# Investigation of the Molecular mechanisms of garlic oil as antifungal agent

الآلية الجزيئية لزيت الثوم كعامل مضاد للفطريات


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**This Thesis submitted in partial fulfilment of the requirements for the Master's Degree in Clinical Laboratory Science from the faculty of Graduate Studies at Birzeit University Palestine**

January, 2021

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Master thesis submitted and accepted on Tuesday January 5th, 2021.

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January, 2021

*I would like to dedicate my work to*

*my father Yaser, my mother Amal and*

*my brothers Baha'a and Ehab Who*

*gave me the power to continue*

*Until I reach the end.*

### *Acknowledgments*

I would like to acknowledge my thesis supervisor Dr. Mohammad Farraj who provided me with guidance and supported throughout my work. I would also like to acknowledge the Program Director Dr. Mahmoud Srour who provided me with great support. Thanks also go to the genetic research assistant Mr. Israr Sabri who helped throughout the project with technical directions and optimization of all scientific experiments as well as guidance. In addition, I would like to acknowledge the genetic biology master program department at the Arab American University in Ramallah for their cooperation to use their facilities to conduct some experiments.

Thanks also for everyone who provided me with support and encouragement.

*HYR*

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## List of abbreviations

| | |
|---|---|
| CLSI | Clinical Laboratory Standard Institute |
| PCR | Polymerase Chain Reaction |
| Th17 | T-Helper Lymphocytes 17 |
| FISH , | Fluorescent In-situ Hybridization |
| VVC | Vulvovaginal Candidiasis |
| RVVC | Recurrent Vulvovaginal Candidiasis |
| 2D-DIGE | Two Dimensional Gel Electrophoresis |
| SDA | Sabouraud Dextrose Agar |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| cDNA | Complementary Deoxyribonucleic acid |
| B-ME | 2-Mercaptoethanol |
| MIC | Minimum Inhibitory Concentration |

## Abstract

**Introduction**: Infections caused by Candida spp. constitute a significant health problem with symptoms ranging from mild to severe. *C.albicans* is an opportunistic human pathogen causing diseases in the immunocompromised patients as well as vaginal candidiasis commonly encountered in pregnant women. The treatment of infections with *C. albicans* has been hampered with therapeutic failure. This has been attributed to the evolution of resistance to antifungal drug caused by selective pressure.

**Objectives:** The aim of this project was primarily to determine the susceptibly of *C. albicans* to selected antifungal agents as compared to garlic oil. In addition, we tried to elucidate the molecular mechanisms of garlic oil as antifungal agent against *C. albicans.*

**Materials and Methods:** Ten vaginal swabs were obtained from hospitalized pregnant women with severe vaginal infection from several hospitals in the North areas of the West Bank. The swabs were cultured on fungal media to isolate and identify the presence of *C. albicans*. The antifungal susceptibility testing and minimal inhibitory concentration was performed on Ketoconazole, Nystatin and fresh garlic oil extract. To elucidate the molecular basis of antifungal effect of garlic oil against *C. albicans*, eleven genes were selected and tested by Real time PCR to examine their rate of expression after treatment with fresh garlic oil extract. .

**Results:** Most of the C. albicans isolates tested showed in-vitro resistance to ketoconazole and nystatin. However, the same isolates were highly sensitive with low

MIC to garlic oil. The effects of garlic oil on the expression of 11 housekeeping genes was done by real time PCR. The cycle threshold for both the garlic oil treated samples and untreated controls was determined. The cycle threshold was high (8 cycles) for both indicating low level of expression and no significant effect of garlic oil on gene expression.

In conclusion, *C. albicans* was susceptible for garlic oil with low MICs. The molecular basis of the antifungal effects of garlic oil on *C.albicans* remained to be elucidated.

## ملخص الأطروحة

في وقتنا الحاضر، تشكل العدوى الفطرية واحدة من أخطر أنواع العدوى، وذلك نتيجة للأعراض المصاحبة لها والتي تتراوح بين الخفيفة إلى الحادة المبيضة *(Candida)*، هي من أكثر الفطريات (الخمائر) الانتهازية التي تصيب الإنسان، والتي تسبب عدة أنواع من الأمراض مثل: داء المبيضات المهبلي ( Vaginal Candidiasis ) ، الذي يعد من أكثر الأمراض شيوعاً بين فئة النساء الحوامل ، والتي تحدث بشكل خاص بفعل *المبيضة البيضاء ( C. albicans )*. علاوة على ذلك، مرضى نقص المناعة عند اصابتهم بهذا النوع من الأمراض الفطرية ، يتعرضون لمضاعفات عدة، وذلك قد ينجم بفعل الاستخدام الخاطئ لمضادات الفطريات ، الذي قد يؤدي إلى مقاومة متعددة للكثير من أنواع المضادات الأخرى .

الهدف من هذه الدراسة هو استكشاف التقنية الجزيئية التي تلعب دوراً في عمل زيت الثوم كمضاد لنمو فطر ( *Candida albicans* )، وكذلك أيضاً للتأكيد على أن الاستخدام الخاطئ لمضادات الفطريات، يقودنا في نهاية المطاف للبحث عن بدائل طبيعية المكونات للتخلص من العواقب الوخيمة للعدوى الفطرية الحادة.

قمنا بجمع عشرة مسحات مهبلية من نساء حوامل يعانون من عدوى حادة بشكل عشوائي من مستشفيات في شمال الضفة الغربية ، والتي تضم : مستشفى درويش نزال الحكومي ، مستشفى وكالة الغوث الدولية – قلقيلية ومستشفى رفيديا الحكومي نابلس . قمنا بإجراء فحوصات تحديد نوع الفطر ، وفحوصات مضادات الميكروبات والحد الأدنى للتركيز المثبط لنمو الفطريات لنوعين من مضادات الفطريات الأكثر استخداماً سريرياً ، وهما : الكيتوكونازول والنيستاتين ( Ketoconazole and Nystatin ) .

النتائج تشير إلى أن العينات التي تم الحصول عليها هي من نوع: *المبيضات البيض(C. albicans)* تظهر مقاومة للنوعين المستخدمين من مضادات الفطريات ، لذلك ونظراً للفائدة الطبية الفعالة للثوم وزيت الثوم كتحسين صحة الجهاز الهضمي والدوران والبولي ، قمنا بإجراء فحص مضادات الميكروبات بواسطة زي الثوم ، بعد تحضيره طازجاً وعصره واستحلابه باستخدام الماء ، بالإضافة لإجراء فحص الحد الأدنى للتركيز المثبط لنمو الفطريات.

نتائج دراستنا أظهرت بأن *المبيضات البيض* حساسة لزيت الثوم ( ٩٠ % ) ، وبعد عدة محاولات وجدنا أن استحلاب زيت الثوم بالماء كان الأكثر نجاعة كمضاد للفطريات . كما قمنا بإجراء فحص التقنية الجزيئية لتفاعل البلمرة المتسلسل لمضاعفة الحمض النووي باختيار ١١ تقنية ذات دور مهم في العمليات الخلوية للفطريات ( كتنظيم عملية النسخ ، امتداد وتحسين النمو الخيطي ، وإنتاج الهيفات الخ .) وذلك لمعرفة التقنية التي تتأثر بفعل زيت الثوم تحديداً.

نتائج الدراسة أكدت على وجود علاقة بين استخدام زي الثوم في تثبيط نمو مستعمرات فطر (*C. albicans* ) ، لكن لم نتوصل للتقنية التي تتأثر بزيت الثوم وتتسبب في تثبيط النمو حتى الآن.

# I. Introduction:

## I.1 The prevalence of Fungal Infections

About 33.8–60% of fungal infections caused by *C. albicans,* as it is the major pathogenic *candida* species. Infection rate caused by Candida Spp. other than *C. albicans* has been also considerably increasing [1]. It found that one of the most serious health issues is the invasive fungal infections, especially among immunocompromised patients. The signs and symptoms can range from mild to severe depending on the involvement of several virulence factors such as phospholipase, dimorphic growth, melanin production, mannitol metabolism, superoxide dismutase, rapid growth and invasion to blood stream, heat tolerance and toxin production. Therefore, soon after fungal infections detected, anti-fungal susceptibility should administer as soon as possible [2].

*C. albicans* is the most predominant causative agent, which is a durable organism that has the ability to resist adverse environmental conditions. It has the ability to form biofilm, which makes it hard to eradicate and increase its ability to resist large doses of antibiotics. Many Studies have focused on the relationship between biofilm formation regulation and associated drug resistance. It found that the tendency of C. albicans to form biofilm increases with the increased use of antifungal agents [3, 4].

## I.2 The critical health concern of *C.albicans* among pregnant women

It was found that the prevalence of *Candida* species among the African Northwest Ethiopia is relatively high 25% and the most predominant species were C. albicans, followed by *C. krusei, C. glabrata, C. tropicalis* and other unclassified *Candida species*. The most affected group of people are pregnant women who suffer from symptoms of vaginal infection in age groups range from 26-40 years old [5,6]. Amphotericin B is an antifungal drug which is the most effective antifungal drug was used to treat these Candida species above. Whereas *Candida* species show resistant to itraconazole and ketoconazole [7]. With repeated incorrect use of these antifungal agents, multiple drugs resistant *Candida* species evolved. Therefore, it recommended making regular screening for pregnant women for *Candida* colonization, proper prophylaxis and treatment should provide in correct manner [8].

Because of selective pressure, drug resistance is inescapable. Therefore, intensive research should focus on this critical issue, since its frequent occurrence as the susceptibility pattern of C. albicans to antifungal agents varies with region and constant monitoring of any increase in resistance should be monitored [9].

Studies have shown that high frequency of Candida recovered from Lebanese pregnant women created a major public health concern. Regular screening for candidiasis with antifungal susceptibility testing is highly recommended for better monitoring of the disease and its burden among Lebanese pregnant women, especially among those suffering from vulvovaginal candidiasis [10].

No insights have found according to the impact of intrauterine Candida infection among pregnant women in early terms on fetal health. Nevertheless, this intrauterine *C. albicans* infection will lead to adverse outcomes for the mother and her fetus health, including preterm birth. By using animal models, e.g. sheep using Doppler imagining, to assess the intrauterine *C. albicans* infection consequences at early pregnancy, results revealed that a systemic fetal candidiasis occur in association with systemic inflammatory response and significant cardiac abnormalities [11]. Moreover, Chorioamnionitis caused by intrauterine infection, which is associated with postnatal intestinal pathologies including necrotizing enterocolitis. The intra-amniotic *C. albicans* infections mechanism adversely affecting the fetal gut remains unclear. Intra-amniotic *C. albicans* caused intestinal infection, injury and inflammation. By treatment trials, it observed that treatment with fluconazole decreased mucosal injury but was unable to maintain *C. albicans*-mediated mucosal inflammation, so more applied antifungal therapeutic strategy should be investigated [12].

## I.3 Other Infections caused by *C. albicans*

Candidemia: which manifested with high mortality rate, patients who are at high risk of this infection including; ICUs hospitalized patients, immunocompromised patients e.g. the elderly and surgical requirement cases. For further optimized management, local surveillance of candidemia should continuously do [13].

Invasive fungal infections: in addition to candidemia, invasive fungal infections leads to high morbidity and mortality rates especially, within liver transplant recipients (LTRs) [14] .The invasive candidiasis spectrum ranges from minimally symptomatic candidemia to fulminant sepsis with mortality rate exceeding 70%. Candida spp. is skin and gut commensal organisms, and so the invasive disease relay on the fact of *C. albicans* action on the cutaneous and gastrointestinal barriers destruction. For powerful management and maintenance, diagnosis of invasive candidiasis should early investigate. This will achieve with the development of rapid molecular diagnostics. As this step can reduce the mortality event, which improve the situation furthermore .There are many choices for treatment regimen, including echinocandins and azoles. Whereas, the emergence of multi-drug resistance due to repeated use of the drugs as well as misuse of the available agents makes the condition more complicated [15].

Septicemia: is one of the infectious complications especially among cancer patients, who develop oral and gastrointestinal candidiasis. The invasive infection mechanism caused by interactions between *C. albicans* and endogenous mucosal bacteria. By using animal modeling, it was found that changes of the oral macrobiotics are due to immunosuppression and Candida infection after the administration of antibiotics which resulted in the alteration of these organisms load , attenuate the oral mucosal E-cadherin degradation and *C. albicans* invasion without affecting fungal burdens , which is called in other words " overt dysbiosis "[16].

## I.4 Risk factors for *C. albicans* infection acquiring

Candida spp. is usually normal flora, when activated it causes diseases such as opportunistic fungal infections during immunosuppression status, resulting in surface and invasive infections with high morbidity and mortality rate. Administration of Immunosuppressive therapy, mucous damage and prolonged hospitalization increase the risk of having Candida infection. In addition, genetic factors have a critical participation in Candida infections and pathogenesis [17]**.** In addition, patients at high risk of developing infections by *C. albicans* are those who suffer from diabetes, corticosteroid use, cancer patients, neutropenia, intensive care unit admission, presence of central venous catheter (CVC), presence of shock, prior antibiotic therapy [18].

## I.5 Sources of *C. albicans* transmission

Maternal Transmission; Mothers of children with severe early childhood dental caries (S-ECC) considered the primary source of *C. albicans* acquisition in the oral cavity of children. Studies suggest that there is a symbiotic relationship between *Streptococcus mutans*, which infect the human oral cavity and *C. albicans* infection in relation to ECC cases [19].

Nosocomial transmission of *C. albicans* is particularly among hospitalized patients whose suffer from immunodeficiency status. Studies have repeatedly analyzed Candida isolated from bloodstream of hospitalized patients [20]. Advanced molecular techniques such as fingerprinting by whole genome-based amplified fragment length polymorphism (AFLP) used. The results showed that all isolates have identical genotypes. This confirms hospital transmission among patients during outbreaks. These results indicate the need for further implementation of hospital control measures [21].

## I.6 Antifungal Susceptibility Testing

Antifungal susceptibility testing has frequently given some conflicting results for many researchers in the field of medical mycology. All of them focused on studying the patterns of antifungal susceptibility of Candida species strains, which either colonized or infected hospitalized immunocompromised patients [22].

An example of immunocompromised individuals is pregnant women who attend antenatal units where many trials focused on antifungals identification and susceptibility testing. As the most causative agent of vulvovaginal Candidiasis was *C. albicans. Those pregnant women take many types of antifungal agents,* leading to decline in susceptibility to all drugs, particularly the azoles especially for non-albicans organisms, which used in treating vaginal candidiasis [23].

The care of patients with invasive candidiasis done by performing the susceptibility testing of fungi against antifungal drugs. Two available sources for antifungal susceptibility testing (AFST) which are; Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA: 2008. Approved standard—2$^{nd}$ and 3$^{rd}$ edition.) and European Committee on Antifungal Susceptibility Testing 2017*th* Edition (Arendrup M.C., Version 9.3.). Both developed standardized reference methods and commercial manual/automated phenotypic methods e.g. Sensitivite Yeast-One and E-test methods are widely used for AFST. Nevertheless, AFST improved toward new phenotypic methods, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), which are rapid [24].

In conjugation with the increased immunosuppressive diseases, the fungal infections became diverse and rapidly spreading especially by species, including; Candida, Aspergillus, Cryptococcus, Coccidioides, Pneumocystis, and Zygomycetes species. Increased infections are common among the immunocompromised as seen immunodeficiency virus (HIV) epidemic, Solid organ transplantation medicine with technological improvements, stem cell

transplantation, and neonatology, coupled with the insert of new immunosuppressive drugs. [25].

**Ketoconazole** is belonging to the azole class, act on inhibiting the cytochrome P450 14α-demethylase enzyme, which considered as antifungal agent. This enzyme is specialized in the biosynthesis of triglycerides and phospholipids by fungi. In more specific manner, ketoconazole inhibits the synthesis of lanosterol, a necessary precursor for ergosterol biosynthesis. The role of Ergosterol is to maintain the integrity of the cell membrane of fungi and in its absence the fluidity of the membrane increase, eventually preventing the fungal growth. [26]. On the other hand, taking Ketoconazole in high doses, can competitively bind at androgen receptors, which can decrease the activity of testosterone and dihydrotestosterone in prostate cancer. Furthermore, Ketoconazole negatively affect the activity of the enzyme 21-hydroxylase which is incorporated in mineralocorticoids and glucocorticoids synthesis, so affecting the production of cortisol , and this can be useful in Cushing syndrome treatment [27].

**Nystatin** is a polyene derived from Streptomyces noursei in 1949; it was discovered while conducting research at the Division of Laboratories and Research of the New York State Department of Health, Elizabeth Lee Hazen and Rachel Fuller Brown [28]. In 1955, It was reported that topical nystatin be effective for treatment of noninvasive candidiasis, which is a frequent complication among children undergoing early chemotherapeutic leukemia trials [29].

## I.7 Mode of Resistance

The azoles role as antifungal agents act by inhibiting the cytochrome P450 lanosterol demethylase enzyme (14α-demethylase), which is encoded by Lanosterol 14-alpha demethylase (ERG11), that incorporated in the ergosterol biosynthesis pathway. The azole's ring free nitrogen atom binds an iron atom within the enzyme Heme group. Eventually, this will lead to cell death, as ergosterol is an essential component of fungal cell membranes. Resistance mechanisms for azoles include:

### I.7.a. Increased drug target

The overexpression of ERG11 gene, which encodes for the azoles target, can be in low levels or in conjugation of other resistance mutations, which makes the process of impact identification more complicated [30].

### I.7.b. Alteration in drug target

The coding region of the ERG11 gene undergo point mutations which lead to amino acid substitutions , and so altering the structure of the protein and this will reduce the binding affinity and azole susceptibility [31,32].

### I.7.c. Alteration in sterol biosynthesis

Bypass sterol-pathway production results in resistance to fluconazole, which occurred because of loss-of-function mutations in *ERG3*. This lead to $\Delta^{5,6}$-sterol desaturase enzyme inactivation. The cell will bypass toxic methylated sterols synthesis, in the presence of the azoles and the effect of fluconazole minimized. [33].

### I.7.d. Increased drug efflux

When there is a disruption in the protein transporters, which are responsible for antifungal efflux, the drug cannot accumulate intracellular, resulting in resistance. [34].

### I.7.e. Aneuploidy and other chromosomal abnormalities

Alteration in gene-copy number leads to genetic variation, this occurs in routine cultures and genetic manipulation of the laboratory strains or clinical isolates of *C. albicans* [35].

## I.8 Treatment Trials of recurrent fungal infections

Many trials attempted to test the ability of conventional antifungal drugs to eliminate the biofilms formed by certain microorganisms. These attempts were unsuccessful due to the rapid formation of the biofilm, which made the action of the tested antifungal agents insignificant and ineffective. The challenge to eradicate biofilms dictates that more knowledge needed about the organisms that form biofilms. It could be necessary to further understand the mechanism of biofilm formation and identify agents that could damage the extracellular substance forming the matrix which protect the biofilm [3].

## I.9 Antifungal resistance Overcoming Strategies

According to several studies, researchers began to think of new strategies to overcome the biofilm association with antifungal resistance [36].This relied on the understanding of the multifactorial aspects of biofilm involved in its antibiotics resistance and other reagents and chemicals. It means that biofilm alone cannot make this effect. The regulatory expression of the drug efflux pumps and the alteration in their metabolic activity will act collectively. Researchers focused on studying the nature of the biofilm formation process, the molecular mechanism of their genetic expression and of their adherence properties, either between the cells of the *C. albicans* and between the biofilm cells and the host surfaces. In addition, the biofilm interaction with the host defense protective factors, can aid in

finding new therapeutic protocols and strategies with higher effectiveness. Other studies focused on studying the usefulness of overcoming the drugs resistance by using them in combination to target ergosterol biosynthesis and β-1, 3-glucan synthesis that was recently investigated [37]. The biofilm formation can also play a critical role in *C. albicans* bloodstream infections, which continue to be a critical challenge in intensive care units, as prolonged exposure to antifungals. Therefore, the formation of biofilms resulted in selection of spontaneous mutations that increase expression or decreased susceptibility of the target, which reduce the antifungal responsiveness [38].

In addition to the biofilm formation by *C. albicans*, it has been distinguished from other Candida species in terms of its yeast-to-hyphae transformation. This feature in *C. albicans* forms a critical point in its pathogenesis acting as a significant virulence factor. A group of regulatory pathways, including the environmental sensing, signaling, transcriptional modulators and chromatin modifications, triggers the regulation of the hyphal formation [39].

Through studying the fungal morphogenesis, it found the replication independent HIR histone chaperone complex, which operates as a crucial modulator of hypha development. This observed by the reduced response to the shape plasticity modifying because of the HIR complex subunit Hir1 deletion. As the Hir1 affect the early stages of hypha initiation, so to resolve the hir1Δ/Δ filament destructive cells, there are many trials in inducing Ume6 gene transcription expression ectopically, as it has a vital role in hyphae maintenance [40].

The golden method for diagnosing fungal infection is by observing the fungus in the infected tissue by fungal specific stains and by culturing patient's clinical samples to isolate the causative agent. Whereas these methods have disadvantages as low sensitivity, researches tend to find new improved techniques with higher sensitivity based on serological and molecular basis; e.g. galactomannan antigen test for aspergillosis and the glucan test for invasive Candida spp. and molds. In addition, Polymerase Chain Reaction (PCR) and other molecular techniques such as the fluorescence in situ hybridization (FISH) has introduced providing that they need experts to perform the tests and interpret the results [41].

*C. albicans considered* as one of the human normal flora, which colonize several body tissues without causing any clinical issues. When the human immune system weakened, they act opportunistically leading to various infections ranging from superficial mucosal to disseminated candidiasis. With time, infection with this opportunistic pathogen may progress to involve several virulence factors such as its ability to form a biofilm. Studies indicated that biofilm plays a role in the protection process from harsh conditions. *C. albicans* can form biofilms on inanimate surfaces such as medical devices and implants (catheters, pacemakers, dentures, and prosthetic joints). This and other factors collectively play a significant role in *C. albicans* resistance to many antifungal agents [42].

There are many types of antifungal agents used for treating fungal infections; the selection of the appropriate agent depends on factors related to patient, pathogen, drug and the infection site, and the ability of certain agents to cause side effects and other complications [43].

## I.10 The Host Immune defense against the fungal infection

The immune defense against *C. albicans* infections initiated by the innate immunity through recognition of antigens of *C. albicans* cell wall components. In contrast, *C. albicans* has several virulence factors against the action of the host immune system; e.g., it can escape from the phagocytes and invade tissues then spread through circulation by its multiple shapes modifying ability. According to this morphological flexibility, it found that the *C. albicans* is unique in their negative effect on the immunological response against the hypha formation including cytokines secretions. Furthermore, the immune response dose relayed such as the fungal invasion pattern and the fungal load level on some factors. The cell-mediated immunity activated because of the *C. albicans* mucocutaneous and systemic infections after its migration through the skin barriers [44]. In addition to

the activation of the innate immunity, Humoral immunity also plays a critical role as e.g. Systemic Th17 inflammation driven by CD4+ T cells responsive to tonic stimulation by commensal *C. albicans*, which encourage the improved host defense against extracellular pathogens, but with potentially harmful immunological consequences [45].

## I.11 New therapeutic solutions for the antifungal resistance issue

One of the major health concerns is the fungal resistance to antifungal agents, especially among patients with weak immunity, which becomes a big challenge to find alternative effective drugs. However, researchers used several techniques such as antifungal agents targeting the nucleic acids and modifying them e.g. targeting the histone deacetylase ( HDAC ) , which have several roles in cell cycle stages including proliferation and death , all of this under the term of " Epigenetic therapy " . Figure 1; shows the antifungal agents' targets including; cell integrity, metabolic pathways and signal transduction pathways [46].

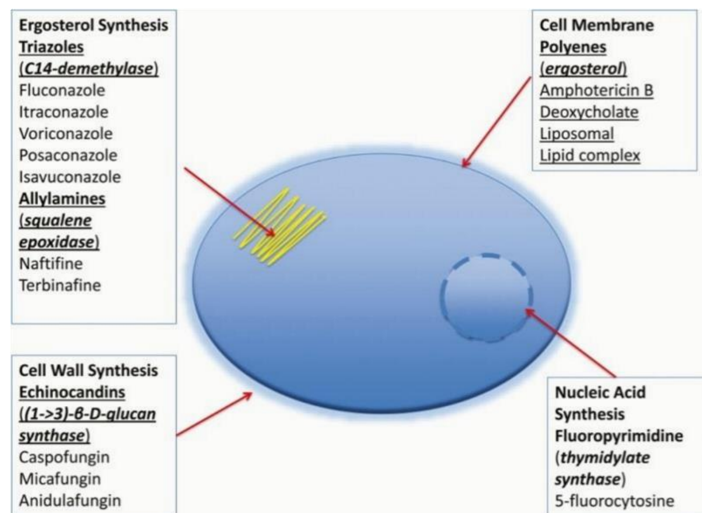


Figure 1: This figure shows the antifungal agents cellular and biochemical targets. (Updated by Matthew W McCarthy, 2017).

There was high prevalence of invasive fungal infections in those with underlying invasive procedures requiring immune suppression. Several investigations have found that the combination of two synergistic antifungal agents is necessary to treat these invasive fungal infections [47, 48, and 49].

In 2016, echinocandins, antifungal agents targeting fungal cell wall synthesis successfully used to treat fungal infections. However, the recurrent use of the static forms of the antifungal agents resulted in the evolution of resistance due to selective pressure. These issues increased the need to explore for more new effective agents [50, 51].

Many global studies have shown that many women have suffered from frequent Vulvovaginal candidiasis (VVC). Initially, they treated by local administration of antifungal agents or by oral antifungal tablets. Some women have frequently developed this recurrent condition, vulvovaginal candidiasis (RVVC), (more than four times yearly). The recurrence of this infection, which causes unpleasant and sometimes severe symptoms, indicates therapeutic failure due to resistance to antifungal agents [52].

Many cases of recurrent vulvovaginal candidiasis (RVVC) documented worldwide among women before the use of certain menstrual replacement hormones. Complains disappeared when women reached the menopause age. The most commonly fungal agent responsible for this condition is *C. albicans.* In order to control RVVC, the causative agent should be isolated and treated properly with effective antifungal agents. In addition, the predisposing factors including genetic factors, vaginal flora and the immune status considered. Since RVVC is a

recurrent condition due to therapeutic failure, trials to develop a vaccine are being developed [53].

It became necessary to find new antifungal agents, particularly naturally derived from herbal sources. Garlic and its constituents have used since ancient times, and may be considered for this purpose. Reports indicated the potential role of garlic in treating several medical conditions as oil or extract administered in low toxic doses. One of the therapeutic constituents of garlic is Sulfur compounds.

Although used to treat many conditions such as cardiac, vascular, malignancies, blood pressure, diabetes, atherosclerosis and fungal infections, it's mode of action remains unknown and needs to be elucidated [54].

## I.12 Medical evidence of garlic oil

Among immunocompromised HIV-tested patients who have opportunistic mycoses. As the antifungal resistance from opportunistic fungal pathogens in HIV-infected patients is rapidly growing, the need for an alternative therapy increased, so the garlic essential oil and extracts might represent a promising future solution for this infection [55].

Many benefits have been observed with prolonged use of garlic oil, which includes adjusting the distribution of immune cells, elevated levels of Serum Tumor Necrosis Factor- α (TNF- α ) and Interleukin-6 (IL-6) prevention, lowering the level

of low density lipoprotein- Cholesterol (LDL-C) in obese individuals in order to avoid the prognosis of the related chronic diseases [56].

## I.13 Garlic oil destructive mechanism and Chemical composition

Garlic oil is one of the most effective antifungal agents. Researches indicate that garlic oil has cellular demolition activities on fungi, which begins with hypha penetration and subsequent cytoplasmic infiltration and cellular destruction. However, some researchers have suggested that there is a negative effect of garlic oil on the level of certain vital proteins, which incorporated into fungal cells and affect their physiological and metabolic activities [57, 58].

The chemical composition of garlic oils has characterized by Gas chromatography and Mass spectrum. It found that the major constituents of garlic essential oil is diallyl trisulfide and diallyl disulfide, their percentage differ according to the type of the extracted plant-skin cultivar color. The garlic oil dissolved in isopropanol evaluated against bacteria and fungi. The results showed a dose dependent activity of the garlic oil. However, water- based extracts showed better effects on the microorganisms, which are tested [59].

## I.14 Molecular Investigations of the garlic oil antifungal effectiveness

Wen-Ru Li and his team found that the garlic oil play a critical role as antifungal agent through organelle damage and eventual cell death. Garlic oil effects on *C. albicans* found through the penetration of garlic oil in the cellular membrane and the membrane of other organelles as mitochondria. On the molecular level, garlic oil acts on the genetic level by increasing oxidation-reduction responsible for gene expression. In addition, it affects pathogenesis and cellular drug response related genes. RNA sequencing technique, as these genes clustered within the 19 Kyoto Encyclopedia of Genes and Genomes (19 KEGG) pathways, can be used for analysis. Two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) analysis and mass spectrometry, have shown that some of the produced responsible proteins are upregulated and others are downregulated regarding to their function. This can lead to critical dysfunction in the biological activities of the fungal cells. The up and down regulation of the cellular proteins remains unidentified and therefore further work is needed to clarify their function [58].

## II. Literature Review

Several studies focused on the antifungal resistance and revealed that the fungal virulence factors play a significant role in developing this resistance. It was found that biofilms formation is the major virulence factor , especially among the *C. albicans* species, as it is form superficial condensed cellular adherent communities .These biofilms have many defense functions including ; intrinsic antifungal resistance , protection against the host immune system and other environmental factors [60,61]. The development of this biological feature is controlled by several regulatory mechanisms such as; the mitogen-activated protein kinase (MAPK) and Cyclic adenosine monophosphate (cAMP) signaling pathways , transcriptional regulators such as Biofilm and Cell regulator 1 (Bcr1) and Transcription activator (Tec1) and morphological transitions and sexual reproduction [62].

On the other hand, *C. albicans* as a filamentous fungi characterized by its morphological flexibility, it has the ability to change its shape from an oval yeast form to a filamentous hyphal form in order to adapt or to be pathogenic [63].

In other studies , It was found that *C. albicans* secrete fungal cytolytic peptide toxin used in epithelial membranes destruction and serious signaling pathway enhancing [64,65] .This type of toxin which is called candidalysin is secreted only

by the invasive form of *C. albicans* , as it's incorporated in the mucosal and systemic infections development . Otherwise, candidalysin activate the host cells by recruiting the granulocytes as neutrophils and T-helper 17 cells of humoral immunity [66]. Other studies discover the candidalysin function as a conventional virulence factor through its contribution process including; immune evasion, immune cell membranes damage, NLRP3 inflammasome activation enhancement, in addition to pro-inflammatory response cells protection [67, 68].

In 2015, Leah E. Cowen and her team studied the emergence of antifungal drug resistance, as there are several classes of antifungal drugs, which are in the management of acute and chronic mycoses. The selection between these agents should be in proper manner, in order to get rid of the emergence of antifungal drug resistance, which restricts the treatment option chances. The resistance of these agents can inherit or acquired, and both have the same mechanisms. They found that the molecular mechanisms of this resistance include altered drug affinity and target abundance, reduced intracellular drug levels caused by efflux pumps, and formation of biofilms [69].

A study in 2016, focused on the molecular mechanism of antifungal resistance, e.g. Candida species resistance to echinocandins mediated by the FKS gene, which undergoing homozygous mutation in *Candida tropicalis* leading to an amino acid substitution corresponding to a well-known S645P in *C. albicans* . According to the mutation type, the level of resistance will be different. Another example, loss of susceptibility to long-structured azoles caused by A61E change in ERG11 [70].

Other study in 2017 has evaluated most fungal species that developed an increased resistance to antifungal agents, under the limited number of remained classes e.g. Candida and Aspergillus species. It has found that the resistance among species of low susceptibility is naturally occurring but it acquired among the susceptible organisms. They also make a recommendation to set a control programs to monitor drug resistance, which act on rapid fungal diagnosis, therapeutic drug in order to keep the drug effective [71].

According to the results of Elisa Bona's study and her team in 2016 , which aim to compare between essential oils (oregano, winter savory and rosemary , lavender, tea tree, basil, anise, laurel and grapefruit essential oils) and conventional antifungal drugs (clotrimazole, fluconazole and itraconazole ) effect on *C. albicans* vaginal isolated strains . This essential oils extracted from aromatic plants have well-known antifungal characteristic. The obtained results of this study and previous ones (Rasmus Hare Jensen, 2016 and David S Perlin, 2017) revealed that the typical interaction pathway of inhibition of essential oil is unknown. This is obvious in relation to the used antifungal drugs, which inhibit the production of specific enzymes (azoles, allyl amines and amorolfine), bind to certain molecules in the cell membrane (polyenes) or components of the cell wall (e.g. caspofungin). Nowadays in Europe, United States and China, the researchers are collectively observing the rising of resistance to antifungal agents [72].

As a result, the researchers have increased use of the essential oils as an alternative to antifungal agents. Essential oils have many advantages characterized by high therapeutic efficiency and low toxicity in the treatment of vaginal candidiasis [73].

Several studies then started to test the antifungal capabilities of several essential oils; one of these effective oils in particular was the garlic oil. In one study conducted in 2014, it found that the minimum fungicidal concentrations were 0.125 in agar medium and 0.0313 % in broth medium, which means that garlic oil had powerful antifungal activity. The dominant antifungal factors in garlic oil are mainly composed of sulfides, as shown by in the results of gas chromatograph-mass spectrometer (GC/MS). They also used the transmission electron microscope (TEM) and scanning electron microscope (SEM) and suggested that garlic oil leads to the death of *Penicillium funiculosum* by damaging multiple sites on the hyphae [57].

In 2016, the studies started to be oriented toward *C. albicans* , by investigating the antifungal activity, kinetics, and molecular mechanisms of action of garlic oil by using multiple methods, including; poisoned food technique, minimum inhibitory concentration (MIC) of garlic oil and transmission electron microscopy. The obtained results indicated that garlic oil MIC was 0.35 μg/mL and act on the level of cellular membrane and other organelles membrane destruction.

By using the RNA sequencing analysis, it has revealed that garlic oil induced critical genes differential expression such as; oxidation-reduction processes, pathogenesis, and cellular response to drugs and starvation. Otherwise, several normal metabolism and physical functions proteins inhibited, but the exact mechanism is still unclear [58].

## III. Statement of the problem

In the fields of pharmaceutical and food industries, attention has focused to use herbal natural extract as preservatives as well as antifungal agents. The reason behind this is the growing number of resistant pathogens to the currently used antifungal agents.

It has observed lately the emergence of multidrug resistant bacterial and fungal isolates. In the face of lacking new antifungal agents to deal with the issue of antifungal resistance, and the poor contribution of using antifungal combination to address this problem, it became essential to look for new natural herbal extracts as alternative to the antibiotics. One advantage is to avoid the toxicity of many of these antibiotics such as amphotericin B and others. Another advantage is to avoid the use of artificial flavors, which may cause irritation to some patients.

The use of substances extracted from herbs such as thyme, rosemary, cloves, garlic and their essential oils plays a significant role for their antifungal potential. These herbs contain many compounds such as polyphenols and others that have several therapeutic potentials such as anti-inflammatory, antioxidants, therapeutic and antifungal potential among others.

Therefore, in this project we tried to test freshly obtained garlic oil by micro-emulsion technique, for its antifungal potential (particularly antifungal potential). In addition to determine the molecular mechanisms responsible for its mechanism of action.

## IV. Objectives of the study

### Objectives:

- To identify the most causative agent of vaginal candidiasis among pregnant women in the northern area of the West Bank region in Palestine
- To test the *C. albicans* isolated against conventional antifungal agents.
- To investigate the correlation between garlic oil and the potential of inhibiting the growth of Candida colonies according to 11 cellular fungal mechanisms.
- To compare between the Fresh garlic oil and the purchased ones and analyze their activity against fungal pathogens.

**Specific Aim**: The aim of this project was to gain an understanding of the molecular mechanisms of garlic oil as antifungal agent. The clinical isolates obtained by vaginal swabs from pregnant women suffering from vaginal candidiasis.

## V. Materials and Methods:

### V.1 Study Materials:

**Table 1: List of the study materials, Lot numbers and sources**

| No. | Material | Lot no. ,Source |
|---|---|---|
| 1. | Sabouraud Dextrose-Agar (SDA) | (BD 8227587), Difco |
| 3. | Corn Meal media | (0000221285),HIMEDIA |
| 4. | Tween 80 | (39H00921),SigmaUltra , Polyoxyethylene-Sorbitan-Monooleate Tween 80 |
| 5. | Ketoconazole antifungal agent | (PR070111),BPC |
| 6. | Nystatin antifungal agent | (PR110151),BPC |
| 7. | Muller Hinton Broth | (0000332457),Himedia |
| 8. | Muller Hinton Agar | (0000357521),Himedia |
| 9. | D(+)-Glucose | (78H0097),SIGMA |
| 10. | ISOLATE II RNA Mini Kit | (IS509-B053430), Bioline, meridian Bioscience, USA/ |
| 11. | Tetro CDNA Synthesis kit | (Bio-65043)m Bioline, meridian Bioscience, |

| | | USA |
|---|---|---|
| 12. | Agarose powder | (A-9539), SIGMA |
| 13. | Gel Loading Dye | (B7024S),BioLabs |
| 14. | Real time PCR or quantitative PCR (qPCR), GoTaq Master Mix A6001 and A6002 | (A6002),Promega kits from Labtech company in West Bank |
| 15. | qPCR primers | (3027177),Labtech company –Integrated DNA Technologies |

## V.2 Study Plan:

This was an experimental study conducted from October 2018 until December 2019. The study samples collected from women with infections caused by *C. albicans.* Ten clinical vaginal swabs were collected from pregnant women with severe infection, obtained from randomly selected hospitals in the North areas of the West Bank including; Darwish Nazzal Hospital, Qalqilia UNRWA hospital and Rafidia Hospital). The selected patients suffer from fungal infections caused by *C. albicans* but they were free of other chronic disorders.

## V.3 Data Collection and Sample criteria:

Vaginal swabs obtained from married women diagnosed with Candidiasis. The samples obtained by a physician with the consent of the patient. The physicians provided information about the specific antifungal treatment of these women.

Numbers coded the samples obtained. The collected samples cultured on Sabouraud Dextrose agar (SDA) plates. Identification of the fungal isolates confirmed by the germ tube test and fungal morphology on Cornmeal agar plates. The antifungal susceptibility tests performed by disc diffusion on agar and the minimal inhibitory concentration (MIC). Also, antifungal activity of the garlic oil has determined by the disc diffusion on agar and the MIC following the guidelines of the Clinical Laboratory Standards Institute (CLSI). RNA then extracted from the two isolates and cDNA synthesized to be used for analysis by using the real time PCR.

## V.4 Inoculation and biochemical identification tests:

V.4.a Inoculation of yeast colonies done on Sabouraud Dextrose-Agar (SDA) (Difco BD, LOT: 8227587).

**V.4.b** Identification Tests were by :

**- Germ Tube**: By using rabbit serum or human plasma , to study the ability of yeast to form "germ tube form" as distinguished from other non-germ tube forming yeast as no constriction of the mother cell; Germ tube was performed on candida species colonies growing on SDA for 24-48 hrs. A total of 1-2 large colonies or 3-4 small colonies added into tubes containing 200µl rabbit serum, incubated for 3-4 hrs. At 37°C (Clinical Laboratory Science Institute-28$^{th}$).

- **Cornmeal agar testing** (HIMEDIA- M 146-500G , LOT:0000221285) One liter of media was prepared by adding 17.6 g of the CMA to one liter of distilled water supplemented with 10 ml Tween 80 ( SigmaUltra , Polyoxyethylene-Sorbitan-Monooleate Tween 80, lot ;39H00921) heated to boil then sterilized in the autoclave at 121$^{o}$ C and 15 minutes before pouring in sterile Petri dishes. Cornmeal agar testing used to detect the formation of chlamydospores, which are characteristic of *C. albicans.* A portion of the yeast colony streaked on one quadrant of the CMA agar surface, the media cut in two adjacent parallel lines and then covered with a sterilized cover slide. A positive control (*C. albicans*) and negative control (*Saccharomyces cerevisiae*) were included each time the test was done.

The plates then incubated at room temperature and examined after 48-72 hrs. C. *albicans* produces pseudohyphae, regular clusters of blastoconidia along the points of septation, and large, spherical chlamydospores (Clinical Laboratory Science Institute-28***th***).

## V.5 Antifungal Susceptibility Testing by Disc diffusion plate method

To determine the susceptibility of the *C. albicans* clinical isolates, a suspension equivalent to two McFarland standards was prepared and spread on the surface of the agar plates using sterile cotton swabs. After 10 to 15 minutes, sterile filter paper discs placed at proportional distances of each other and different concentration of the antifungal agents and garlic oil added to the filter papers. The plates incubated at $37^{o}$ C for 48 hours, and the zones of inhibition measured using Vernier Caliper.

Two antifungal agents belonging to different classes used; the first one was Ketoconazole (PR070111, BPC), an Azole antifungal class; the second was Nystatin (PR110151, BPC), a polyene macrolide class. Each antifungal agent prepared by dissolving in a suitable solvent; here in our study we are used Dimethyl Sulfoxide (DMSO); both the ketoconazole and Nystatin are considered as water insoluble drugs, which can be dissolved in alcoholic compounds to yield stock solutions of 1000 µg/ml.

The antifungal susceptibility testing was carried out using Muller Hinton agar supplemented with 2% Glucose (as for Candida enhancement factor) and 0.5µg/ml Methylene Blue (for clear zones discrimination); the PH was adjusted to 7.3. Sterile Filter papers of 6 mm in diameter used. After preparing the needed

concentrations, 5µl of each antifungal agent or garlic oil added to the filter paper disc (Clinical Laboratory Science Institute-28th).

## V.6 MIC determination of antifungal agents:

Muller Hinton broth (MHB) supplemented with 2% Glucose used. Stock solutions of ketoconazole and Nystatin of 1000µg/ml was prepared. MIC done using the microplate method in flat 96 well sterile ELISA plates. The experiment done as follows:

MH broth of 100 µl added to wells number 2 through well number 10. Then a concentration of 256 µg/ml of the antifungal agent prepared in MH broth was placed in wells number 1 and number 2. This was followed by doing a serial dilution starting from well number 2 throughout number 10 by transferring 100 µl of well mixed suspension from one well to the next. Finally, the 100 µl from well number 10 discarded.

The inoculum of the *C. albicans* to be tested was prepared as follows: a suspension of the fresh cultured yeast on SDA equivalent to 0.5 McFarland standards was prepared in Muller Hinton broth. Then diluted 1:20 in the same broth (according to CLSI instructions), then 100µl of the yeast suspension was added to all wells and mixed by shaking the plate on the bench. The final concentration of the antifungal agent in each well will then be 256,128, 64,

32,16,8,4,2,1, 0.5 and 0.25μg/ml. These steps followed for each antifungal agent and each C. *albicans* isolate following aseptic technique.

The plates incubated at 35$^{o}$ C for 24 hours. Interpretation of the plate done by comparing growth of the yeast in the Enzyme linked Immunoassay (ELISA) wells with the positive control. In addition, we confirmed the absence of growth in the un-inoculated broth medium. The last tube that has no growth considered as the MIC. (Clinical Laboratory Science Institute-28$^{th}$).

## V.7 Extraction and MIC of garlic oil :

It was published that fresh garlic oil is more effective as antifungal agent in comparison with commercially available oils (Al-Alsalam Factory). We found that there are several extraction methods to obtain the oil of garlic , one of these methods is concentrated on extracting oil from white and purple skin cultivars of garlic and delivering it in isopropanol as organic solvent , but they show some disadvantages through its antifungal activity against fungi spp. [59].So, we tend to search about other alternative extraction method , for obtaining sufficient yield of garlic oil with high potency against *C.albicans* with keeping on its chemical composition such as diallyl disulfide and diallyl trisulfide . We found many researches which made a comparison between garlic from various geographic locations, they act on extracting their oil by using hydro distillation method, garlic of different site of origin show qualitative similarity but there is quantitative difference

in their chemical compositions of organosulfur compounds in these extracted oils , their results are confirmed by using Gas chromatography technique [74]**.**
On other hand, other researchers act on making a combination of different extraction processes including; steam distillation, heat exchange and emulsion filtration [75]. We performed our extraction procedure according to these trials, as this method ensure the yield nature and consistency of garlic oil and their intact constitute of sulfur containing compounds, which are presented in the sufficient quantities for its antifungal role.

We followed the same exact procedure for all experiments conducted on the garlic oils. The same procedure conducted with antifungal agents repeated here for disc diffusion plate method and broth microdilution testing for assuming the minimum inhibitory concentration for garlic oil against C. albicans. The results analyzed in a similar manner as mentioned above. (Clinical Laboratory Science Institute-28$^{th}$).

## V.8 RNA Extraction Method:

RNA extraction done using the ISOLATE II RNA Mini Kit (Bioline, meridian Bioscience, USA):

At first, sample homogenization done by a washing step from the Muller Hinton broth or the oil suspension treatment using sterile distilled water. This followed by adding 350µl of Lysis Buffer RLY and 3.5 µl ß-ME to the sample pellet with the addition of some glass beads to each tube and vortexed vigorously and incubated at 48 ◦C.

In summary, RNA Extraction procedure done as follows:

Cell lysis, Filter the lysate, Adjust RNA binding conditions, Bind RNA, Desalt the Silica membrane, Digest DNA, Washing and drying silica membrane and Elution of the RNA and the determination step performed by using Nano-drop instrument. The steps shown in Figure 2. (Bioline Company/ Isolate II RNA Mini Kit Protocol).

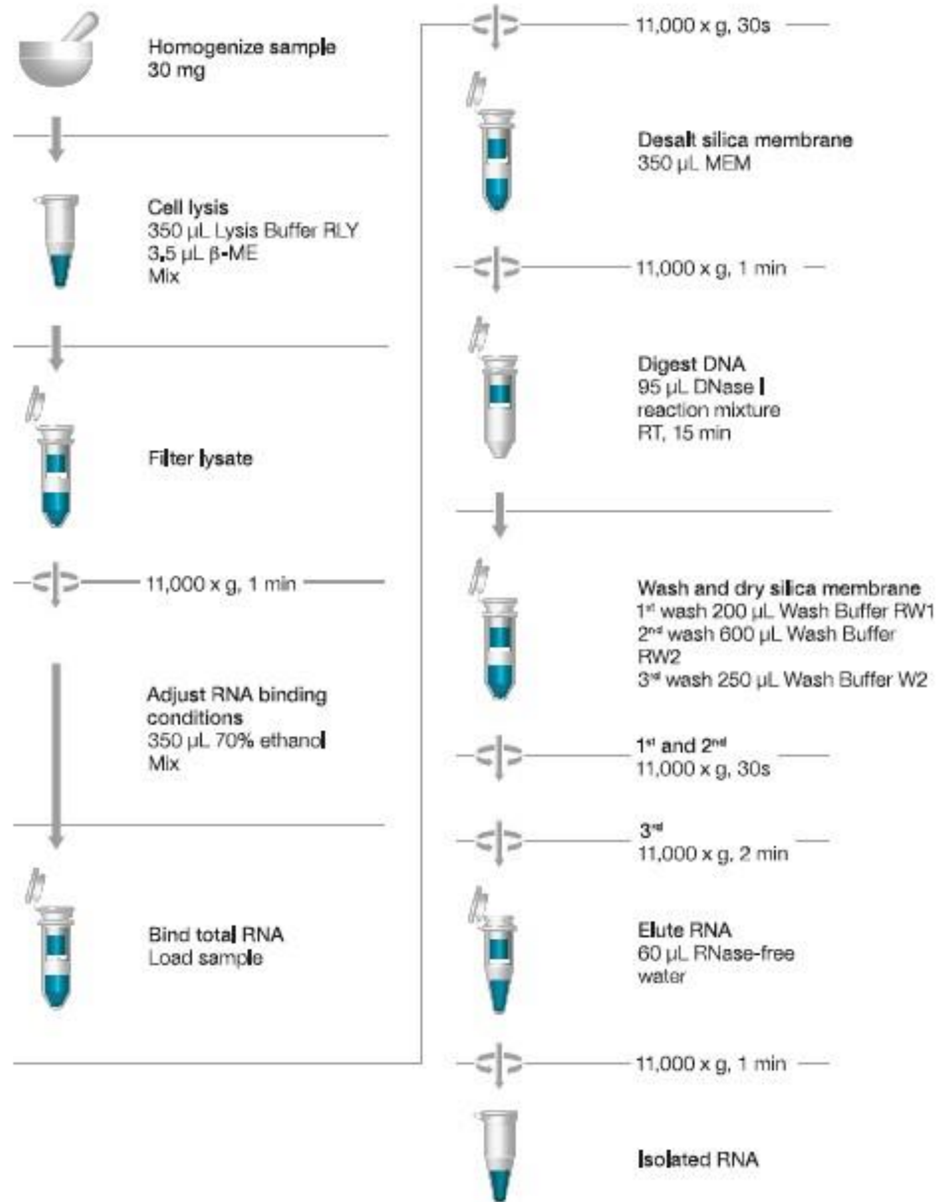


Figure 2: stepwise illustration of purifying total RNA from C. albicans (Bioline Company/ Isolate II RNA Mini Kit Protocol).

## V.9 Complementary DNA (cDNA) Synthesis:

by (Tetro CDNA Synthesis kit , to generate cDNA from an RNA template, that is intended for the use in the PCR applications as gene specific primers ( e.g. Real time PCR ) , optimization of cDNA synthesis of the yeast samples was done as follows in Table 2:

**Table 2:** Optimization conditions of cDNA synthesis for the yeast samples

| | |
|---|---|
| Water ( DEPC-treated water ) | 9.5 µl |
| Primer 1 ( Oligo dt $)_{18}$ | 1 µl |
| Primer 2 ( Random Hexamer ) | 1 µl |
| RT Buffer 5x | 4 µl |
| RiboSafe RNase inhibitor | 1 µl |
| 10mM dNTP mix | 1 µl |
| RNA template | 2.5µl |
| Total | 20µl |

Two primers were used; the Oligo dt primer which stands on the presence of poly-A tail of the 3' end of most eukaryotic mRNAs. Whereas the Random Hexamers bind randomly to the whole sequence of RNA, so cDNA pool of several lengths. After preparing the master mix of certain volume according to the no. of RNA templates, we prepared the quantity required of cDNA required; we mixed the mixture and then

using a thermocycler (BIO RAD, T100 Thermal Cycler) for cDNA program as follows:

The starting incubation point due to the use of Random Hexamer primer was 25°C for 10 min. then at 45°C for 30 min – 45 min. The reaction finished by raising the temperature to 85°C for 5 min. followed by chilling on ice. The resulted cDNA was determined by using the housekeeping gene (18s rRNA) and C. *albicans* genes by gel electrophoresis. The cDNA put at -20°C for long-term storage or used directly for the real time PCR application. (Bioline Company/ Tetro cDNA Synthesis Kit Protocol).

## V.10 Gel electrophoresis:

determination of RNA and cDNA products with the housekeeping gene18s rRNA and specific primers of *C. albicans* genes: 0.5g agarose (***SIGMA Agarose powder*** A-9539) was added to 27ml TAE 1x buffer and heated to boil in the microwave. After cooling, 2µl of ethidium bromide stain added to the gel, mixed and poured in a tray with a comb. After solidification, the comb gently removed, and the wells were loaded with two µl of each sample containing a loading dye, except the first well where 2ul of the molecular weight markers placed. (*SIGMA-individual solution for reliable identification-electrophore*sis2000).

## V.11 Comparative Real time PCR :

For Real time PCR or quantitative PCR (qPCR), the GoTaq Master Mix A6001 and A6002 of Promega kits used. The BRYT Green Dye is a Fluorescent DNA binding dye wadded to all samples. The used reference dye is the carboxy-Xrhodamine (CXR). After qPCR primers reconstitution (20X) we prepared a working concentration of 200nM to 1µM (1X).

The used BRYT dye is excited at 493nm and emitted at 530nm, similar to the excitation and emission the SYBR green dye.

The reagent has low level of carboxy-X-rhodamine (CXR) reference dye, GoTaq Hot Start Polymerase, $MgCl_2$, dNTP and reaction buffer. The real time PCR instrument that we used is" the Applied Biosystems 7500 Real Time PCR system".

- After GoTaq Master Mix and Nuclease-Free Water thawing at room temperature and 3-5 seconds mixing by vortex at low speed. The final reaction volume was 20µl. After preparing the number of reactions including waste to avoid pipetting errors, 16µl of the master mix into each well of Real Time PCR wells and 4µl of each sample to the sample wells and water for the blank, no template wells added, the plate was sealed and centrifuged. The running program was 40 cycles of 95°C Hot Start polymerase activation and denaturation steps for 1 minute and 15 seconds/cycle, respectively. Then 55°C (as it's the suitable mean annealing temperature for each qPCR primers that we used) for annealing and

extension steps for 1 minute/cycle.( Real Time PCR/Go Taq qPCR Master Mix/Promega protocol).

Primers that are used in our study are chosen on the base of their specific genes incorporation through the *C.albicans* cell cycle mechanisms ,referring to previous articles which focused on studying the responsible genes which increase the *C.albicans* capacity and tolerance to the harsh environments and it's ; e.g. PH changes , osmotic stresses , temperature variation ,etc., this can be applicable by its morphogenesis transition [76], and we make google alignment for each gene role and their related regulatory genes e.g. EFG1 gene which involves in cell wall dynamics regulation during yeast to hyphae transition as well as CPH1 gene . EED1 gene involved in hyphal extension on solid surfaces and during host cells interaction. CST20 serine/threonine protein kinase expression, play a role in conjugation with HST7 gene in mating pheromone response and cell polarity and cycle regulation. CYR1, involved in filamentation regulating process, phenotypic switching and mating. The activation of RAS1 gene is essential in cells pathogenicity through inducing the yeast-hypha transition. In addition, adhesion and invasion mechanisms of *C.albicans* is critical pathogenic factors which initiated as a response of hypha-specific gene ; ALS3 , which is also involved in early biofilm formation and so drug resistance . Another hypha specific G1 cyclin related protein 1 which expressed by HGC1 gene , as it regulated the hyphal morphogenesis of *C.albicans* species.HWP1 gene express the hyphal wall protein 1 , which incorporates in the benign and invasive *C.albicans* to host interaction. Whereas, the filamentous growth gene expression level and duration is controlled

under the action of UME6 gene, which expressed to transcriptional regulatory protein. Table 3 shows the used primers names, melting point, Sequence and their G+C content (Purchased by Labtech company in West Bank).

-

**Table 3:** The used primers names, melting point, Sequence and their G+ C content Labtech Company –Integrated DNA Technologies (Order No. 3027177)

| Name | Sequence | Tm | GC content |
|---|---|---|---|
| 18S rRNA (F) | 5'CGATGGAAGTTTGAGGCAATA3' | 52.9 | 42.9 |
| 18S rRNA (R) | 5'CTCTCGGCCAAGGCTTATACT3' | 56.4 | 52.4 |
| RAS1 (F) | 5'AGAACCGGTGAAGGGTTTTT3' | 54.7 | 45 |
| RAS1 (R) | 5'GCCAATGCTAATCCATCTTGA3' | 53.1 | 42.9 |
| CYR1 (F) | 5'CCAACAAACGACCAAAAGGT3' | 53.9 | 45 |
| CYR1 (R) | 5'TCTTGAACTGCCAGACGATG3' | 54.9 | 50 |
| EFG1 (F) | 5'CCCCCATACCTTCCAATTCT3' | 54.2 | 50 |
| EFG1 (R) | 5'CTCGTGGTCTGATTCCTGGT3' | 56.5 | 55 |
| HWP1 (F) | 5'TCTACTGCTC CAGCCACTGA3' | 57.8 | 55 |
| HWP1 (R) | 5'CCAGCAGGAATTGTTTCCAT3' | 53.3 | 45 |
| ALS3 (F) | 5'AATGCTGT TTTGGGTTGGTC3' | 54 | 45 |
| ALS3 (R) | 5'TCACCTGCCTGAAATTGACA3' | 54.4 | 45 |

| | | | |
|---|---|---|---|
| CST20 (F) | 5'ATGGGAAAAAGCGGAAAAGT3' | 53 | 40 |
| CST20 (R) | 5'TCCCACTCTATTGGCAAACC3' | 54.8 | 50 |
| HST7 (F) | 5'GTCAAACATTGAGCCGACCT3' | 55.5 | 50 |
| HST7 (R) | 5'TGGTTGTTGGAAGATGGTGA3' | 54.1 | 45 |
| CPH1 (F) | 5'TATGACGCTTCTGGGTTTCC3' | 54.8 | 50 |
| CPH1 (R) | 5'ATCCCATGGCAATTTGTTGT3' | 53.1 | 40 |
| EED1 (F) | 5'AGCAACGACTTCCAAAAGGA3' | 54.6 | 45 |
| EED1 (R) | 5'CGGTTTCTGGTTCGATGATT3' | 52.9 | 45 |
| UME6 (F) | 5'AGCACCAAATTCGCCTTATG3' | 53.6 | 45 |
| UME6 (R) | 5'AGGTTGAGCTTGCTGCAGTT3' | 57.5 | 50 |
| HGC1 (F) | 5'GCTTCCTGCACCTCATCAAT3' | 55.4 | 50 |
| HGC1 (R) | 5'AGCACGAGAACCAGCGATAC3' | 57.2 | 55 |

# VI. Results:

Ten High Vaginal Swabs (HVS) obtained from women suffering from fungal infection; the identification of *C. albicans* done on typical colonies growing on SDA plates. Germ tube and the morphology on Cornmeal agar done on all isolates. In addition to gel electrophoresis optimization of the *C. albicans* RNA and cDNA with the housekeeping gene (18s rRNA) and the *C. albicans* specific genes which were selected here for the study. Three samples remained as they collectively have the same characteristics to the *C. albicans* strain. We selected one of them for the RNA extraction, synthesizing its cDNA and for Real time PCR runs after treating it by using the fresh garlic oil suspension.

## VI.1 Colonies morphology on SDA agar:

We inoculated the samples by using Sabouraud Dextrose Agar at 37°C, creamy-white pasty colonies with distinct budding yeasts were resulted as ***shown in figure 3:***

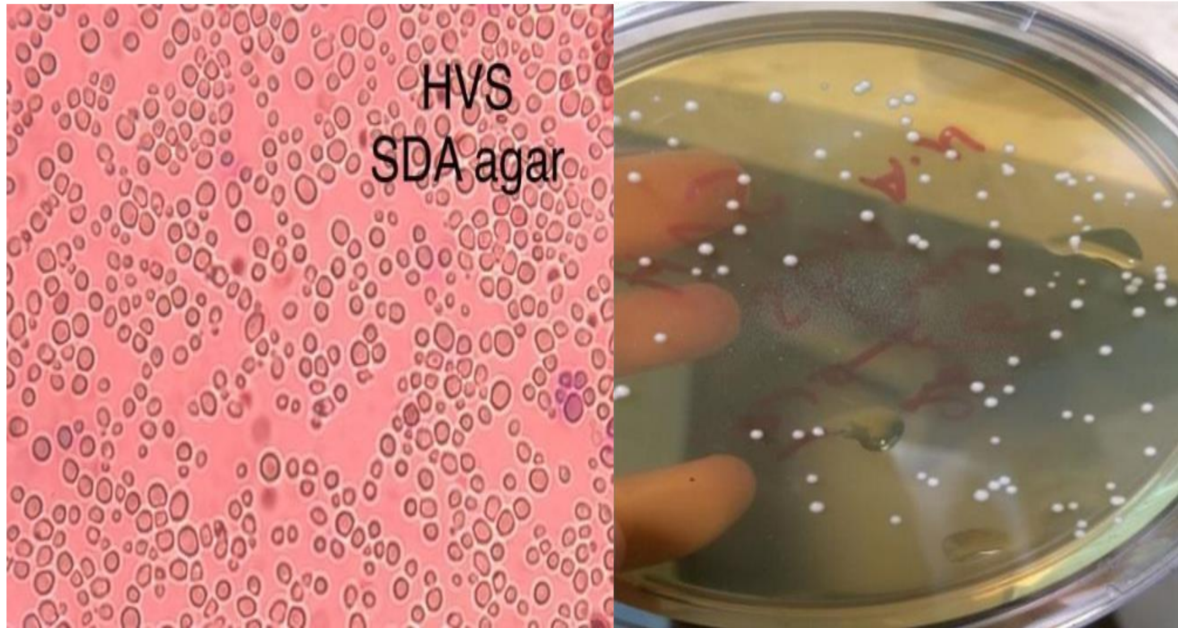


**Figure 3:** Shows the High Vaginal Swabs (HVS) colonies, which inoculated on SDA under the high power field microscope *on the left* and the characteristic colonies of the *C. albicans* on SDA agar *on the right.*

## VI.2 Germ Tube testing:

Typically, *C. albicans* forms Short germ tube when inoculated into rabbit serum and incubated for few hours at 37°C. This test used to identify *C. albicans* compared to other species [17]. The germ tube has parallel walls and no constriction at the point of origin at the blastospore mother cell. We found that some of the samples form germ tube without any constriction; as shown in Figure 4:

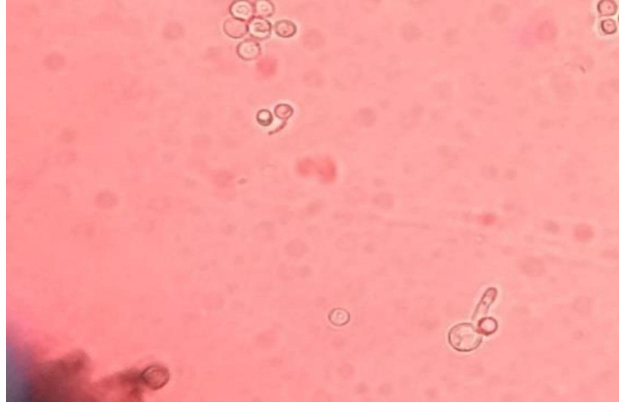

**Figure 4**: shows the germ tube forming without any constriction for *C. albicans.*

## VI.3 Cornmeal agar test:

We inoculated the germ tube positive samples on Cornmeal agar and placed them at room temperature for 24-72 hours, the formation of pseudohyphae, chlamydospores, elongated blastoconidia (blastospore) and other structures observed by examining the CMA plate under the light microscopic objective lens using reduced illumination.

As shown in Figure 5 shows the results of the germ tube positive samples on Cornmeal agar.

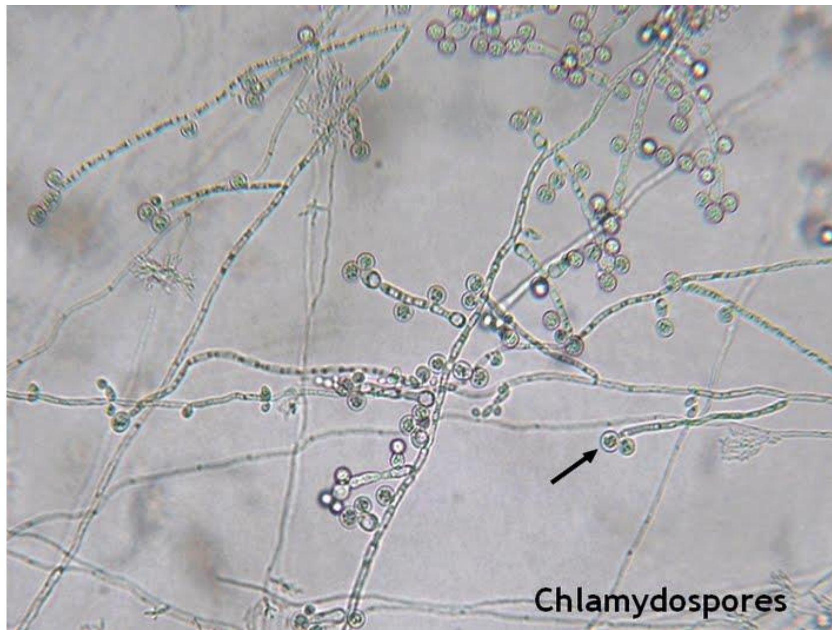


**Figure 5:** shows the appearance of chlamydospores production.

## VI.4 Antifungal Susceptibility Testing by Disc Diffusion:

After performing the previous identification tests for detecting *C.albicans,* we found that the three clinical isolates which are numbered as; 3, 6 and 8 have the correct features and characteristics for *C.albicans.* By antifungal susceptibility test using disc diffusion technique, We notice that sample no. 6 showed resistance for all Nystatin concentrations, while it was sensitive for Ketoconazole concentrations. The zone diameter of each concentration sensitivity for sample no.6 against Ketoconazole drug showed about 23mm, whereas the remaining samples (sample no. 3 and 8) showed resistance for all concentrations of both ketoconazole and Nystatin drugs. Sample no.5 excluded because it was not Candida species.

## VI.5 Broth microdilution antifungal Susceptibility Testing by Minimum Inhibitory Concentration by ELISA microreader wells:

From this test, we found that our selected samples were resistant to the all concentrations for both drugs, at even under low concentrations of them, the clinical isolates show resistance, and this is shown in Table 4 below:

**Table 4:** The Minimum Inhibitory Concentration of antifungal agents (both ketoconazole and Nystatin) against samples no. 3, 6 and 8:

| **Drug dilution concentration (µg/ml)** | **sample no. 3** | **sample no. 6** | **sample no. 8** |
|---|---|---|---|
| 256 | + | + | + |
| 128 | + | + | + |
| 64 | + | + | + |
| 32 | + | + | + |
| 16 | + | + | + |
| 8 | + | + | + |
| 4 | + | + | + |
| 2 | + | + | + |
| 1 | + | + | + |

## VI.6 Anti-fungal susceptibility testing and MIC of garlic oil:

Here, we found that the remaining samples (no. 3, 6 and 8) showed high sensitivity for all garlic oil concentrations and clear wells have been observed without any growth, sample no. 8 was sensitive even to the lowest concentrations whereas the other samples no. 3 and 6 showed resistance to the last two dilutions, MIC of 2µg/ml of the serial dilution, as we notice in table 5a. However, for purchased garlic oil we found that C. *albicans* samples showed sensitivity to its high concentrations but showed resistance to the lower concentrations, approximately MIC of 32µg/ml of the serial dilution, as shown in Table 5b.

**Table 5:** The Minimum Inhibitory Concentration of Fresh Garlic oil (a) and Purchased Garlic oil (b) against samples no. 3, 6 and 8:

| **a. Garlic oil ( Fresh ) Concentration (µg/ml)** | **Sample no. 3** | **Sample no. 6** | **Sample no. 8** |
|---|---|---|---|
| 256 | S | S | S |
| 128 | S | S | S |
| 64 | S | S | S |
| 32 | S | S | S |
| 16 | S | S | S |
| 8 | S | S | S |
| 4 | S | S | S |
| 2 | S | S | S |
| 1 | R | R | S |
| **b. Garlic oil (Purchased) Concentration (µg/ml)** | **Sample no. 3** | **Sample no. 6** | **Sample no. 8** |
| 256 | S | S | S |
| 128 | S | S | S |
| 64 | S | S | S |
| 32 | R | R | S |
| 16 | R | R | R |
| 8 | R | R | R |

| | | | |
|---|---|---|---|
| 4 | R | R | R |
| 2 | R | R | R |
| 1 | R | R | R |

**** R: Resistant, S: Sensitivite. Instead of R and S we can put + for resistant (growth) and – for S (no growth).**

## VI.7 RNA Extraction

RNA was extracted from *C. albicans* sample no. 8 that was treated and untreated with fresh garlic oil, as it has the highest sensitivity level under garlic oil treatment, so we can suggest that the treatment effect will be more obvious.

## VI.8 cDNA synthesizing (cDNA and PCR amplification of cDNA result on gel electrophoresis):

The cDNA was synthesized from the RNA product of the sample no.8 in its untreated and treated forms by normalization process as we make the RNA product concentration for both treated and untreated forms at an equal level of approximately 100 ng/µl as a template before cDNA synthesis, resulted cDNA was optimized with the housekeeping gene at gel electrophoresis as ***shown in figure 7 below:***

**Figure 7**: shows the electrophoretic image of the cDNA in the untreated (***band 1 and 3)*** and treated (***band 2 and 4)*** forms in an optimization process with the 18s rRNA as housekeeping gene.

## VI.9 Comparative Real time PCR; Curves, readings and Δ ΔCT

Because of RNA template normalization to a constant concentration, we obtained the same number of copies of the cDNA. We use the 18s rRNA as a reference gene which is not affected by any treatment processes ,as both the treated and untreated sample (no.8) forms has CT number of approximately 12 cycles, which confirm that the 18s rRNA is acting as housekeeping gene in all samples . Then we select 11 mechanisms of crucial roles through the fungal cellular processes ;( e.g. transcription regulation, extension and maintenance of filamentous growth, hypha production etc.) , which included EFG1, EED1, CST20, CYR1, RAS1, ALS3, HGC1, HWP1, CPH1, UME6 and HST7. In order to test behaviors under

treatment action by analyzing them. We use the comparative Real time PCR on 7500 Real Time PCR system of applied Biosystems AB instrument, at the same conditions with 18s rRNA housekeeping gene, the CT and delta CT values of each mechanism, in relation to the housekeeping gene of each treated and untreated sample forms are shown in table 6. The results calculated by an Excel program sheet design of equations 1, 2 and 3 for ∆Ct, ∆∆Ct and fold change, as shown below;

**∆Ct = Ct (gene of interest) – Ct (housekeeping gene)** eq. 1

**∆∆Ct = ∆Ct (Treated Sample) – ∆Ct (Untreated sample average)** eq. 2

**Fold gene expression = 2^-(∆∆Ct)** eq. 3

According to these equations, the ∆∆Ct and the fold change of each mechanism illustrated in Table 7. We found that there is no significant variation between the treated and untreated samples in respect to the housekeeping gene. As the fold change of each did not exceed the 1.5 fold, so we concluded that the garlic oil treatment did not affect any of the selected mechanisms. For more confirmation, we repeated the run by using the sample in several dilutions e.g. 1:10 and 1:100, and obtained the same results.

The amplification plots for the 11 primers separately; RAS1,HST7,HWP1,UME6,ALS3,CYR1,CST20,CPH1,EED1,HGC1and EFG1, respectively of both forms; treated and untreated forms of the sample in 1:10 dilution shown in Figures (8-19), as the x-axis represents the cycle numbers and y-axis represents the instrument fluorescent intensity, according to these curves we found that each of the two forms have approximately the same Ct value ,as the two curves are close and when we drop a vertical line from the horizontal line of the point that the fluorescent signal cross the threshold line ,we determine the Ct cycle value , eventually the amplification plot for the water blank shown in Figure 19, as there is no template presented , so we won't detect any florescent signal .

**Table 6:** The Ct and delta Ct values of the treated and untreated sample

| Target Gene | Treated sample CT | Untreated Sample CT | Treated sample Delta CT | Untreated sample Delta CT |
|---|---|---|---|---|
| EFG1 | 22.64 | 21.95 | 10.23 | 9.55 |
| HGC1 | 22.80 | 21.92 | 11.41 | 10.41 |
| EED1 | 23.99 | 22.77 | 8.52 | 8.18 |
| HWP1 | 21.10 | 20.54 | 14.23 | 13.72 |
| CST20 | 26.80 | 26.09 | 8.28 | 7.76 |
| CPH1 | 20.861 | 20.121 | 7.63 | 8.06 |
| UME6 | 20.20 | 20.43 | 9.20 | 8.29 |
| ALS3 | 21.78 | 20.65 | 10.36 | 10.12 |
| HST7 | 22.94 | 22.48 | 14.39 | 14.48 |
| RAS1 | 26.97 | 26.85 | 13.70 | 13.84 |
| CYR1 | 26.27 | 26.21 | 10.07 | 9.59 |

**Table 7:** The calculated ∆∆CT values and Fold change of each targeted mechanisms

| Target gene | ∆∆CT | Fold Change |
|---|---|---|
| EFG1 | 0.48 | 0.72 |
| HGC1 | 0.68 | 0.63 |
| EED1 | 1.00 | 0.50 |
| HWP1 | 0.35 | 0.79 |
| CST20 | 0.51 | 0.70 |
| CPH1 | 0.53 | 0.69 |
| UME6 | -0.44 | 1.35 |
| ALS3 | 0.91 | 0.53 |
| HST7 | 0.24 | 0.84 |
| RAS1 | -0.09 | 1.07 |
| CYR1 | -0.14 | 1.10 |

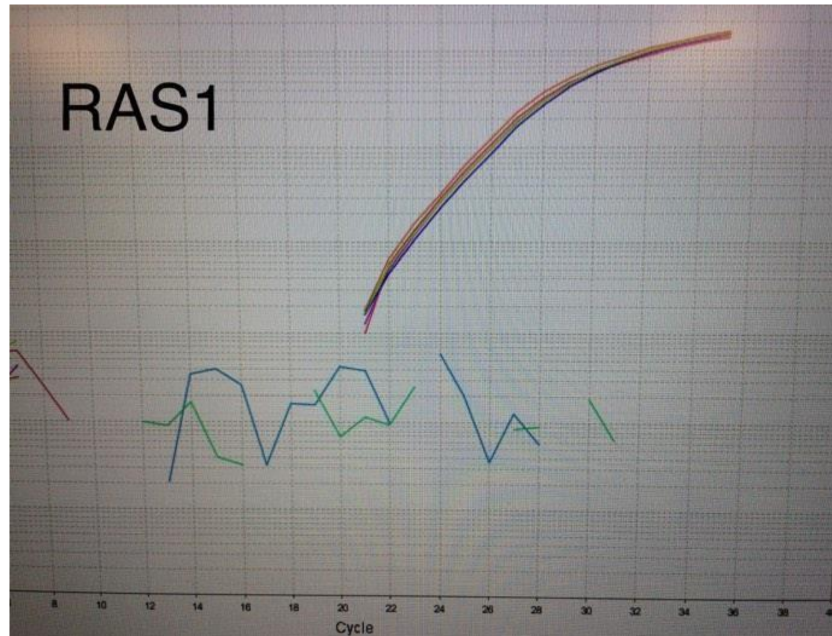


Figure 8: illustrates the amplification plot for the RAS1 primer of the treated (Left) and untreated (Right) sample forms.

Figure 9: illustrates the amplification plot for the HST7 primer of the treated (Left) and untreated (Right) sample forms.

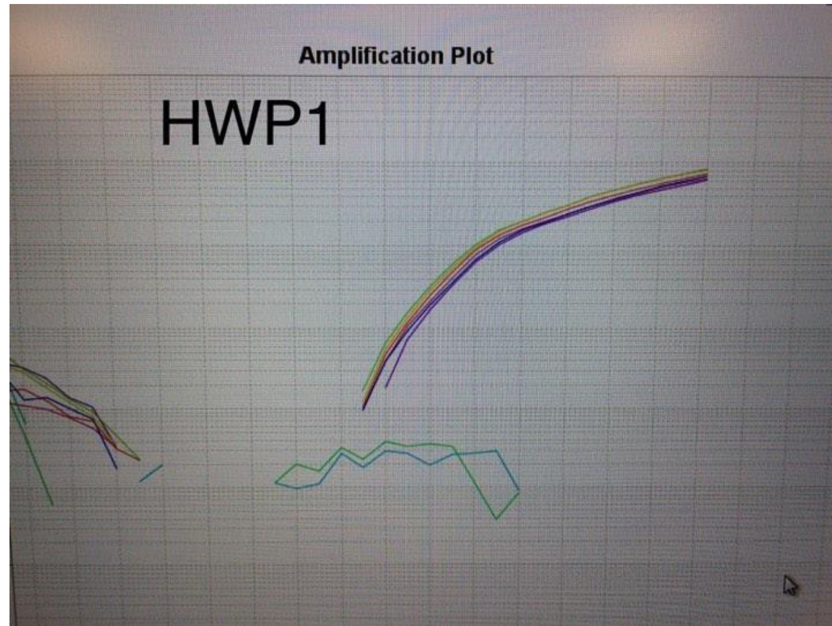


Figure10: illustrates the amplification plot for the HWP1 primer of the treated (Left) and untreated (Right) sample forms.

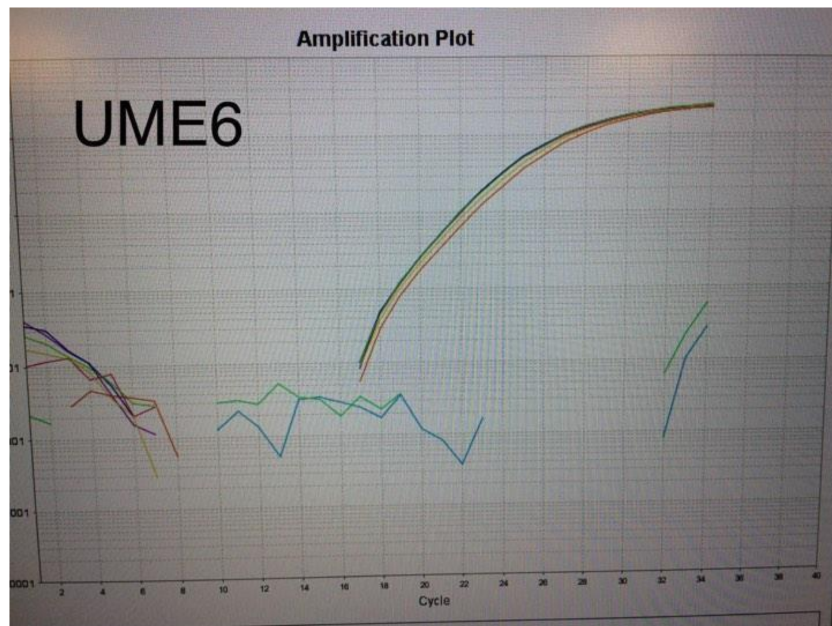

Figure11: illustrates the amplification plot for the UME6 primer of the treated (Left) and untreated (Right) sample forms.

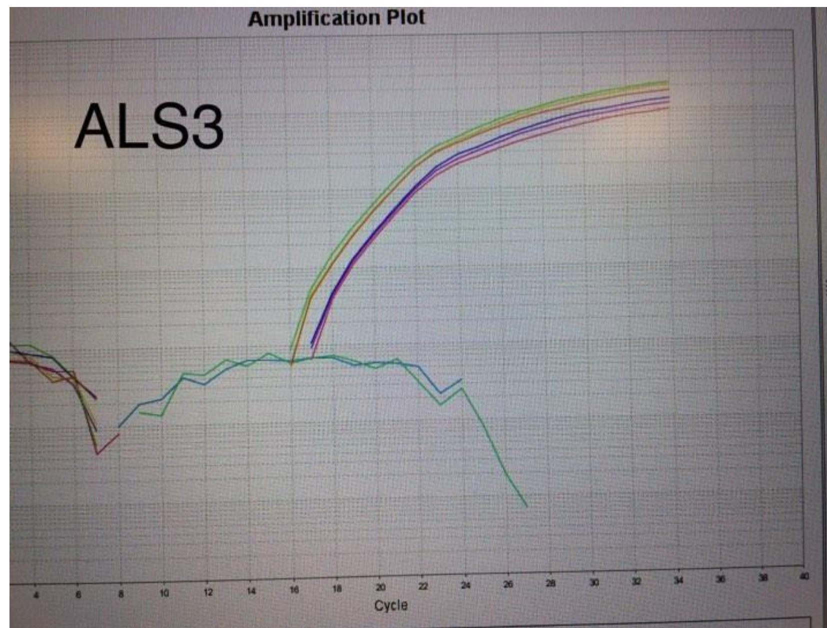


Figure12: illustrates the amplification plot for the ALS3 primer of the treated (Left) and untreated (Right) sample forms.

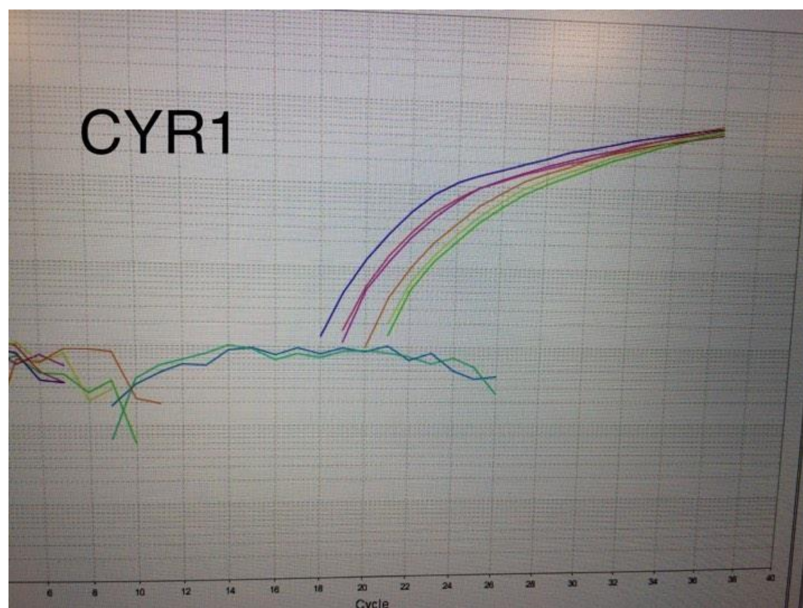

Figure13: illustrates the amplification plot for the CYR1 primer of the treated (Left) and untreated (Right) sample forms.

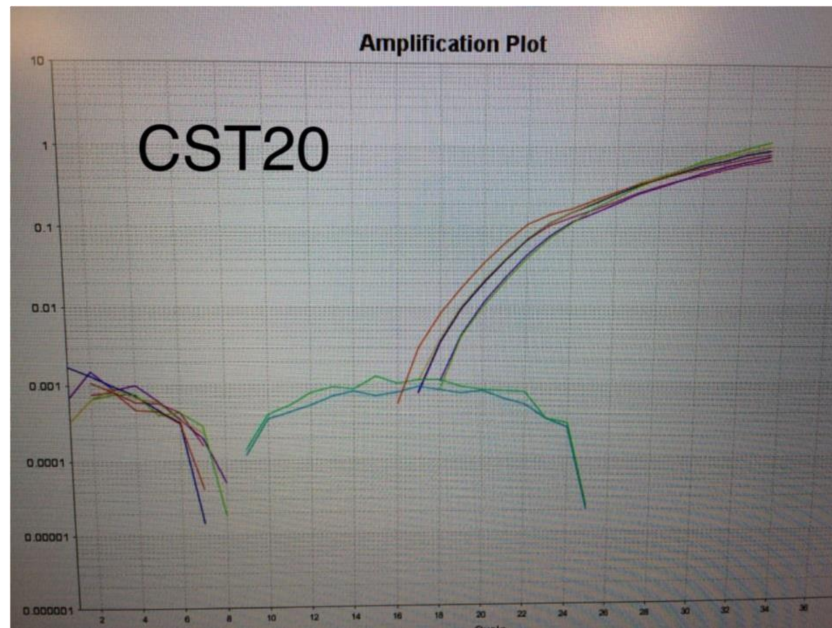


Figure14: illustrates the amplification plot for the CST20 primer of the treated (Left) and untreated (Right) sample forms.

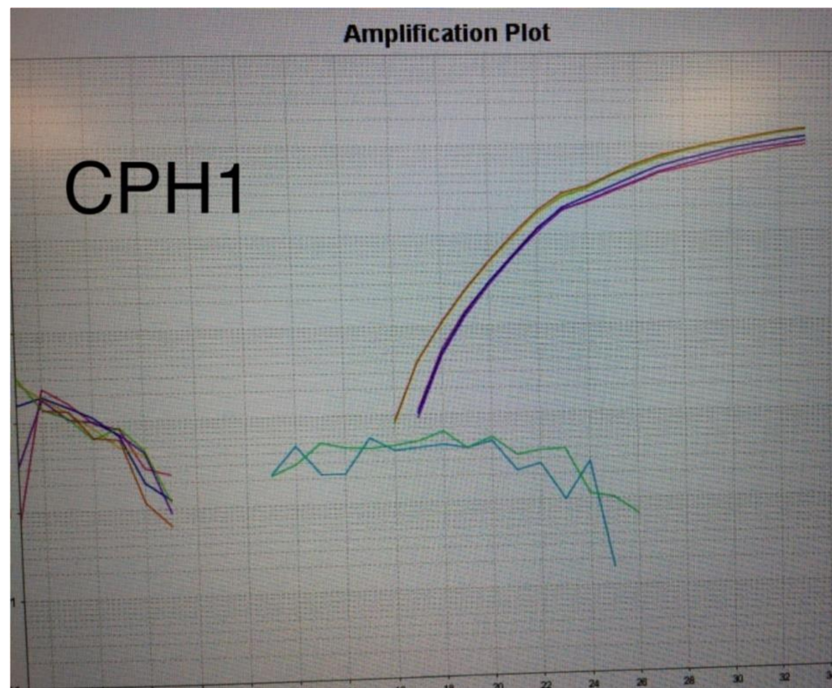

Figure15: illustrates the amplification plot for the CPH1 primer of the treated (Left) and untreated (Right) sample forms.

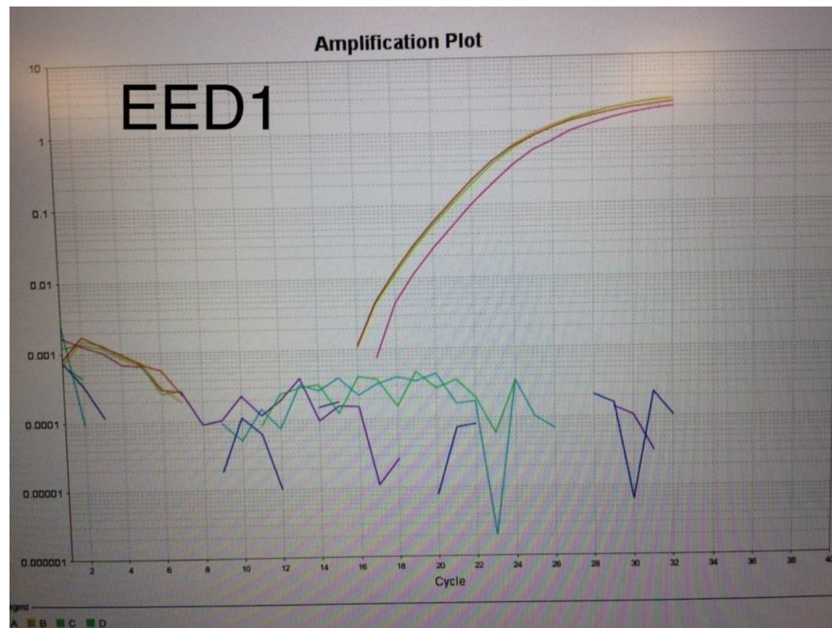


Figure 16: illustrates the amplification plot for the EED1 primer of the treated (Left) and untreated (Right) sample forms.

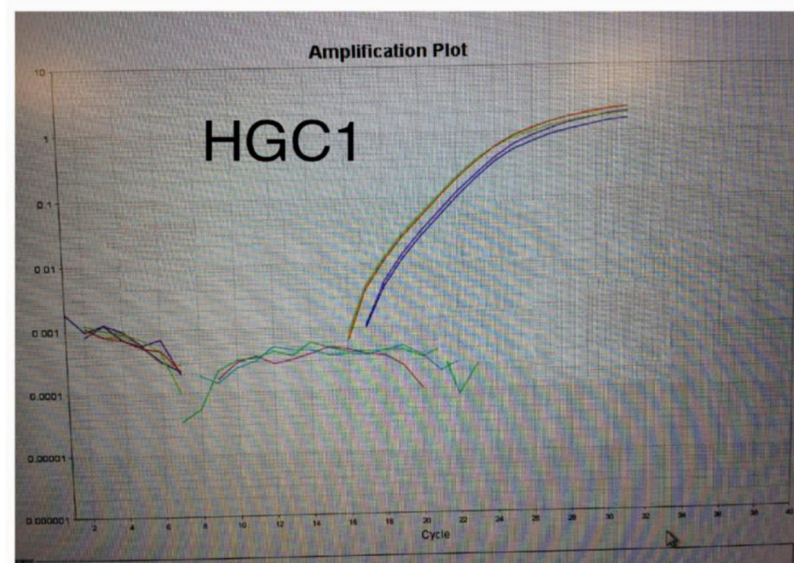

Figure 17: illustrates the amplification plot for the HGC1 primer of the treated (Left) and untreated (Right) sample forms.

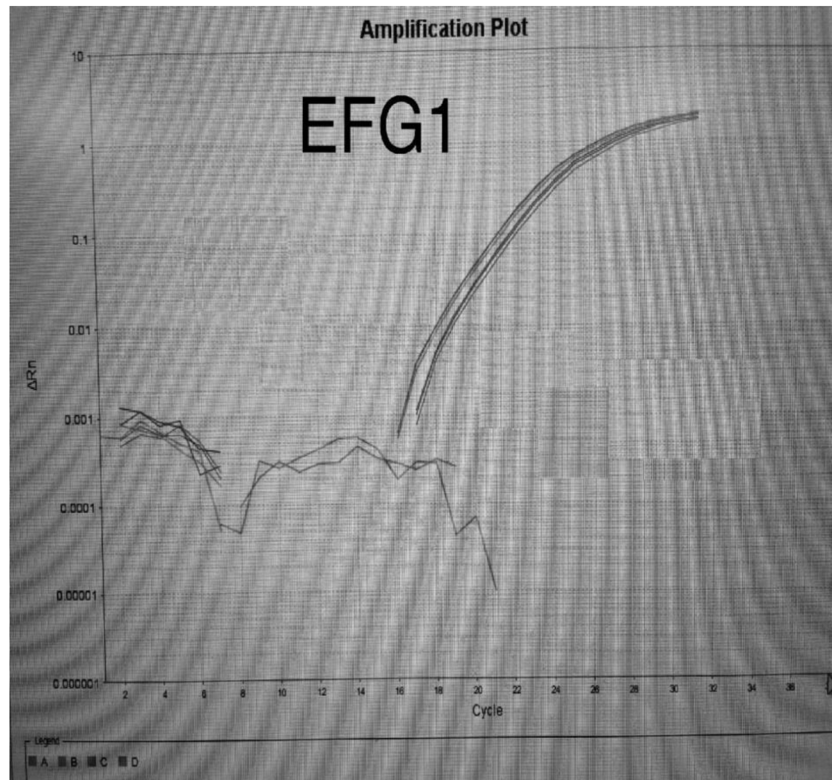


Figure 18: illustrates the amplification plot for the EFG1 primer of the treated (Left) and untreated (Right) sample forms.

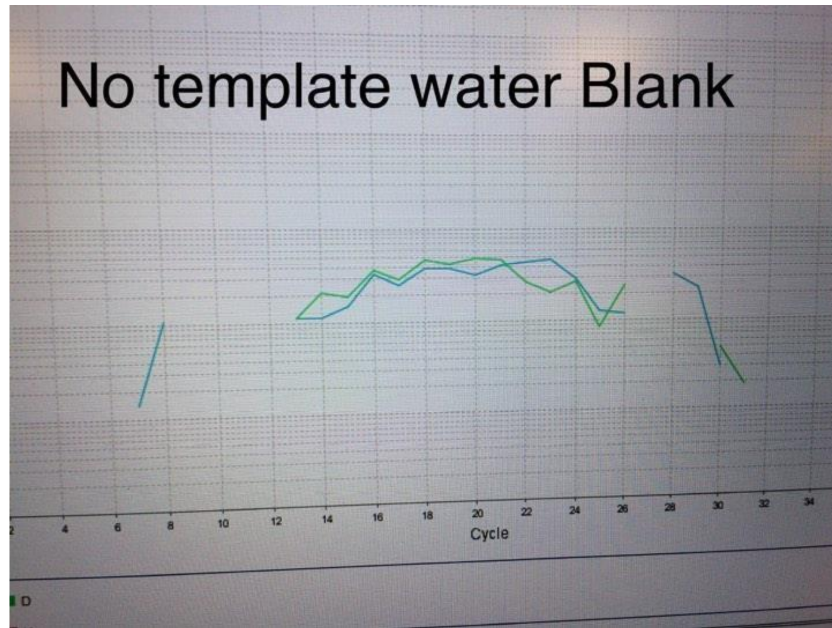


Figure 19: illustrates the amplification plot for water Blank.

# VII. Discussion:

This study demonstrates a correlation between the garlic oil use as a therapeutic agent and the down growth of the *C. albicans* strains but the exact molecular mechanism is still unclear. In agreement with a study through this year about essential oils used as antifungal agent against *C. albicans* and other species as it reduces the hemolytic activity in *C. albicans* and *C. auris* strains. It was revealed that even at minute levels, essential oils have effective antifungal and anti-hemolytic activities in vitro against *C. albicans* and *C. auris* by using the leaf essential oils [77].

Similarly, other study in 2019 carried out the comparison of the minimum inhibitory concentrations of garlic essential oil which showed that *C. albicans* was the most susceptible *Candida* species to this plant's essential oil. In other words, garlic essential oil inhibited the fungal growth at the lowest concentration. As they used Fresh Garlic (*Allium sativum*) which consist of a sulfur compound that is composed of an amino acid (cysteine) called Alliin. The compounds in garlic divided into two groups of sulfur and non-sulfur-containing types. In this study, the standard specimens of different Candida species were cultured on Sabouraud dextrose agar and chromogen agar *Candida*. The extracted essential oil was stored in sterile-container .This happened by using lid at 4°C for laboratory analysis purposes. The extraction was accomplished using hydro-distillation by means of Clevenger apparatus [78].

In this study, all the *C. albicans* that obtained from the respondents had similar degree of resistance to the standard antifungal and the test antifungal in garlic oil.

In our study, Creamy-white pasty colonies with distinct budding yeasts isolated from clinical vaginal swab of pregnant women with severe infection, which showed germ tube formation with no constriction in rabbit serum, and the formation of chlamydospores, blastoconidia (blastospore) and pseudohyphae on CMA. These findings confirmed the identification of the *C. albicans* species. The samples treated by using several antifungal agents. We tested these isolates against antifungal agents (Nystatin and ketoconazole), the samples showed high resistance to them all.

6 of the 10 clinical isolates from pregnant women with severe infections have been found that the causative agent was *C. albicans.* This corresponds to Martha F. Mushi team study on pregnant women with clinical features of vaginitis attending antenatal clinic in Mwanza, Tanzania , the obtained results showed that about two-third of pregnant women was confirmed by laboratory tests to have Candida vaginitis, the main causative agent was *C. albicans* . By returning to the history of these pregnant women of having antibiotic administration [79].

The widespread resistance of many antifungal drugs has been the result of recurrent misuse of antifungal agents. However, the colony pathogenic forms were susceptible to ketoconazole and garlic oil at all concentrations. Ketoconazole is polysaccharides and glycoprotein receptors on the fungal cell wall, the colony forming isolates were susceptible to this topical azole. Similarly, the garlic oil contains oil derivatives that can solubilize the polysaccharide backbone of colony forming fungi, therefore having an antifungal effect on the fungi isolates obtained from the study [80, 81].

The disc diffusion and broth microdilution susceptibility testing considered as the simple applicable methods for testing the garlic oil effectiveness against the *C. albicans* strains growth. We found that the agar plates and the microdilution methods have the same results, which inhibit the growth of *C. albicans* although at high concentrations of the oil which confirm the previous researches findings [82]. Previous studies act on investigating the effect of these antifungal agents either in separate manner or in combination investigated by many researchers as the incidence of invasive fungal infections has been elevated, specifically among patients who undergo transplantation procedures, hematological malignancies and immunosuppression drug administration.

The trials has focused on the field of combination treatment to study the possibilities of synergism between many antifungal agents as a result of there is no consistency between the rate of antifungal resistance and the development of new antifungal agents. Even though these trials have many limitations including; undefined efficacy and safety of combination of antifungals, the high cost, the reduced numbers of cases, but the worse complications of the invasive candidiasis revealed of little useful studies suggest that by using the drugs in combination with different concentrations results in either antagonism or synergy effect. However, this is still under investigation to estimate the level of effectiveness or toxicity [83].

We attempted to use the garlic oil which was freshly prepared, garlic peeled and crushed into garlic puree. Emulsified with water, filtering the emulsion by using a micro-filtration membrane. We made a comparison between the fresh garlic oil and purchased ones from markets. We found that the clinical isolates show susceptibility (90%) to the fresh oil at its high and even low concentrations. But the results obtained from purchased oil showed that the clinical samples were resistant to this oil at low concentration. According to the fact of organosulfur compounds that are found at highly unstable levels, and the potency of garlic oil will be impaired as it's bioactive components of natural sensibility is mostly attributed to thermal degradation. [84].

Hoda S. EL-Sayed and her team aimed to study the chemical composition of garlic essential oils (GEOs) by using Gas chromatography and Mass Spectrum GC-MS analysis. Their trials focused on evaluating its antifungal activity, by comparing between three methods of oil treatment including organic solvent (isopropanol). Water-based emulsion and water-based microemulsion , the results revealed that the use of organic solvent leads to dose dependent antifungal activity against the tested pathogenic bacteria and fungi .The second method totally suppressed the antifungal activity of GEO .Eventually, the third one showed better antifungal activity than emulsions at the same concentration of GEOs [59].

It found by Wen-Ru Li and his team in 2016, that the garlic oil has a critical role in the fungal cells death as seen with *C. albicans*, through damaging their organelles by membrane destruction. These data collected by RNA sequencing technique through the 19KEGG pathway cluster genes such as; oxidation-reduction response genes and pathogenesis genes. They concluded that some of the critical expressed proteins have been downregulated and the others upregulated without determination of their identity by using the two dimensional fluorescent difference gel electrophoresis (2D-DIGE) analysis and mass spectrometry [58].

In our study , we ran specific primers of the *C. albicans* genes which included ;*18s rRNA, EFG1,EED1,CST20,CYR1,RAS1,ALS3,HGC1, HWP1,CPH1,UME6 and HST7* evaluating them by real time PCR in synchronization with gel electrophoresis examination. We found that there was no significant variation between the treated and untreated samples in respect to the housekeeping gene, as the fold change of each did not exceed the 1.5 fold, so we concluded that the garlic oil treatment does not affect any of the selected mechanisms.

Further investigations are required to understand the exact molecular mechanism of the garlic oil as antifungal agent, which act as powerful natural alternative compound instead of the traditional antifungal agents, which show no effective action on overall fungal species.

# VVI. Limitations:

There may be some possible limitations in this study. First, there are very little prior researches on our specific topic. The second limitation concerns the ethical and personal issues especially pregnant women, which affect the data gathering process. In addition, our research is performed on specimens of limited time among pregnant women in 2019, as after our undergoing crisis with Coronavirus, people tend to use the natural crops e.g. garlic, onion, etc. which can make a big improvement at their health. So, through this time we lack the opportunity to measure these changes.

# IX. Conclusion:

Currently, there is an increasing misuse of antifungal agents concerning the recurrent cases of fungal infections. This observed in particular, among married women with frequent miscarriage and various others complains. Such misuse has eventually leaded to the evolution of high resistance rate to the available antifungal agents by many fungal species. This directs the medical researchers toward studying the effectiveness of the natural compounds according to their availability, cost, low toxicity and their fungicidal effect specifically on the fungal cells. One of these natural compounds that we studied is the garlic oil. We found that fresh form of this oil has a high affectivity against the *C. albicans* cells. However, the molecular mechanisms of the fungicidal effect of the garlic oil still need further investigations. We did not find any significant variation between the garlic oil treated and untreated sample with respect to the tested *C. albicans* primers in our study.

# X. Recommendations:

Recently, extensive research conducted to test the antifungal activity of natural herbal extracts. The results of such research showed promising results. In our research, we showed antifungal activity of garlic oil against C. albicans using disk diffusion and MIC according to the recommendations of CLSI. However, we were not able to elucidate the molecular basis of resistance using several genes and real time PCR. This could be due to the small sample size among other things.

Therefore, we recommend further testing of garlic oil extract against fungal isolates including yeasts and molds. In addition, we recommend choosing genes that encode enzymes that have various inhibitory targets of the fungal isolates. This important field deserves more attention and further research, by more studying efforts on genes down or upstream to the *C.albicans specific* cell cycle genes, which are regulated by them e.g. EED1 gene which is play an important regulatory role in hyphal extension, regulate the expression of ECE1, AGP2, NRG1, SOD5, etc., as these information gathered by Global clustering of transcriptional profiles of C. albicans project [85].

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