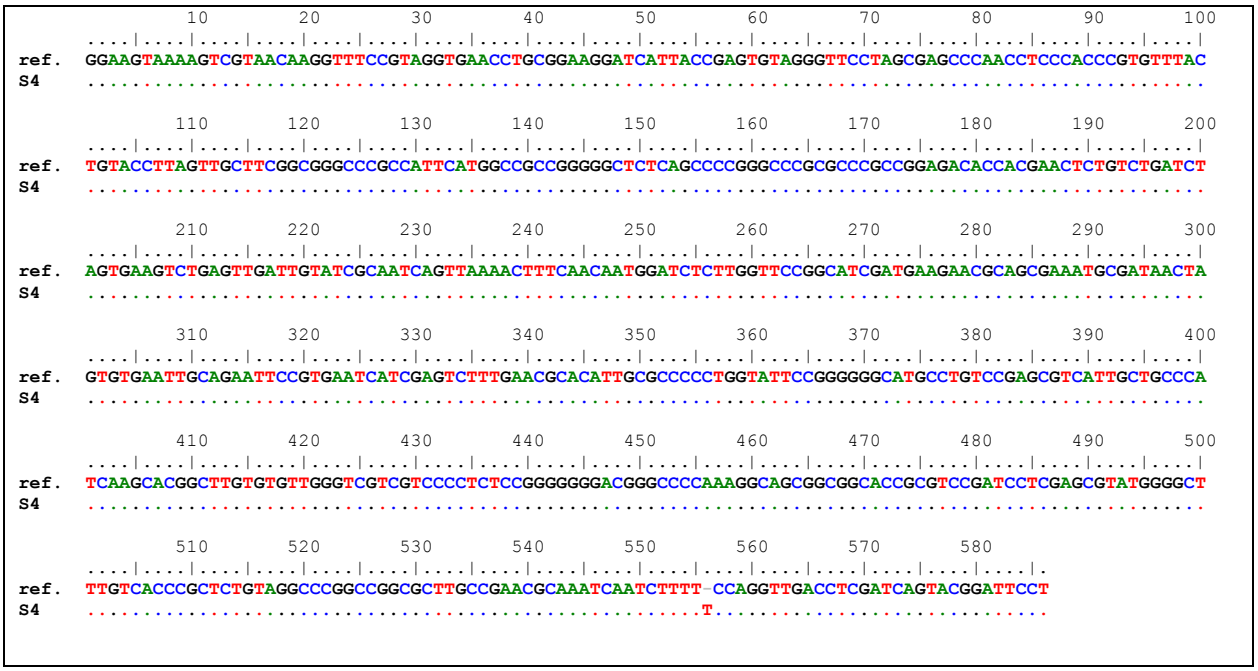


Figure (4-14): DNA sequences alignment of one varieties of *Aspergillus flavus* with its corresponding reference sequences of the 609 bp amplicons of the internal transcribed region of the ITS4-ITS5rRNAgenetic DNA sequences. The symbol "ref " refers to the NCBI referring sequence, "S4" refer to the samples no.4.

Only one nucleic acid substitutions (NCBI) was observed in all of the investigated specimens, were T 555-556 ins, in S4 The sequencing chromatogram of the identified variation region, as well as their detailed annotations, were documented, and the chromatogram this sequence was shown according to its position in the PCR amplicons (Figure 4-15).

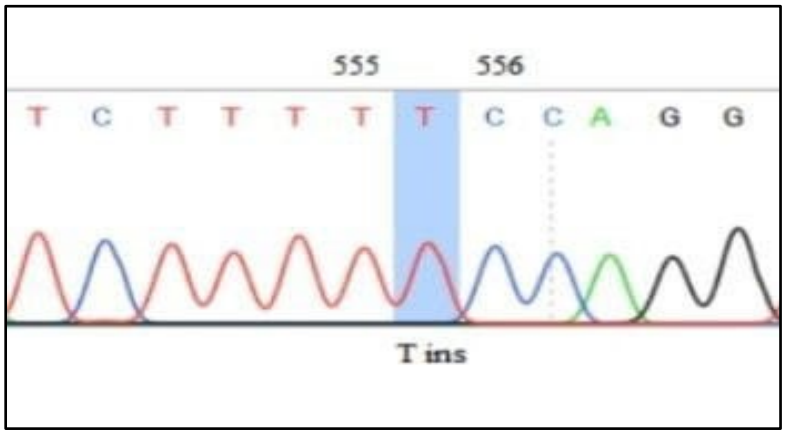


Figure (4-15): The pattern of the detected one insertion mutation in the DNA chromatogram of the targeted 585 bp amplicons of the ITS4-ITS5 rRNA gene. The identified SNP were highlighted according to its position in the PCR amplicons. The symbol "ins"‖ refers to"insertion"‖ mutation.

5.GROUP-5; Sequencing of ribosomal amplicons of one (S8) *Curvularia hawaiiensis*

Within this locus, one samples was detected in this study. These samples was screened to amplify internal transcribe regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these

PCR amplicons. Concerning the supposed 556 bp amplicons, the NCBI BLASTn engine shown about high 100% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. MT065820.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure 4-16).

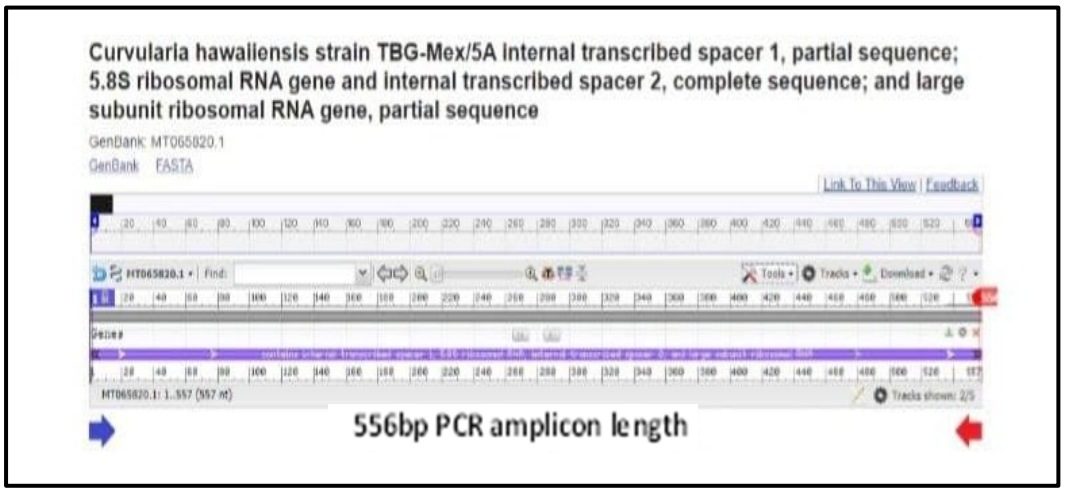


Figure (4-16): The exact position of the retrieved556pb amplicon that partilly covered a portion of the rRNA gene within *Curvularia hawaiiensis* (GenBank acc. MT065820.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint. The letter "S" refers to sample numbers.

After positioning the 556bp amplicons' sequences within the the details of its sequences we *Trichophyton mentagrophytesre* highlighted, in terms of the positioning of both forward and reverse primers of the 556 bp amplified amplicons (Table 4-8).

Amplicon	Reference locus sequences (5′ - 3′	length
DNA sequences within the internal transcribed region of the rRNA gene	TGCGGAGGGATCATTACACAATAAAATACGAAGGCCGTTCGCGGCTG GACTATTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCG GGTTCGCTCGCCACCAGGACCACAATATAAACCTTTTTTATGCAGTT GCAATCAGCGTCAGTATAACAAATGTAAATCATTTACAACTTTCAAC AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG ATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA CGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCG TCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCCCC CCCAAAAAGGGGACTCGCCTTAAAAGGATTGGCAGCCGGCCTACTGG TTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGAC GGCAATCCATCAAGACTCCTTCTCACGTTTGACCTCGGATCAGGTAG GGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA	556 bp

Table (4-8): The positions and length of the556 pb PCR amplicon used to amplify a portion of the internal transcribe regions rRNA gene within *Curvularia hawaiiensis* (GenBank acc. MT065820.1). the highlighted sequences refer to the positions of the forward and reverse primers respectively.

The alignment results of the 556bp samples revealed the Absence of any mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-17)

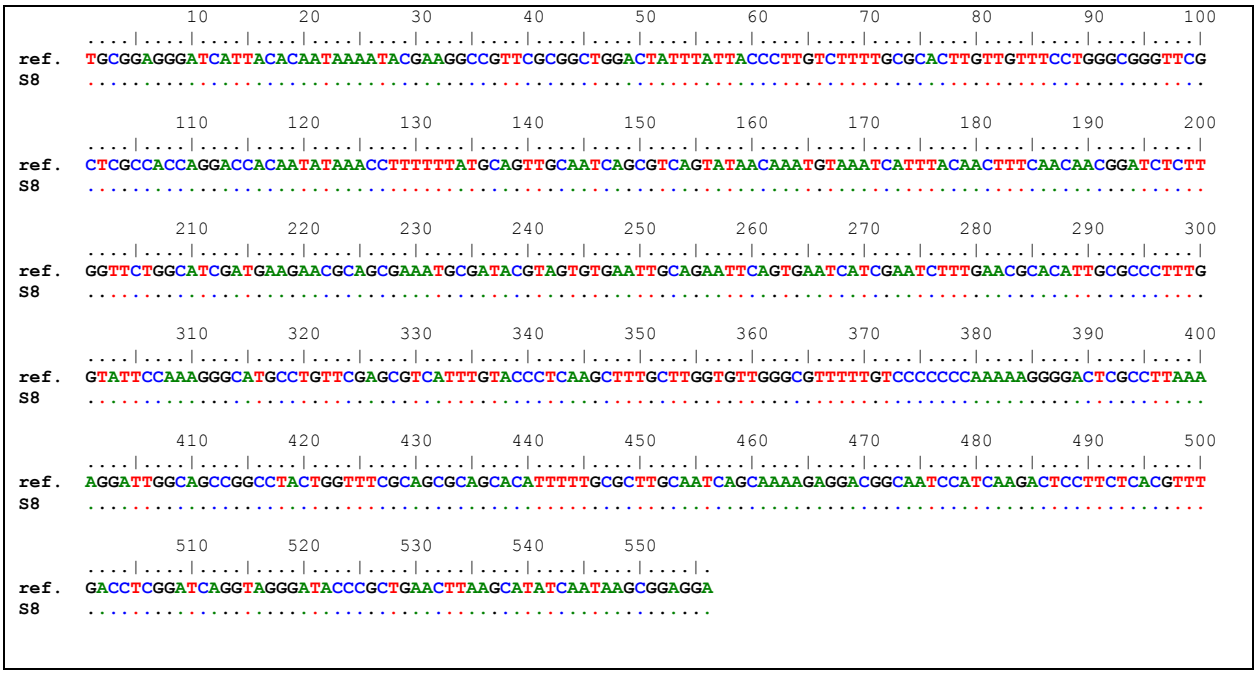


Figure (4-17): DNA sequences alignment of Curvularia hawaiiensis with its corresponding reference sequences of the 556 bp amplicons of the internal transcribed region of the ITS4-ITS5rRNAgenetic DNAsequences. The symbol "ref" refers to the NCBI referring sequence, "S8," refer to the samples no. 8.

4.3.Phylogenetic Results:

A comprehensive phylogenetic tree was generated, which was based on the investigated rRNA nucleic acid sequences in the analyzed fungal samples. Along with the other deposited DNA sequences, this phylogenetic tree contained the currently investigated samples (S1 to S9) aligned with its highly related sequences in a neighbour-joining mode. In the currently constructed tree, the total number of aligned nucleic acid sequences was 106 sequences. This comprehensive tree entailed the presence of only five organisms, which represents the only incorporated nucleic acid sequences within the tree. These sequences were *Trichophyton mentagrophytes*, *Cladosporium limoniforme, Curvularia hawaiiensis, Aspergillus flavus, and Penicillium chrysogenum*. Based on the analyzed genetic sequences, our rRNA sequences were clustered into five major adjacent phylogenetic clades, which entailed a particular range of diversity of these fungal sequences in terms of our analyzed rRNA sequences Figure 4-18).

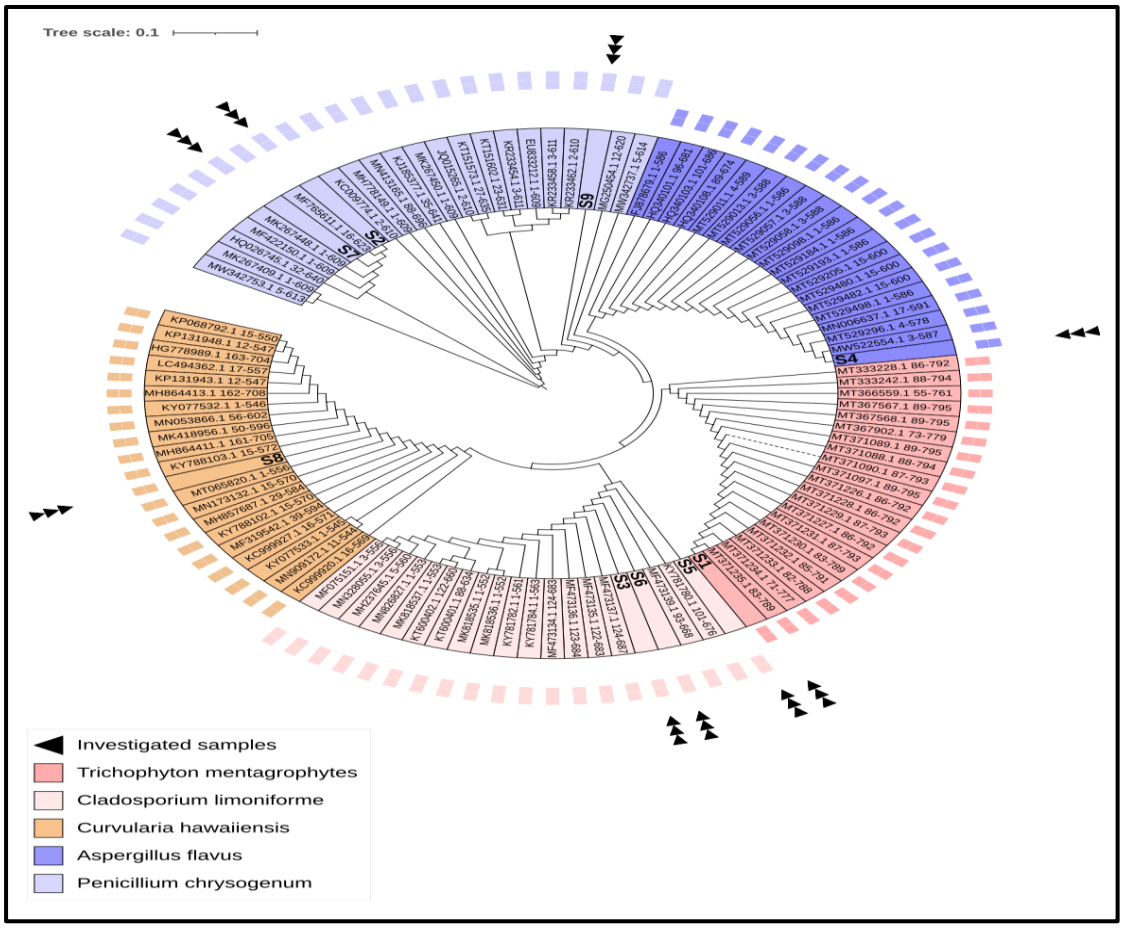


Figure (4-18): The comprehensive phylogenetic tree of the five amplified fragments that partially covered the rRNA sequences within five fungal genomic sequences. The variable colors refer to the variable grouping of the analyzed variants, within their Genbank deposited sequences. The number "0. 1" at the top left portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The described numbers in the tree refer to the degree of phylogenetic distances among the investigated fungal organisms. The letter "S" refers to the code of the investigated samples in this study.

One of these adjacent clades was represented by *Trichophyton mentagrophytes* clade, in which the currently investigated S1 sample was incorporated. However, this sample was positioned in the vicinity of the GenBank accession number of MT371235.1, which was belonged to an

Iraqi strain of the same species. S3, S5, and S6 samples were incorporated within the *Cladosporium limoniforme* clade. In this clade, S3 and S6 were situated beside the GenBank accession numbers of MF473139.1 and MF473137.1, which were belonged to Australian and American isolates of the same organism. Meanwhile, S5 was positioned in the vicinity of the GenBank accession numbers of KY781780.1 of *Cladosporium limoniforme* that belonged to an isolate from Qatar. However, this slight tilt in the phylogenetic positioning of S5 was attributed to the genetic variations (C>T 186, T>A 239, T>C 349, and G>C 358) observed in this investigated sample. S2, S7, and S9 samples were incorporated within the *Penicillium chrysogenum* clade. In this clade, S2 and S7 were situated beside the GenBank accession numbers of MF765611.1 and MK267448.1, which were belonged to Chinese and American isolates of the same organism. Meanwhile, S9 has positioned within the same *Cladosporium limoniforme* clade in the vicinity to the GenBank accession number of MG250454.1 that belonged to an African isolate from Namibia. However, this remarkable deviation in the phylogenetic positioning of S9 was attributed to the genetic variations (C>T 84, G>T 109, and C>A 535) observed in this investigated sample. For this observation, it is rational to consider a remarkable role for the S9 variations in inducing this considerable phylogenetic deviation. S4 was positioned within the *Aspergillus flavus* clade. Within this clade, S4 resided in the vicinity of the GenBank accession number MW522554.1, which was belonged to an Egyptian sample of the same organism. However, this sample was also positioned in the vicinity of the GenBank accession number of MT529296.1, which was belonged to a strain isolated from China. S8 was positioned within the *Curvularia hawaiiensis* clade. Within this clade, S8 resided in the vicinity of the GenBank accession number of MT065820.1, which was belonged to a strain isolated from Mexico of the same organism. However, this sample was also positioned in

the vicinity of the GenBank accession number of KY788103.1, which was belonged to a strain isolated from Iran.

DNA Sequencing of PCR amplicons

2-Yeast samples

1.GROUP1-; Sequencing of ribosomal amplicons of two (S10,S11) *Candida glabrata*

Within this locus, two samples was detected in this study. These samples was screened to amplify internal transcribe spacer regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons. Concerning the supposed 878 bp amplicons, the NCBI BLASTn engine shown about high 99% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. KP675348.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure 4-19).

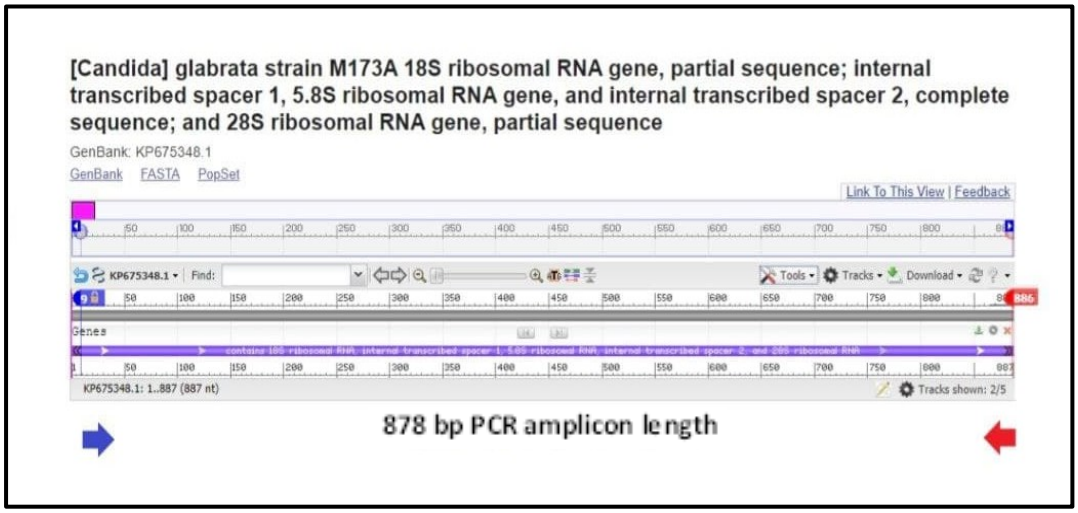


Figure (4-19): The exact position of the retrieved878pb amplicon that partially covered a portion of the rRNA gene within *Candida glabrata* (GenBank acc. KP675348.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint. The letter "S" refers to sample numbers.

After positioning the 878bp amplicons' sequences within the the details of its sequences we *Candida glabrata* highlighted, in terms of the positioning of both forward and reverse primers of the 878 bp amplified amplicons (Table 4-9).

Amplicon	Reference locus sequences (5′ - 3′	length
DNA sequences within the internal transcribed region of the rRNA gene	AGGGGTGACCTGCGGAAGGATCATTAAAGAAATTTAATTG ATTTGTCTGAGCTCGGAGAGAGACATCTCTGGGGAGGACC AGTGTAGACACTCAGGAGGTTCCTAAAATATTTTCTCTGC TGTGAATGCCATTTCTCCTGCCTGCGCTTAAGTGCGCGGT TGGTGGGTGTTCTGCAGTGGGGGGAGGGAGCCGACAAAGA CCTGGGAGTGTGCGTGGATCTCTCTATTCCAAAGGAGGTG TTTTATCACACGACTCGACACTTTCTAATTACTACACACA GTGGAGTTTACTTTACTACTATTCTTTTGTTCGTTGGGGG AACGCTCTCTTTCGGGGGGGGAGTTCTCCCAGTGGATGCA AACACAAACAAATATTTTTTTAAACTAATTCAGTCAACAC AAGATTTCTTTTAGTAGAAAACAACTTCAAAACTTTCAAC AATGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCG AAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCG GGGGGCATGCCTGTTTGAGCGTCATTTCCTTCTCAAACAC GTTGTGTTTGGTAGTGAGTGATACTCTCGTTTTTGAGTTA ACTTGAAATTGTAGGCCATATCAGTATGTGGGACACGAGC GCAAGCTTCTCTATTAATCTGCTGCTCGTTTGCGCGAGCG GCGGGGGTTAATACTGTATTAGGTTTTACCAACTCGGTGT TGATCTAGGGAGGGATAAGTGAGTGTTTTGTGCGTGCTGG GCAGACAGACGTCTTTAAGTTTGACCTCAAATCAGGTAGG GTTACCCGCTGAACTTAAGCATATCATGAGCGGGGGGA	878 bp

Table 4-9: The positions and length of the878pb PCR amplicon used to amplify a portion of the internal transcribe regions rRNA gene within *Candida glabrata* (GenBank acc. KP675348.1), the highlighted sequences refer to the positions of the forward and reverse primers respectively.

The alignment results of the 878bp samples revealed the percent of two mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-20).

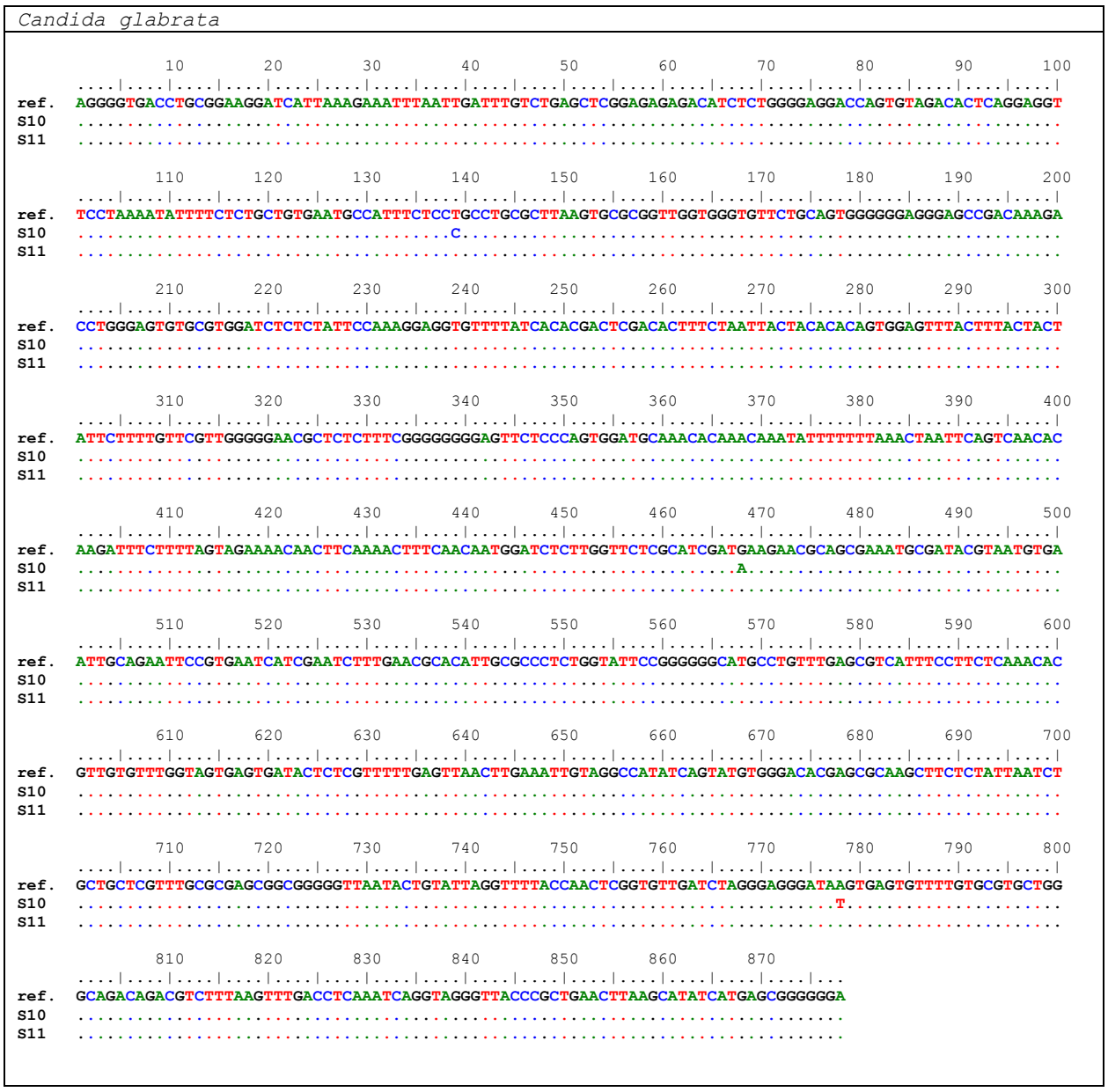
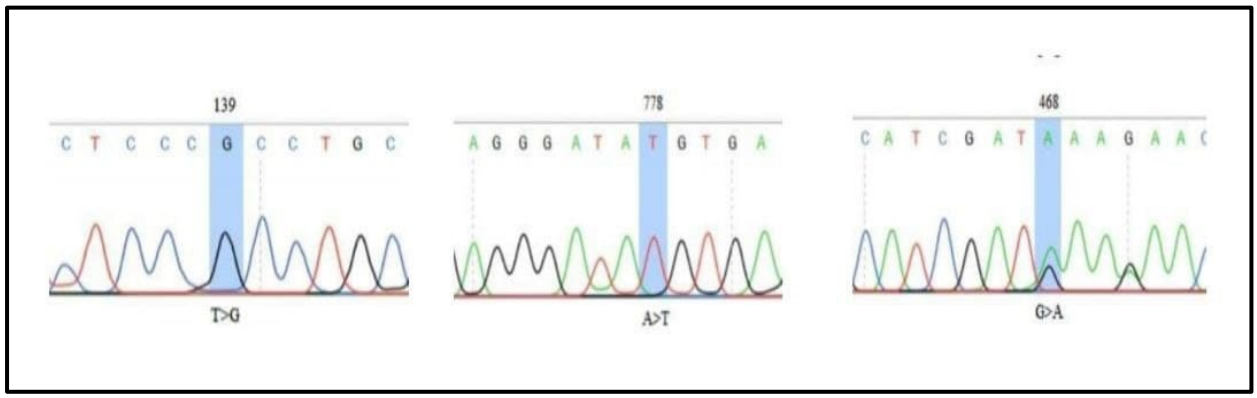


Figure (4-20): DNA sequences alignment of with *Candida glabrata* its corresponding reference sequences of the 878 bp amplicons of the internal transcribed region of the ITS4-ITS5rRNAgenetic DNA sequences. The

symbol “ref” refers to the NCBI referring sequence, “S10, S11,” refer to the samples no. 10,11.

Three nucleic acid substitutions (NCBI) was observed in all of the investigated specimens, were T139.G,G468A,A778T,in S10,S11 The sequencing chromatogram of the identified variation region, as well as their detailed annotations, were documented, and the chromatogram this sequence was shown according to its position in the PCR amplicons (Figure 4-21).



Fig(4-21): The pattern of the detected three mutation the DNA chromatogram of the targeted 878bp amplicons of the ITS4-ITS5 rRNA gene. The identified SNP were highlighted according to its position in the PCR amplicons. The symbol ”>”‖ refers to”substitution”‖ mutation.

2.GROUP2-; Sequencing of ribosomal amplicons of two (S12,S13) *Candida albicans*

Within this locus, two samples was detected in this study. These samples was screened to amplify internal transcribe regions, or ITS4-ITS5, rRNA genetic sequences in the targeted fungal organism. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicon . Concerning the supposed 561 bp amplicons, the NCBI BLASTn engine shown about high 100% sequences similarities between

the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. KJ651874.1), the approximate positions and other details of the retrieved PCR fragments were identified (Figure 4-22)

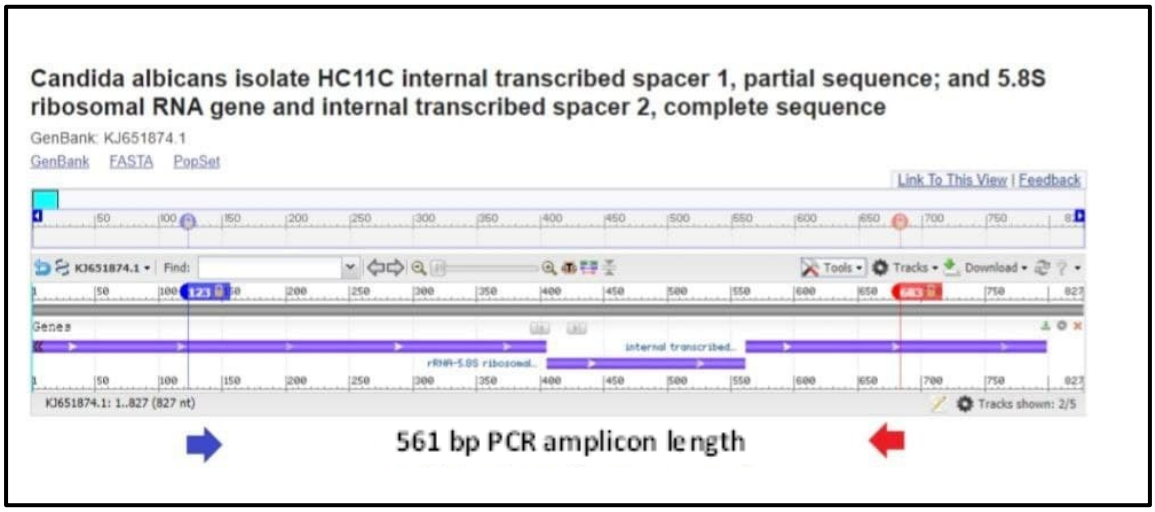


Figure (4-22): The exact position of the retrieved561pb amplicon that partilly covered a portion of the rRNA gene within *Candida albicans* (GenBank acc. KJ651874.1), The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint. The letter "S" refers to sample numbers

After positioning the 561 bp amplicons' sequences within the the details of its sequences we *Candida albicans* highlighted, in terms of the positioning of both forward and reverse primers of the 561 bp amplified amplicons (Table 4-10).

Amplicon	**Reference locus sequences (5′ - 3′**	**length**
DNA sequences within the internal transcribed region of the	GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTG CGGAAGGATCATTACTGATTTGCTTAATTGCACCACATGT GTTTTTCTTTGAAACAAACTTGCTTTGGCGGTGGGCCCAG CCTGCCGCCAGAGGTCTAAACTTACAACCAATTTTTTATT AACTTGTCACACCAGATTATTACTAAATAGTCAAAACTTT CAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTG	561 bp

rRNA gene	AATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTAT TCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCCCTCAA ACCGCTGGGTTTGGTGTTGAGCAATACGACTTGGGTTTGC TTGAAAGACGGTAGTGGTAAGGCGGGATCGCTTTGACAAT GGCTTAGGTCTAACCAAAAACATTGCTTGCGGCGGTAACG TCCACCACGTATATCTTCAAACTTTGACCTCAAATCAGGT AGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGG A	

Table (4-10): The positions and length of the561pb PCR amplicon used to amplify a portion of the internal transcribe spacer regions rRNA gene within *Candida albicans* (GenBank acc. KJ651874.1), the highlighted sequences refer to the positions of the forward and reverse primers respectively

The alignment results of the 561bp samples revealed the absent of any mutation in the analyzed sample in comparison with the referring reference DNA sequences (Figure 4-23).

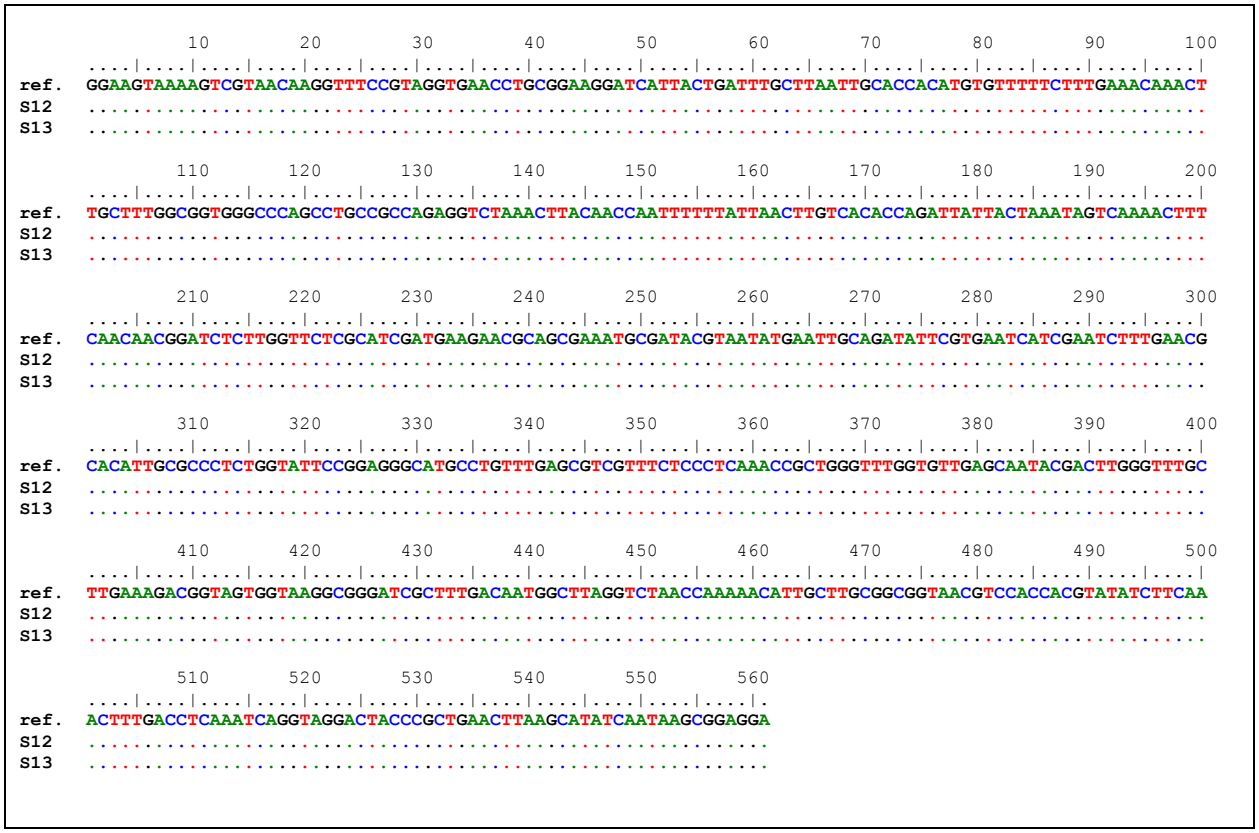


Figure (4-23): DNA sequences alignment of with *Candida albicans* its corresponding reference sequences of the 561bp amplicons of the internal transcribed region of the ITS4-ITS5rRNAgenetic DNA sequences. The symbol "ref" refers to the NCBI referring sequence, "S12, S13," refer to the samples no. 12,13.

4.4.Phylogenetic Results:

As in the case of fungal samples, another comprehensive phylogenetic tree was generated. The generated cladogram was based on the investigated internal transcribed spacer 4 and 5 (ITS4 and ITS5) sequences in the analyzed yeast samples. Along with the other deposited DNA sequences, this phylogenetic tree contained the currently investigated samples (S10 to S13) aligned with its highly related yeast sequences in a neighbour-joining mode. In the currently constructed tree, the total number of the incorporated nucleic acid sequences was 104. This comprehensive cladogram entailed the presence of only two yeast organisms, *Candida glabrata,* and *Candida albicans*, which represented the only incorporated nucleic acid sequences within the tree. Based on the analyzed genetic sequences of both *Candida glabrata* and *Candida albicans*, our analyzed rRNA sequences were clustered into two main phylogenetic clades, the *Candida glabrata*, and the *Candida albicans* clades. In both main clades, several diverse minor clades were observed for both identified organisms in our analyzed rRNA sequences (Figure 4-25) However, both samples of S10 and S11 were incorporated in the *Candida glabrata* clade. Concerning S10, a slight tilt from the positioning of S11 was observed. this observed minor deviation in the phylogenetic positioning of S10 was attributed to the observed genetic variations (T>G 139, G>A 468, and A>T 778) identified in this sample. However, this tilt was only a minor deviation within the

same observed phylogenetic tree as no remarkable genetic difference was revealed from these variations.

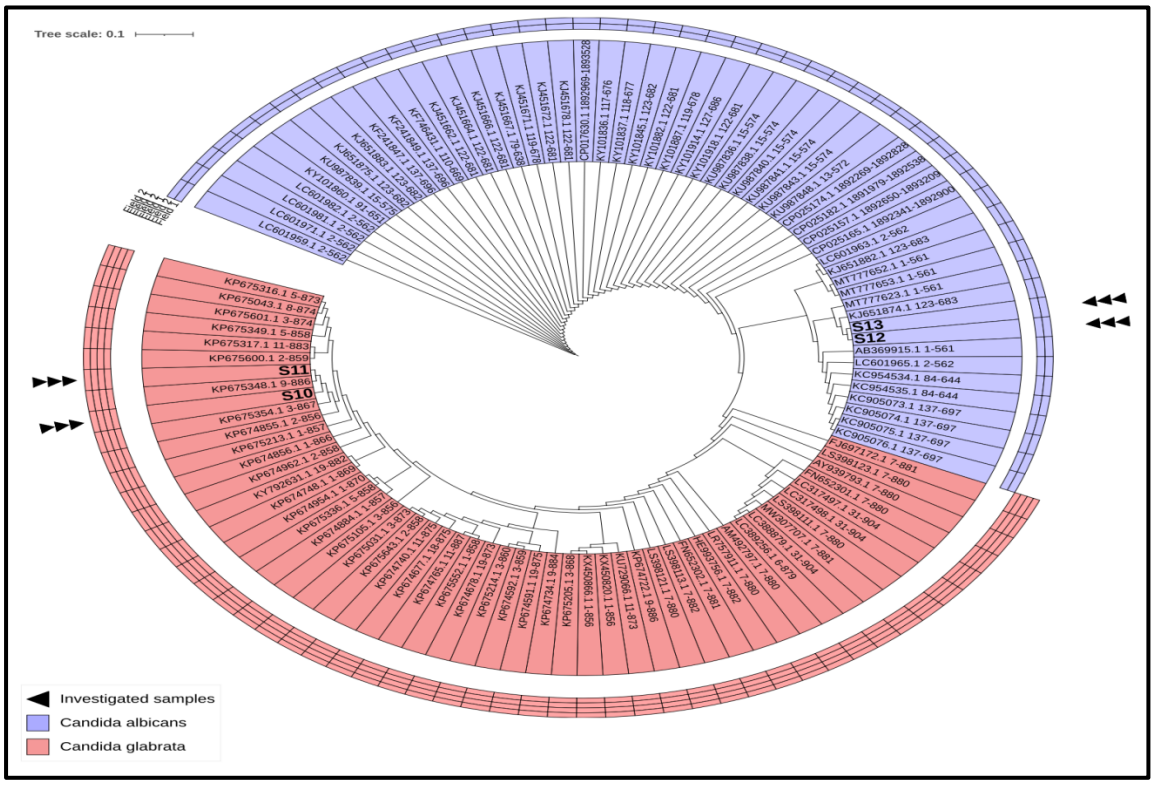


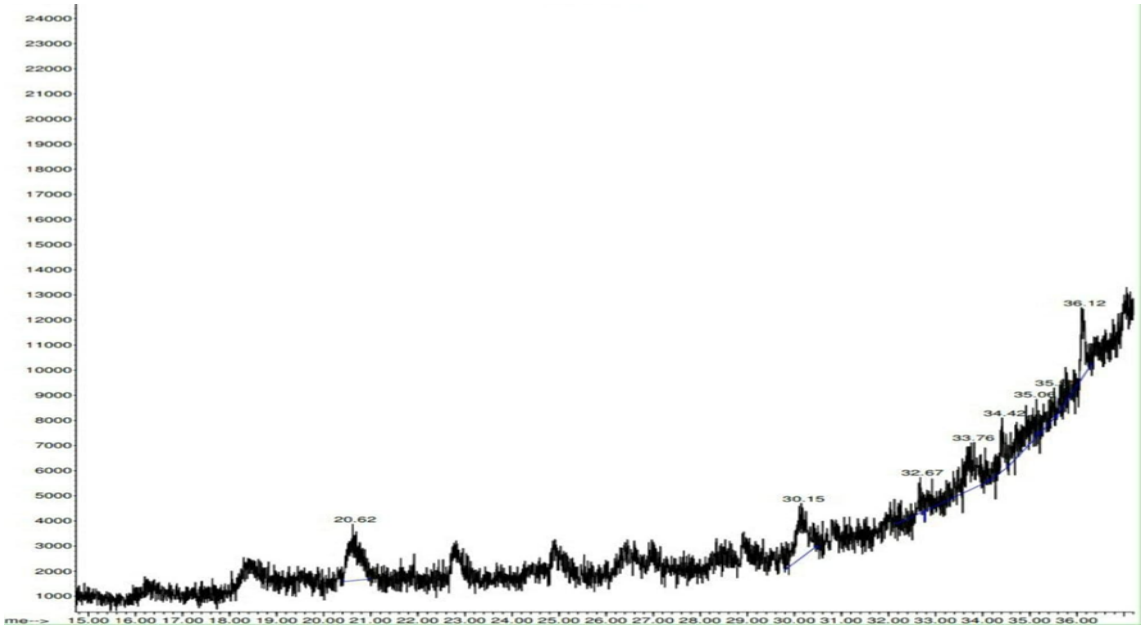
Figure (4-24): The comprehensive phylogenetic tree of the yeast amplicons that partially covered the rRNA sequences within two types of yeast (*Candida glabrata and Candida albicans*) genomic sequences. The variable colors refer to the variable grouping of the analyzed variants, within their Genbank deposited sequences. The number "0. 1" at the top left portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The described numbers in the tree refer to the degree of phylogenetic distances among the investigated fungal organisms. The letter "S" refers to the code of the investigated samples in this study.

This data suggested a non-remarkable role for these variations in inducing a noticeable tilt in the phylogenetic positioning of S10 concerning S11 in the *Candida glabrata* clade. Noteworthy, both S10 and S11 resided in the vicinity of the GenBank accession numbers KP675354.1 and

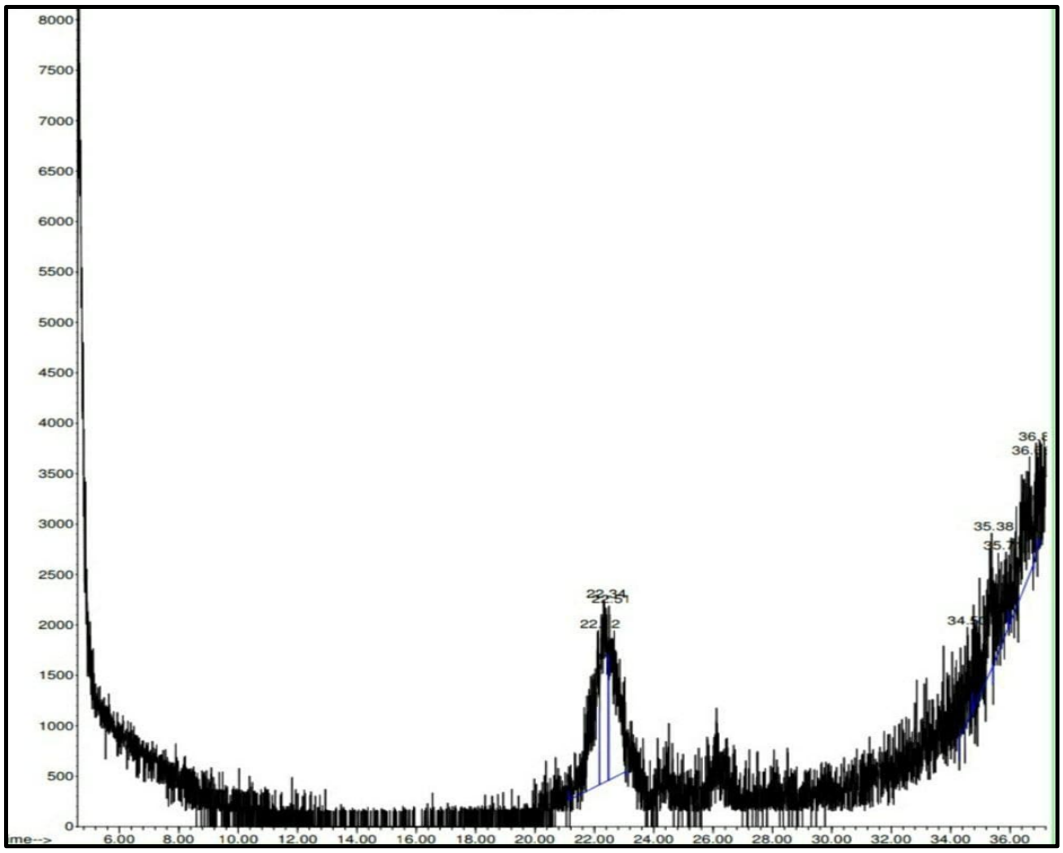
KP675348.1, which were belonged to two different strains of *Candida glabrata* isolated from China. It was deserved to note that the *Candida glabrata* clade was exhibited a noticeable phylogenetic diversity due to the incorporation of many minor clades of the same organism with variable genetic distances in the same major clade of the same organism. In addition to the observed high biological diversity of *Candida glabrata*, another biological diversity of *Candida albicans* was also revealed to a less extent. However, both S12 and S13 were incorporated within this clade. No differences were observed between both S12 and S13 in the main *Candida albicans* positioning since no genetic variations were observed for both investigated samples. however, both samples were suited in the vicinity to the GenBank accession number KJ651874.1, which was belonged to one strain of *Candida albicans* isolated from Brazil. Furthermore, both samples were also positioned beside the GenBank accession number MT777623.1, which was belonged to another strain of *Candida albicans* isolated from Tunisia. Thus, the multinational distribution of both investigated S12 and S13 was also revealed. These sorts of S11 – S13 genetic distribution referred to the sensitivity of the utilized rRNA-based one the ITS4-ITS5 amplicons in the accurate discrimination among the investigated yeast samples. Accordingly, it may be possible to entail the ability to utilize the ITS-based amplicons in the accurate detection of the analyzed samples of fungi and yeast in the constructed ITS-based tree. This rRNA comprehensive tree has presented an extremely inclusive tool about the high ability of such sequences to efficiently differentiate among different samples using the extremely high discriminative power provided by these ribosomal sequences.

4.3 Gas Chromatography – Mass Spectrum Analysis(GC MS)

The results of GC-MS analysis of the Bioactives of plant extract that have pharmacological actions are presented in figure (4-25) and figure(4-26) Bioactives are the chemical compounds often referred to eight bioactive compounds were produced from *Oregnum vulgare* and eight bioactive compounds were produced from propolis were identified in the ethanolic extract**.**



Figure(4-25): GC-MS analysis of the Bioactives of *Oreganum vulgare* extract.



Figure(4-26): GC-MS analysis of the Bioactives of propolis extract.

The identification of bioactive chemical compounds is based on the peak area, retention time, molecular weight, and molecular formula. The GS-MS chromatogram results show more than 8 peaks of *Oreganum vulgarae* and more than 8 peaks for propolis as compounds detected but only 8 detected compounds which have bioactivity , table(4-11) and (4-12).

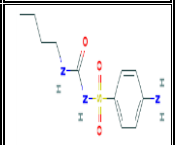
The bioactive chemical compounds produced by *Oreganum vulgare* isolate are Ethidimuron,10-Octadecenoic acid(Z)-,Iron, Tricarbonyl, phenol, bromodime thyle ,pregna, Anthracenediol, Cis-Vaccenic acid, Trifluoroacetic acid, Show different pharmacological properties Antivirals , Antifungal, Antibacterial, Antimicrobial, Anti flammatory, and properties. The different pharmacological properties also found in bioactive chemical compounds propolis isolate such as Carbutamide, Citric acid, Carbonic acid, Octadecanol, Phenol,4-Bromo-2-(1,2-dimethyl),pyridate,

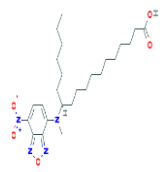
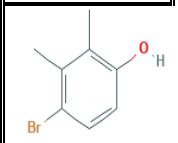
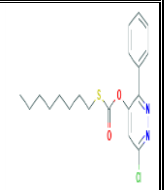
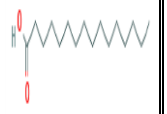
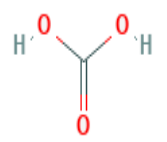
Octadecanoic acid, Iron, Tricarbomnyl, and other chemical commands which show pharmacological properties such as Antibacterial, Antioxidants, Anticancer, Antifungal, Antiflammtry, Insecticide, and other properties.

To compound in propolis , Carbutamide, Octadecanol, were found to be major in this fraction with 18.12% and 16.77% peak area respectively. As explained in table(4-11) and other compounds were shown in the different peak area.

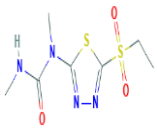
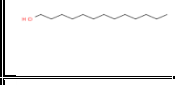
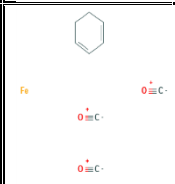
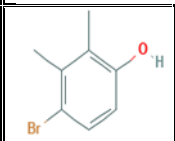
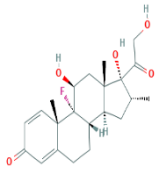
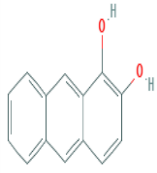
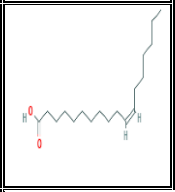
Also, the current study found that two compounds in *Oreganum vulgare* phenol, bromodime ethyl, and 9-octadecenoic acid(Z)- were found to be major in this fraction with 20.51% and 20.36% peak area respectively. many minor constituents were also identified in different fraction areas as explained in the table (4-12).

Table(4-11) The major phytochemical compounds of propoles extract detected by GC-MS analysis that have pharmacological action.

No.	Compounds	RT (min)	Area %	Molecular Weigh (gm/ml)	Formula	Nature of the compound	Bilogacal active	Chemical structure
1	Carbutamide	22.34	١٨.١٢	271.34	$C_{11}H_{17}N_3O_3S$	Phenols	Anti microbal, Anti fungle, Insecticide,	
2	Citric acid	22.51	16.77	192	C_{6H8O7}	Fatty acid	Anti microbal/	

3	Octadecanoic acid	22.11	21.38	284	$C_{18}H_{36}O_2$	Fatty acid	Antibacterial, Anti fungal,	
4	Octadecanal	34.50	5.17	252	$C_{18}H_{38}O$	Fatty alchol	Anti fungal, Antibactral, Anti larva,	
5	Phenol,4-bromo-2-(1,2-dimthyl)-	35.70	11.19	295	$C_{11}H10BrONO2$	Phenols	Anti fungal, Anti tumor, Anti oxdant,	
6	pyridate	35.38	7.36	378	$C_{19}H_{23}CIN_2O_2S$	Phenols	Anti microbile	
7	Iron,Tricarbonyl	36.89	16.36	220	C_9H8feO_3	Phenoles	Anti flammtary Anti fungle, Anti microbile	
8	Carbonic acid	36.89	3.18	62.025	CH_2O_3	Organic citrus	Anti micerbal, Anti flammatry	

Table(4-12) The major phytochemical compounds of *Oreganum vulgare* extract detected by GC-MS analysis that have pharmacological action.

NO.	Compounds	RT (min)	Area %	Molecular Weigh (gm/ml)	Formula	Nature of the compound	Pharmacological actions	Chemical Strcture
1	Ethidimuron	20.62	18.38	264	$C_7H_{12}N_{14}O_3S_2$	Alkaloids	Herbicids Antiviral Antiflmmatray	
2	10-Octadecenoic acid methyl	30.15	7.36	282	$C_{18}H_{34}O_2$	Unsaturated-fatty acid	Antioxdiant Anti cancer	
3	Iron, Tricarbonyl	32.67	4.37	220	$C_9H_8FeO_3$	Phenols	Anti fungal Anti bacteral	
4	Phenol.bromodimethyl	33.76	20.51	201.06	C_8H_9BrO	Phenols	Anti bacteral Anti fungal Anti flammtray	
5	Pregna	34.42	7.36	374	C22H30O5	Steroids	/	
6	Anthracenediol	35.6	12.99	210.23	$C_{14}H_{10}O_2$	Flavonoids	Anti fungal Anti bacteral	
7	Cis-Vaccenic acid	35.51	4.37	282	$C_{18}H_{34}O_2$	Fatty acid	Antivirials, Antipsoria, Antibacteral,	
8	Trifluoroacetic acid	36.12	13.30	114.02	$C_2HF_3O_2$	Organic Citrus	Antiflammtary Antivrial	

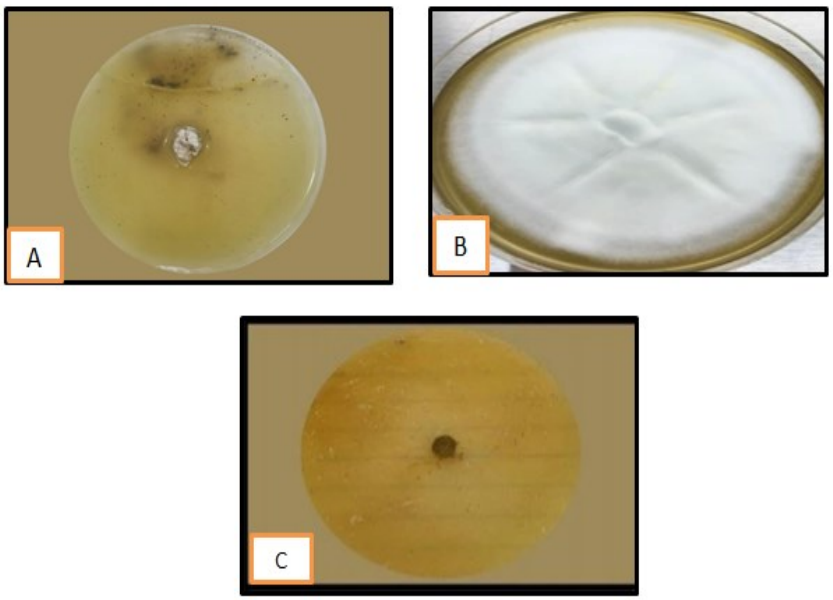
4.3 Antifungal activity of the (*propolis and Oreganum vulagre L*) against Fungal species isolated from various clinical cases

The results of antifungal activity of ethanolic extract of propolis against Fungal species isolated in the study are presented in tables(4-13) *Oreganum vulagre* was screened by food poisoning method.

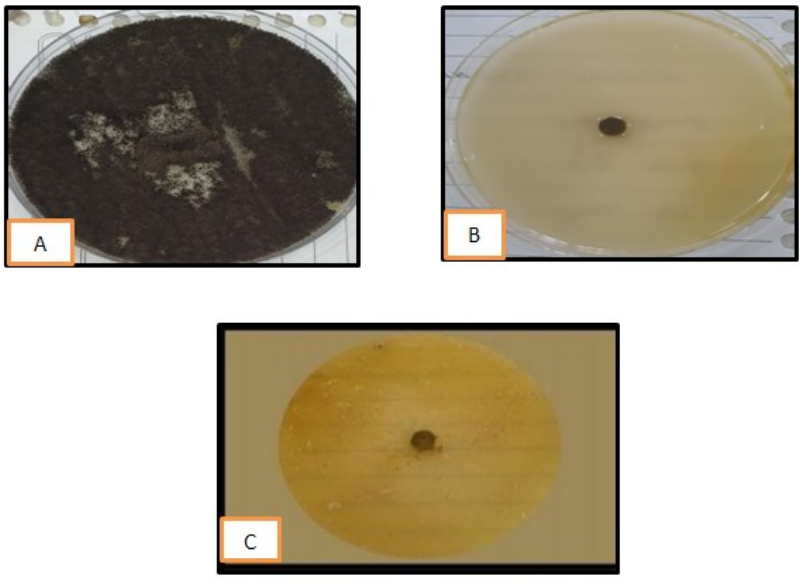
The results revealed that ethanolic extracts of Oregano showed significant reduction at P <0.05 in the growth of fungi isolated antifungal activity was applied at (5,10,and15) mg/ml. Mycelial inhibition ranging *T.mentagrophytes* (58.7%, in 5mg/ml ,81.7% in 10mg/ml, 100% in 15mg/ml)(figur4-27,A) compared with positive control(B) and negative antifungal ketoconazole 5mg/ml control (C) the rate of inhibition was in the fungus *A.niger* (38.5% in 5mg/ml,56.59% in 10mg/ml,100% in 15mg/ml) (figur4-28,A) compared with positive control (B) and negative control (C) the rate of inhibition was in the fungus *A. flvaus* (51.11% in 5mg/ml, 74.29% mg/ml, 100% in 15)(figur4-29,A) compared with positive control (B) and negative control (C) the rate of inhibition was in the fungus *P.chrusogenum* (38.88% in 5 mg/ml ,63.26 in 10 mg/ml, 71.48% in 15 mg/ml) (figur4-30,A) compared with positive control(B) and negative control(C) and the rate of inhibition was in the fungus *Cladosporium limoniforme* (67.70 in 5 mg/ml, 76.57 in 10mg/ml, 92.18 in 15 mg/ml) (figur4-31,A) compared with positive control (B) and negative (C) were inhibition percentage was (0.00% for negative control and 100% for positive control).

Table 4. 13: Antifungal activity of phytochemical compounds alcoholic extracted from *Oreganum vulgare* against fungi isolated

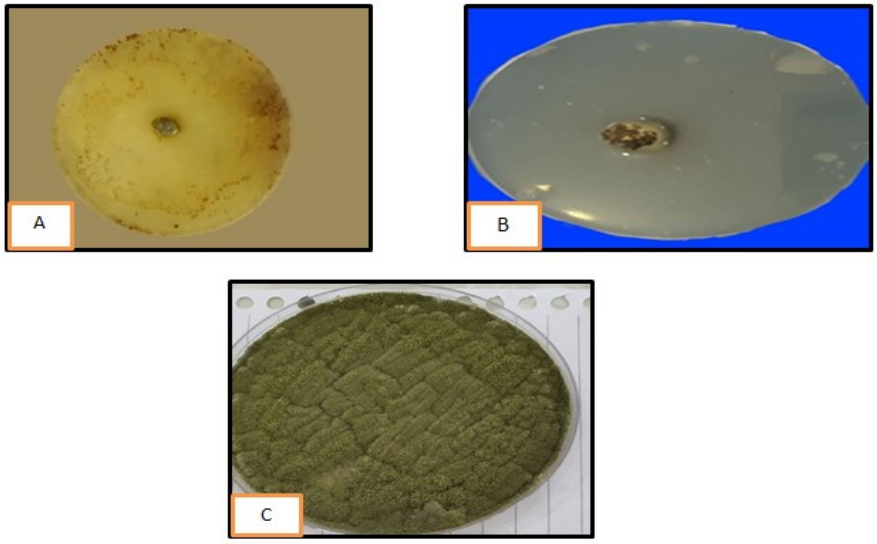
Concentrations Mg/ml	T.mentagrophytes	A.niger	A.flavus	P.chrysogum	C.limoniform
Control(-)	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
5mg/ml	58.7±0.06	38.5±0.64	51.11±0.00	38.8±0.00	67.7±0.06
10mg/ml	81.07±0.06	56.59±0.063	74.29±0.17	63.26±0.06	76.57±0.028
15mg/ml	100±0.00	100±0.00	100±0.00	71.48±0.317	92.18±0.06
ketoconozol +5mg/ml	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
L.S.D	0.32				
*Mean± standard deviation					



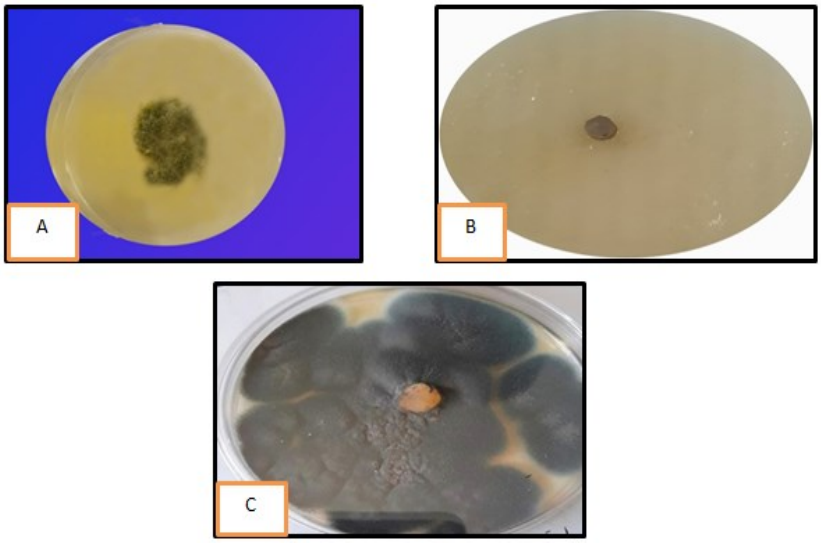
Figure(4-27): Antifungal activity of phytochemical compounds alcoholic extracted from *Oreganum vulgare* against *T.mentagrophyts* A:extract of oregano 15 mg/ml , B: negative control(10% DMSO treatment), c: positive control (ketoconazole 5mg/ml)



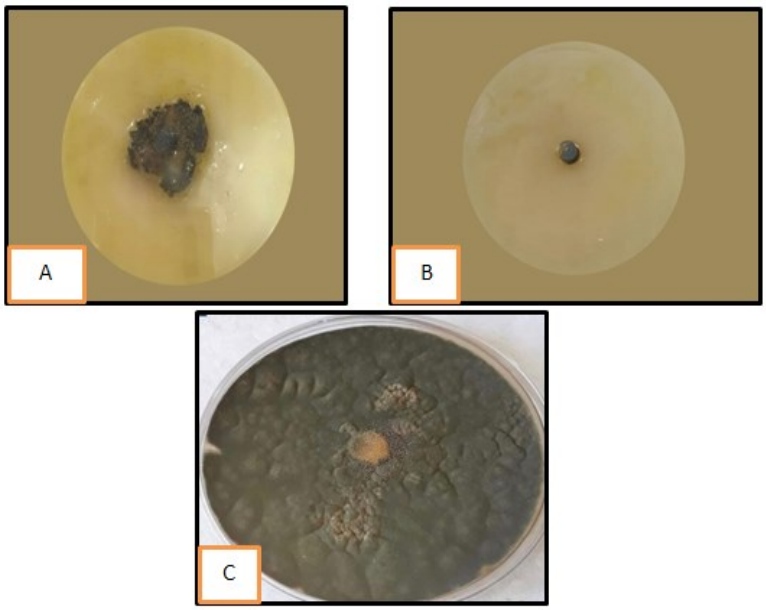
Figure(4-28): Antifungal activity of phytochemical compounds alcoholic extracted from *Oreganum vulgare against A.niger* A: negative control(10% DMSO treatment), B: positive control (ketoconazole 5mg/ml), c: extract of oregano 15 mg/ml



Figure(4-29): Antifungal activity of phytochemical compounds alcoholic extracted from *Oreganum vulgare against A. flavus* A: extract of oregano 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control(10% DMSO treatment)



Figure(4-30): Antifungal activity of phytochemical compounds alcoholic extracted from *Oreganum vulgare against P.chrusogenum* A: extract of oregano 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control(10% DMSO treatment

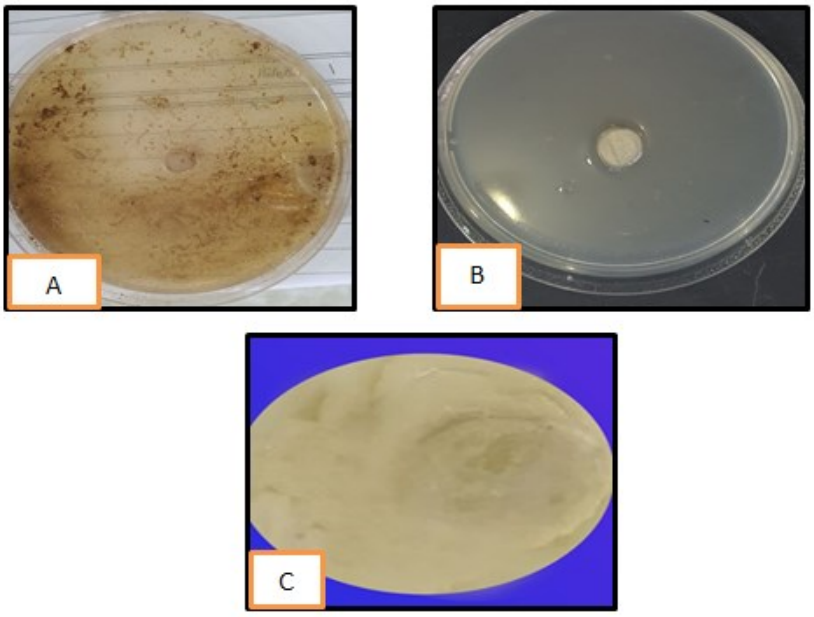


Figure(4-31): Antifungal activity of phytochemical compounds alcoholic extracted from *Oreganum vulgare against C.limoniforme* A: extract of oregano 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control(10% DMSO treatment

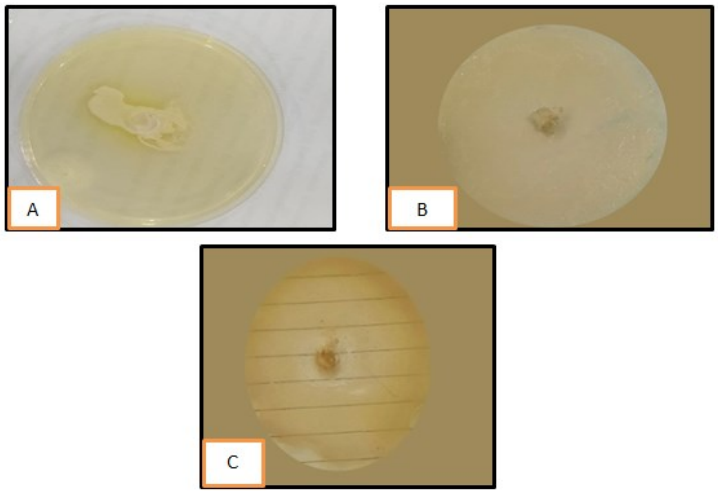
C. albicans and C. glabrata anti-fungal activity were applied (5,10,and15mg/ml) as explained in table (4-14) mycelial inhibition ranging of Eethanol extract *C. albicans* showed (68.14%, in 5mg/ml and 74.81%, in 10mg/ml and 100%,in 15 mg/ml) (figur4-32,A) compared with positive control(B) and negative control antifungal ketoconazole 5mg/ml (C) and the rate of inhibition was in the fungus *C.glabrata* (63.33%,in 5mg/ml72.96,in 10mg/ml and 91.85% in 15mg/ml)(figur4-33) compared with positive control(B) and positive control (C).

Table 4.14: Antifungal activity of phytochemical compounds alcoholic extracted from (*Oreganum vulgare*) against *c.albicans and c.glabrata*

Concentrations Mg/ml	***C.albicans***	***C.glabrata***
Control(-)	0±0.00	0±0.00
5mg/ml	68.14±0.64	63.33±0.00
10mg/ml	74.81±0.64	72.96±0.64
15mg/ml	100±0.00	91.85±1.28
Ketoconozol(+) 5mg/ml	100±0.00	100±0.00
L.S.D	4.487	
*Mean± standard deviation		



Figure(4-32):Antifungal activity of phytochemical compounds alcoholic extracted from *(Oreganum vulgare)* against *C..albicans* A: extract of oregano 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control(10% DMSO treatment

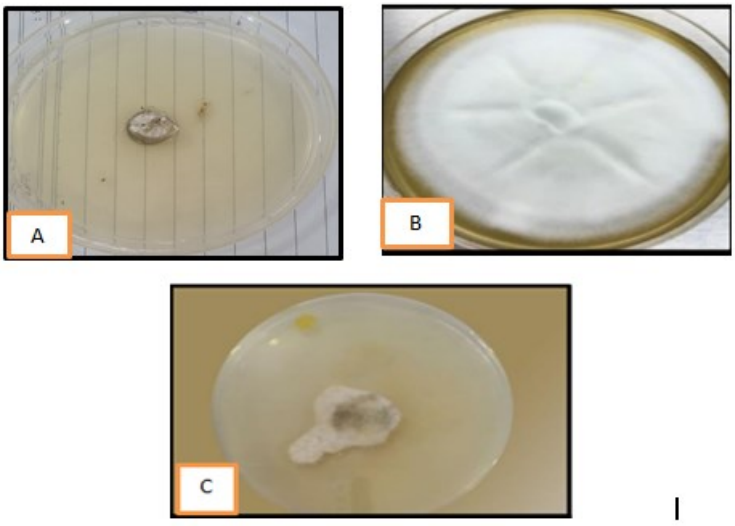


Figure(4-33):Antifungal activity of phytochemical compounds alcoholic extracted from *(Oreganum vulgare)* against *C.glabrata* A: extract of oregano 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control(10% DMSO treatment

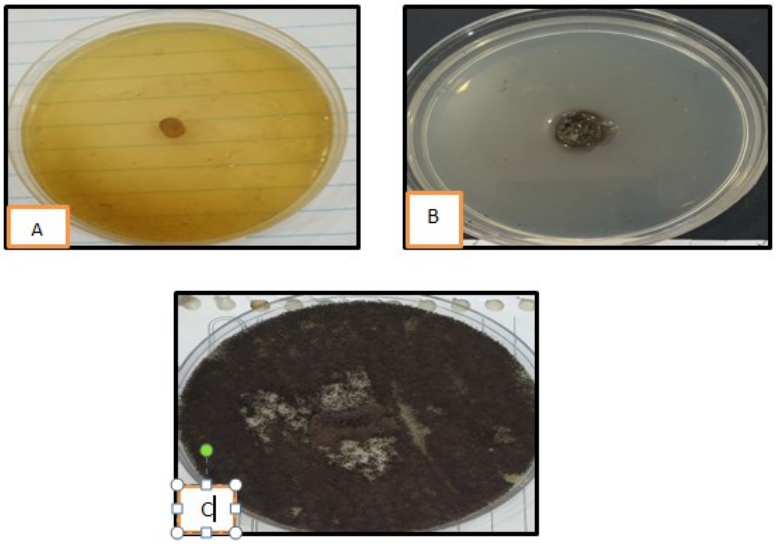
The results revealed that in table (4.15), the extracts of ethanolic of Propolis showed significant reduction at $p < 0.05$ in the growth of Fungi isolated antifungal activity was applied at (5,10,and15) mg/ml. Mycelial inhibition ranging *T.mentagrophytes (*47.4%, in 5mg/ml ,87.7% in 10mg/ml, 72.2% in 15mg/ml)(figur8,A) compared with positive control (B) and negative control ketoconazole 5 mg/ml(figure 9,A) the rate of inhibition was in the fungus *A.niger* (32.2% in 5mg/ml,78.8% in 10mg/ml,100% in 15mg/ml) (B) compared with positive control (B) and nagative control ketoconazole (C) the rate of inhibition was in the fungus *A.flaus* (40.7% in 5mg/ml, 69.6% mg/ml, 96.2% in 15) compared with negative control (B) and compared positive control(C)the rate of inhibition was in the fungus *P.chrusogenum (*67.4% in 5 mg/ml ,87.2 in 10 mg/ml, 100% in 15 mg/ml)(figur10,A) compared with positive control (B)and nagative control(C) the rate of inhibition was in the fungus *Cladosporium limoniforme* (60.7 in 5 mg/ml, 82.9 in 10mg/ml, 92.4 in 15 mg/ml)(figur11,A) compared with positive control (B) and negative control(C) compared were inhibition percentage was (0.00% for negative control and 100% for positive control).

Table 4.15: Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against fungi isolated

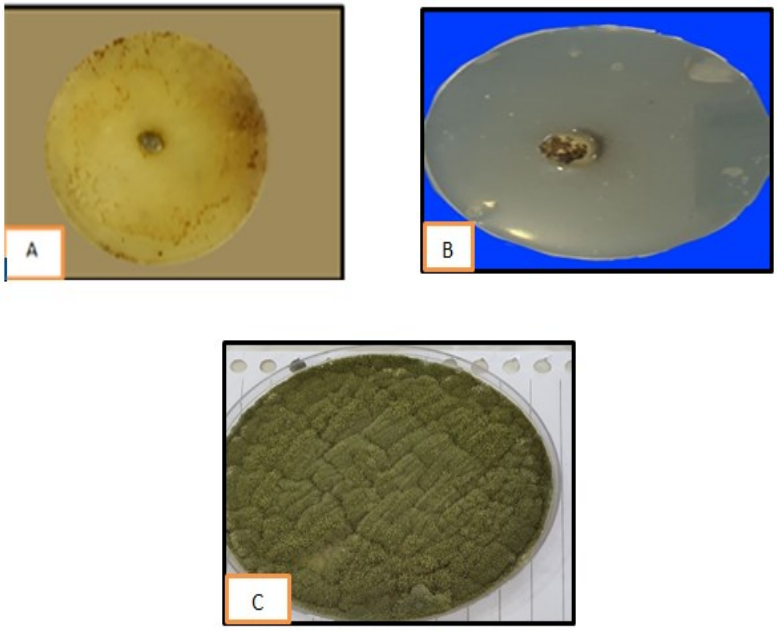
Concentrations Mg/ml	T.mentagrophtes	A.niger	A.flavus	P.chrysogenum	C.limoniforme
Control(-)	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
5mg/ml	47.4±0.64	32.2±1.11	40.7±0.64	67.4±0.71	60.7±0.64
10mg/ml	87.7±1.11	78.8±0.00	69.6±0.64	87.2±0.44	82.9±0.61
15mg/ml	72.2±0.00	100±0.00	96.2±3.56	100±0.00	99.4±0.56
ketoconozol +5mg/ml	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
L.S.D	**1.839**				
*Mean± standard deviation					



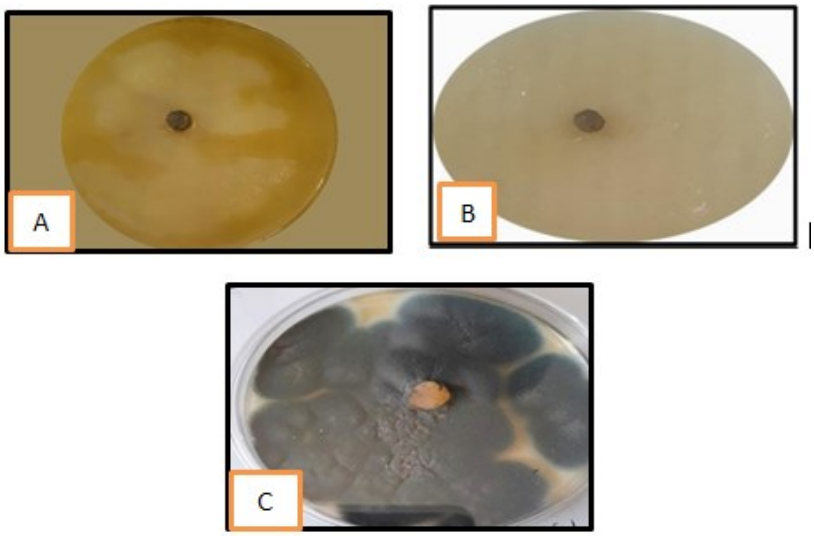
Figure(4-34): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *T.mentagrophytes* A: positive control (ketoconazole 5mg/ml), B: negative control(10% DMSO c: extract of propolis 15 mg/ml



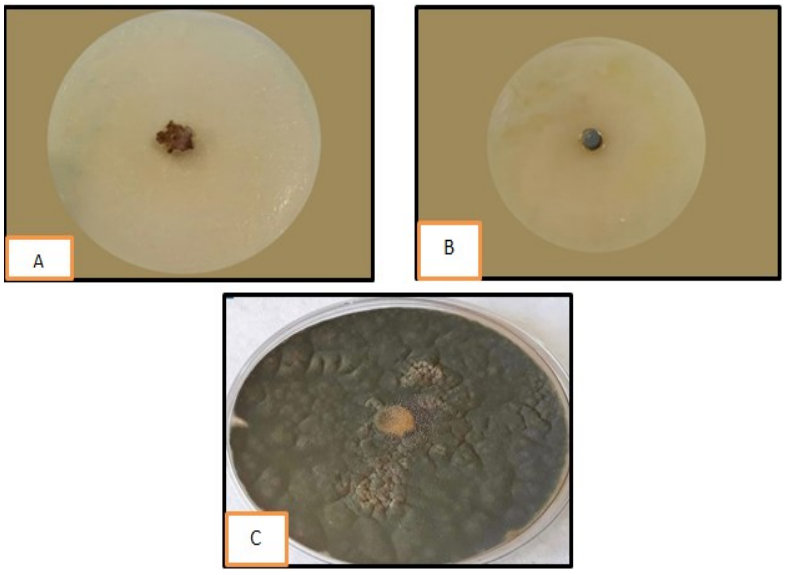
Figure(4-35): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *A. niger* A: extract of propolis 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control 10% DMSO treatment



Figure(4-36): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *A. flagus* A: extract of propolis 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control 10% DMSO treatment



Figure(4-37): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *P.chrysogenum* A: extract of propolis 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control 10% DMSO treatment

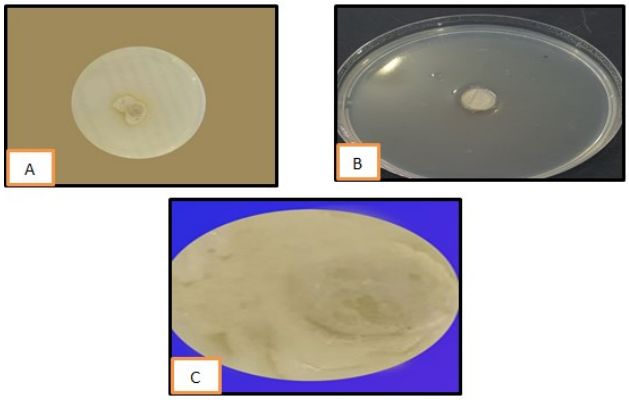


Figure(4-38): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *C.limoniforme* A: extract of propolis 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control 10% DMSO treatment

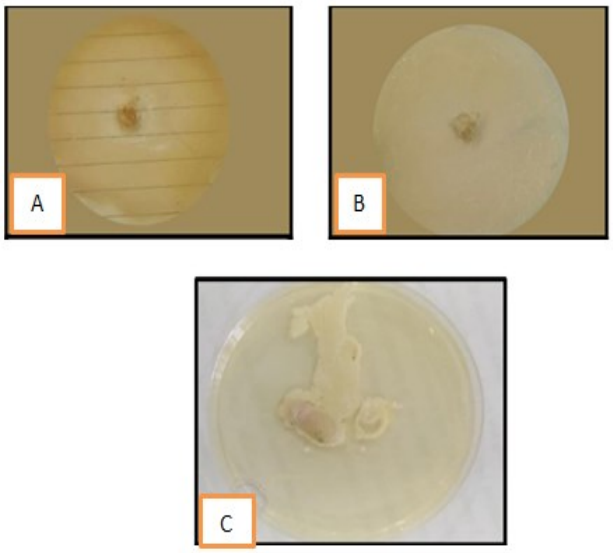
C. albicans and c. glabrata anti-fungal activity were applied (5,10,and15mg/ml) in table (4-16) mycelial inhibition ranging of ethanol extract showed(56.6%,in 5mg/ml and 65.5%, in 10mg/ml and 92.07%, in 15 mg/ml)(figur12,A) compared with positive control(B) and negative (C). and the rate of inhibition was in the fungus *C.glabrata* (22.1% in 5mg/ml,28.8%,in 10mg/ml and 64.4% in 15mg/ml).(figur13,C) compared with negative control (A) and positive (B) were inhibition percentage was (0.00% for negative control and 100% for positive control).

Table 4.16: Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *c.albcans and c.glabrata*

Concentrations Mg/ml	***C.albicans***	***C.glabrata***
Control(-)	0±0.00	0±0.00
5mg/ml	56.6±0.63	22.1±0.63
10mg/ml	65.5±0.00	28.8±0.63
15mg/ml	92.07±0.127	64.4±0.56
Ketoconozol(+) 5mg/ml	100±0.00	100±0.00
L.S.D	1.571	
*Mean± standard deviation		



Figure(4-39): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *C.albicans* A: extract of propolis 15 mg/ml B: positive control (ketoconazole 5mg/ml), c: negative control 10% DMSO treatment.



Figure(4-40): Antifungal activity of phytochemical compounds alcoholic extracted from (propolis) against *C.glabrata* A: negative control 10% DMSO treatment B: positive control (ketoconazole 5mg/ml), c: extract of propolis 15 mg/ml

The results showed that the inhibitory effect of plant extracts the tested fungi were depended on the type of extract (alcoholic) and its concentration in addition to the type of fungal isolate, the in case alcoholic extract had a high inhibitory activity and it was evident that the rates of fungal colony diameters increased with the increase in the concentration of the extract used at the time in which the inhibition percentages were directly proportional to the increase in the concentration of the extract. Table (4, 13,14and 15).

This present study is consistent with a numerous studies, Akgül and Kivanc, (1988) which found that, among ten tested spices, only oregano exerted antifungal activity against nine tested fungi. A study by Paster *et al*., (1995) demonstrated the antifungal activity of oregano at concentrations of 2.0 and 2.50 µL/L on mycelium and spores of *A. niger, A. flavus*. Baratta *et al*., (1998), Bouchra *et al*., (2003) and Viuda-Martos *et al*., (2007) implied that essential oil of oregano possesses stronger antifungal activity against *A. niger and A. flavus* in comparison to those of rosemary, sage, thyme and clove.

A study by Kocić-Tanackov *et al*., (2012) revealed that the oregano extract has the ability to reduce mould growth at all applied concentrations. Stronger inhibitory effect on the growth of *Penicillium species*, contrary to *Fusarium*, was determined. A similar study was conducted by Knez Hrnčič *et al*., (2020) which found that the extracts of *Origanum vulgare* possess compounds with antimicrobial and antifungal properties.

Yotova and Ts, (2015), who demonstrated that oregano represents an economic source of natural mixtures of antifungal compounds that can be as effective as modern medicine to combat pathogenic microorganisms and safe alternative to treat infectious diseases. Also, Yotova and Ts, (2015) which found that the existence of antifungal activity of solutions of oregano towards various pathogenic eukaryotic microorganisms. According to the studies conducted by Cushnie and Lamb, (2005) and Peralta *et al*., (2015) which revealed that flavonoids inhibit fungal spore germination and have been proposed to control fungal pathogens. A study by Kanwal *et al*., (2010), who found that some flavonoids have been isolated from mango Mangifera indica leaves, and they inhibit the growth of *Alternaria alternata, Aspergillus fumigatus, Aspergillus niger, Macrophomina phaseolina, and Penicillium citrii.*

Other studies Campos *et al*., 2015; Capoci *et al*., 2015; Shehu *et al*., 2016; Maureira *et al*., 2017and Dezmirean *et al*., 2017 which revealed that in the ethanolic extracts of the propolis demonstrated activity against *C. albicans.* Furthermore, the antibacterial and antifungal activity of bee propolis has been attributed, at least partially, to its phenolic content (flavonoids, phenolic acids, and their esters), such as the flavonoid galangin (Freires *et al*., 2016; Ota *et al*., 2001) , although the composition of the propolis varies depending on the plant and bee species of each location (Ramón-Sierra *et al*., 2019).

Propolis has received the attention of clinicians and researchers because of its diverse pharmacological activities and low toxicity (da Silva Frozza *et al*., 2013; Agarwal *et al*., 2012 ;Bankova, 2009). The another study by Kacaniova *et al*., (2009) which revealed the antifungal activity of Propolis against *Candida* species.

The study Kacaniova *et al*., (2009) was purely based on discs diffusion and reported the fungistatic activity of propolis against *C. albicans*. Also, the current study consistent with the study findings done by Dalben-Dota *et al*., (2010), which revealed that excellent performance in an in vitro test against vaginal yeasts (*C. albicans, C. glabrata, C. guilliermondii and C. parapsilosis*) by inhibiting their growth at a maximal concentration.

Moreover, the finding of another study Oliveira *et al*., (2006) showed a high activity of Propolis against *C. albicans* and *C. non-albicans* isolated from patient with *onychomycoses. Quercetin, kaemphterol, galangin, and pinocembrin* are among the most efficient flavonoids agents found in the Propolis, which contribute significantly to the fungicidal action

of the SBP (Montero and Mori, 2012; Duailibe *et al*., 2007; Sforcin and Bankova, 2011).

The variation in the antifungal activity of Propolis referred to the differences in the chemical composition of Propolis from one area to another. This variation produced variable synergistic effects of the phenolic compounds (Montero & Mori, 2012) (Kujumgiev et al., 1999) (Hegazi, Abd El Hady, & Abd Allah, 2000) (Sforcin & Bankova, 2011).

The study found that the inhibition rates for the fungi have given high percentages and in all concentrations gradually for both oregano and propolis, as explained in the table (4,11 and 4.12). The ability of the alcohol extract understudy to inhibit the growth of fungi is due to the presence of effective compounds, including phenols, flavonoids, alkaloids, fatty acids, and other elements that have been detected by the GC MAS technique for both Oregano and Propolis (table 4.11, and table 4.12).

Where these compounds act as anti-fungal, bacterial and other micro-organisms, and the mechanism of antimicrobial activities of such compounds is attributed to their higher affinity, and this leads to a defect in the activity and growth of fungal cells and also affects the depletion of the cell membrane as well as prevents the manufacture of enzymes necessary to regulate cellular activity, as well as preventing the manufacture of For essential proteins for the cell and to control cell divisions (Farag *et al* ,1989).

Also, from (table 4.11, and table 4.12), there is another explanation for the activity of these extracts against pathogenic fungi also the presence of proven compounds (the second compound in Oregano and the fourth compound in Propolis), which consistent with the study done by El-Hawary *et al*.,(2013) who found these biological compounds are active

as antifungal, bacterial, and microbial. Furthermore, it was found through the study that the alcoholic extract of the leaves of the oregano plant was the best in terms of affecting the growth of the fungi under study. This is due to the presence of phenolic compounds that are secondary metabolites. Of plants, as well as the presence of phenols, which represent the largest group of phenols (Harborne, 1984; Bowsher *et al*., 2008). In addition, Phenols are considered to be anti-fungal and bacterial agents (Bowsher *et al*., 2008).

Conclusions and Recommendation

Conclusions:-

1-The genus *Candida* is the most frequent, followed by the genus *Aspergillus* and then *Penicillium*

2-The alcoholic extract of the *oreganum vulgare* plant showed a high efficiency in inhibiting in 15% Constrain in the growth of the fungi isolated, as well as the alcoholic extract of Propolis was showed inhibition with different proportions .

3-The study revealed that the *Aspergillus spp* is the most sensitive to the extracts within the current study.

4- It was found through the detection with the GC-MS technology that Propolis and *Oreganum vulgare* contain a range of active compounds like phenols, flavonoids, steroids, and fatty acids.

5. DNA sequencing is considered as typical rapid tools for fungi identification.

Recommendations:-

1-Focusing on studying medicinal plants as alternatives to manufactured medicines because of their important and effective role and returning to nature

2-.Extraction of the active substance in the alcoholic extract of oregano and propolis, and a study of its antibacterial and antiviral activities.

3-Study of the antagonistic activity of the extract on other pathogenic fungi

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الخلاصة:-

اظهرت نتائج (١١٥) عزلة للفطريات المؤخوذه من حالات سريرية مختلفة من مستشفيات وعيادات طبية مختلفة في محافظة بابل عن وجود (٦) اجناس من الفطريات وهي:- *Aspergillus spp.(*33.9%) ,*cladosporum* (6.96%), *(candidaspp spp.(34.78)* *Penicillum spp.* (17.4%) .*Trichophyton spp.*(4.35) ,*curvularia(*2.6%) وقد تم عزل ٤ انواع لجنس candida بطريقة الزرع على الوسط التفريقي (chromagar) والمعتمدة على لون المستعمرة وكذلك بالطرائق الجزيئية سجل وجود نوعين من الكانديدا وهي: *(Candida albicans, Candida glabrata)* كما عزل نوعين لجنس (*Aspergillus*) وهي (*A.flvus, A. niger*) حيث تم تشخيص معظم الفطريات التي تم الحصول عليها بالطرق الجزيئية وتتابع الDNA (DNA Sequencing) .

اظهرت الدراسة بان نبات (الاوريكانو) كان الاكثر فاعلية في تثبيط الانواع الفطرية المعزولة مقارنة با (البروبولس) اذ ان المستخلص الكحولي قد احدث نشاط مضاد في المختبر بأستخدام طريقة التسمم الغذائي ضد انواع الفطريات المعزولة من خلال عمل ثلاثة تراكيز (5,10,15)ملغم/ ملم ومقارنة معاملة السيطره الايجابيه الذي يمثله المضاد الفطري كيتوكونازول والسيطرة السالبة المتمثلة بنسبة ١٠% ثنائي مثل سلفوكسيد.

اظهرت النتائج ان المستخلص الكحولي (البروبولس) قد احدث تاثير تثبيطي بنسبة ١٠٠% على كل من فطر *T.mentagrophytes* و *A.niger* مقارنة مع السيطرة ومضاد الفطريات بمستوى معنوية ٠.٠٥ وكانت نسبة تثبيطها (٠.٠٠ للسيطرة السالبة و ١٠٠% للسيطرة الموجبة). وتفاوتت حساسية الفطريات الاخرى تجاه تراكيز المستخلص الكحولي قيد الدراسة.

ايضا لوحظ تاثير المستخلص الكحولي لاوراق نبات الاوريكانو قد احدث تاثير تثبيطي بنسبة ١٠٠% على كل من (*T.mentagrophytes ,C.albicans ,A.niger ,A.flavus*) مقارنة مع السيطرة الموجبة والسالبة على التوالي.

ان فعالية النشاط المضاد لنبات الاوريكانوا والبروبولس تعود الى وجود مركبات فعالة تم التحري عنها بتقنية G.C.ms وهذه المركبات هي الفلافونيدات ، القلويدات ، السيترويدات ,الفينولات واحماض دهنية كان لها الاثر الواضح على نمو الفطريات قيد الدراسة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل / كلية العلوم للبنات
قسم علوم الحياة

الفعالية ضد فطرية لمستخلص العكبر (propolis) والاوريكانوا

(*Oreganum Vulgaris. L*)

رسالة مقدمة الى

مجلس كلية العلوم للبنات، جامعة بابل

وهي جزء من متطلبات نيل درجة ماجستير علوم

في علوم الحياة

من قبل

خديجة سليم شنون الياسري

(بكالوريوس علوم الحياة/ جامعة الكوفة، 2011)

بإشراف أ .م.د نداء شهاب حمد

١٤٤٣هـ **٢٠٢٢م**