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**IN-VITRO EFFECTS OF ORANGE PEEL
EXTRACTS ON CANDIDA ALBICANS, A MAJOR
SOURCE OF FUNGAL INFECTIONS**

Hikmat Ullah Khan USMAN

Master's Thesis

Supervisor

Asst. Prof. Dr. Şebnem GARİP USTAOĞLU

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The thesis titled IN-VITRO EFFECTS OF ORANGE PEEL EXTRACTS ON CANDIDA ALBICANS, A MAJOR SOURCE OF FUNGAL INFECTIONS prepared by HIKMAT ULLAH KHAN USMAN and submitted on 20/12/2022 has been **accepted unanimously** for the degree of Master of Science in Biomedical Sciences.

Assistant Professor- Tuğçe Naime

GEDİK

Co-Supervisor

Assistant Professor – Şebnem Garip

USTAOĞLU

Supervisor

Thesis Defence Committee Members:

Professor - Feride SEVERCAN

Department of Biophysics,
Altınbaş University

Associate Professor – Rafiq
GURBANOV

Department of Bioengineering,
Bilecik Şeyh Edebali
University

Assistant Professor - Şebnem Garip
USTAOĞLU

Department of Medical
Biochemistry,
Altınbaş University

I hereby declare that this thesis meets all format and submission requirements of master's thesis.

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I hereby declare that all information/data presented in this graduation project has been obtained in full accordance with academic rules and ethical conduct. I also declare all unoriginal materials and conclusions have been cited in the text and all references mentioned in the Reference List have been cited in the text, and vice versa as required by the abovementioned rules and conduct.

Hikmat Ullah Khan USMAN

Signature

DEDICATION

First and foremost, thanks to the almighty Allah.

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Finally, I want to dedicate my research thesis to all those Afghan girls who got banned from schools and universities in current Taliban regime in Afghanistan. They have even been deprived of work opportunities and some basic human rights in the society. I am confident that the people of Afghanistan will get rid of Taliban one day.

ABSTRACT

IN-VITRO EFFECTS OF ORANGE PEEL EXTRACTS ON *CANDIDA ALBICANS*, A MAJOR SOURCE OF FUNGAL INFECTIONS

Usman, Hikmat

M.Sc., Biomedical Science, Altınbaş University

Supervisor: Asst. Prof. Dr. Şebnem Garip Ustaoglu

Co-Supervisor: Asst. Prof. Dr. Tuğçe Naime Gedik

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Candida albicans is the part of our natural microflora but also responsible for mucosal and systematic infections that can lead to death in individuals with malnutrition, diabetes and immunocompromised individuals. These are the most common species which cause nosocomial diseases and are capable of developing resistance to antifungal antibiotics. The mechanism of action of antifungal drugs to be used against fungal infections are different from antibacterial drugs as fungi are eukaryotes and bacteria are prokaryotes. It is the first study to identify the dose range with the antifungal effect of various orange peel extracts *in Candida* fungus and the most appropriate orange extract to be studied using infrared spectroscopy with chemometric analysis, to find out the structural and functional effects of orange extract in fungal biomolecules with detailed spectral characterization. Thus, a novel method that can monitor the antifungal effect can be proposed with this study.

Based on the results of disc diffusion and microtubule dilution tests, we determined the concentration of the orange extract that we use for IR spectrum analysis which are OE50%, OE25%, OE12.5%, OE6% and two other groups named control (C) along with *Candida* (CA).

Different orange extract doses can show antifungal effects on *Candida albicans* by effecting the molecular structure and composition of the components of yeast. The most effected components were seen as saturated and unsaturated lipids with triglycerides. In contrast, the protein components of yeast seem to be not affected significantly from the antifungal effects of OE. There

is a dose difference in the antifungal effects of orange extract on *Candida albicans* samples. The best doses for the antifungal activity were determined as medium doses (25 % and 12,5 %) especially 25% OE dose. The highest (50%) and lowest (6%) doses show opposite effects on yeast when compared to the medium doses.

The optimal antifungal effect was seen with medium OE doses (12,5% and 25%) by affecting the saturated and unsaturated lipid concentration and composition of *C. albicans*. The protein changes were related to the changes of lipid metabolism of yeast related to the enzymes and regulatory proteins of that metabolism. There was no dose effect in the changes of nucleic acid and carbohydrate contents except low dose of OE (6%) which shows opposite effect for those parameters when compared to the other doses of OE.

The results of this study also showed that ATR-FTIR spectroscopy has the capacity to distinguish the antifungal effects of different doses of orange extract samples on the composition of *Candida albicans* species.

Keywords: *Candida Albicans*, Orange Peel Extracts, Fungal Infections, FTIR Spectroscopy

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ABBREVIATIONS

VAP	:	Ventilator associated pneumonia
CLABSI	:	Central line associated blood stream infection
CDC	:	Centres for disease and control prevention
MFC	:	fungicidal concentration
MIC	:	Minimal inhibitory concentration
ANOVA	:	Analysis of variance
GC-MS	:	Gas chromatography- mass spectrometry
FC	:	Fractional composition
VCD	:	Vibrational circular dichroism
UV	:	Ultraviolet
FT-IR	:	Fourier transformed infrared
OE	:	Orange Extract
MHA	:	Mueller Hinton Agar
MHB	:	Mueller Hinton Broth
ATR-FTIR	:	Fourier Transform Infrared-Attenuated Total Reflection
IR	:	Infrared

LIST OF SYMBOLS

μl : Microliter

% : Percentage

mg : Milligram

Kg : Kilogram

ml : Millilitre



1. INTRODUCTION AND PURPOSE

1.1. CANDIDA INFECTION

Certain fungal species are infectious to human health including yeast and molds while *Candida albicans* are mostly pathogenic among all (Gary P, & Moran *et al*, 2012), but various species of *Candida* well known reason causing both life threatening and non-life-threatening mucocutaneous ailments (Achkar & Fries, 2010).

Natural habitat of the *Candida* or yeast are the environment, but they are often associated with humans as normal flora colonization in their body as a normal flora. They can be found in different anatomical sites in human bodies including vagina, oral cavities, intestinal tracts and often urethra of males, while the new-borns get these very soon after birth. These species are known as non-harmful in human beings, but their grow is based on the opportunity to find suitable environment and nutrients to cause disease (Ruhnke M, 2006).

Normally they found on the human skin and insides of mucosal layers while they do not cause infection on ordinary level. The infections of the *Candida* are caused out of the control of human body physiology since it is an opportunistic infection when reaching deep into susceptible internal organs including brain, kidney, heart, or blood circulatory system (CDC. gov) as mostly in the case of fungemia. Mba *et al*, 2020).

The opportunistic behaviour of the *Candida* is not only restricted to a particular region or area, but it is opportunistic in the nature on worldwide level. It causes superficial, mucosal to the systemic, as well as reproductive tract and disseminated life threatening infections (Parveen Surain, 2013). Thrush, commonly known as candidiasis or mycosis, is a yeast infection that is caused by highly prevalent *Candida albicans*. Technical names for candidiasis include moniliasis and oidiomycosis. (William *et al*, 2006).

There are many different types of *Candida* species which can cause infection in human beings, and some are mentioned which are implicated in human beings, *Candida albicans*, *Candida lusitanae*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida guilliermondii*,

Candida parapsilosis, *Candida kefyr*. Many types of candidiasis are found during daily life exposure (Parveen Surain, 2013).

An outstanding property of its pathogenicity is capability to develop in yeast, pseudo hyphal and hyphal forms. The hyphal form has a vital role in causing disease by attacking epithelial cells as well as causing injury to tissue. *Candida* composed of many different types of species but the *Candida albicans* are mostly human opportunistic pathogen which can expose its morphology as dimorphic shape based on the incubation period and temperature. It normally causes the infections in yeast form (Lok b *et al*, 2021).

Human internal mucosal linings and anatomical areas are attacked by both *albicans* and *non-albicans* species of *Candida albicans*, but also *non-albicans*. These are also now a vulnerable threat to human beings and colonize human mucocutaneous surfaces (Rao, S.D. 2013). Infection of *Candida* is also affected by the host immune status and the infection differentiation among the individuals of same species is based upon the disease performance and presentations (Parveen Surain, 2013).

Fungi have become a major contributor to human illness, according to changes in how the subjects of interest have been treated medically and surgically during the past thirty years. *Candida albicans* are the cause of invasive infections comparatively higher than other fungal types, making it the most prevalent infection among the disseminated mycoses (Elizabeth, 2009).

To cause an infection of oral cavity, there are three factors which are required including oral mucosal environment, capable strain of the *Candida albicans* and the immune status of host. The pathogenicity is linked to hyphae of *Candida* (Ugun *et al*, 2007) while other yeast species have been progressively recognized, like *non-Candida albicans Candida* (NCAC) species (*Candida glabrata*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, *C. dubliniensis*, and *C. guilliermondii*) (Martins *et al*, 2010).

There are different types of candidiasis, and some common forms are mentioned here. *Candida* infections which are mostly restricted to the vulvovaginal areas, oropharyngeal surfaces and

many non-sterile surfaces are known as mucosal candidiasis (Shao hua *et al*, 2008). 30-50% population carries *Candida* as normal flora (Scardina *et al*, 2007). The common oral candidiasis is oropharyngeal candidiasis found in the patients but often found as well in the HIV infected patients (Dangi *et al*, 2010).

In Cutaneous candidiasis, the skin and nails of the host or immune compromised patients are usually infected with *Candida* as a secondary phase of the infection in both chronic and sub-acute phase. This type of infection can be localized infection as well generalized infection in the nails and the skin of the patients. There are different types of symptoms like intertrigo, diaper rash, paronychia, onychia, and otomycosis types (Mahmoudabadi, 2006).

Cutaneous candidiasis is found the moist and warm regions of the human body like inguinal, intergluteal areas and axillary folds of the body. It is also because opportunistic pathogen causing trauma and softening of the skin surfaces. The underline patients of diabetes are very susceptible and vulnerable people while the obesity is also a bad factor, not only these but the contraceptives and oral antibiotics have a tremendous bad effect (Xiao dong *et al*, 2008).

Currently, non-Albicans *Candida* species are highly causing illnesses (and antifungal-resistant *Candida* isolates) (Arora *et al*, 2011 and Pappas, *et al*, 2018).

Another type of the infection due to *Candida* in the vaginal areas of the female subjects of interest is often known as vulvovaginal candidiasis. During gynaecology consultations in primary healthcare, this illness is frequently taken into account. The condition is frequently linked to significant morbidity, discomfort, pain, increasing healthcare costs, disrupting sexual activity but it is rarely life threatening. (Gonzalez *et al*, 2011).

With systemic or disseminated candidiasis, the *Candida* cells spread throughout practically the whole body and have a propensity to produce abscesses in particularly critical organs, causing their failure and, in 50% of all cases, leading to fatality despite management of severe antifungal therapy (Zaoutis *et al*, 2005).

1.2. IMPORTANCE OF *CANDIDA ALBICANS*

The yeast *Candida albicans* are animal normal flora which lives as an imperfect yeast as a commensal in these animal hosts (Rippon, 1982 and Shepherd *et al*, 1985). The immune compromised individuals who have very weak immune system are the primary host for these microorganisms as they are very soon infected with any type of *Candida* species and pose them to very vulnerable condition of their health (Edwards JE, 1978 and Lerner C *et al*, 1984).

Not only the adults, but the infants are also come under the oral or genital thrush of the *Candida albicans*. If not treated with suitable antifungal drugs, the infection may exceed the pace of time and goes to deteriorate quickly. The *Candida albicans* can be found in both transitioned form hyphae and yeast shape depending on the treatment of temperature and humidity. They have different types of transition phenotypes i.e., cell shape, surface, and permeability. The hyphal genes correlating to hyphal shape are crucial for virulence in several fungal diseases which are being given discussed here.

Eukaryotic epithelial cells are disrupted through mechanical force by hyphae followed by penetration and growth. This is one of numerous processes through which hyphal development is known to improve virulence in *C. albicans* (Kumamoto & Vines 2005).

The conversion mentioned above are vital signs for their pathogenesis to cause (Soll D R, 1988) and is highly important biological procedure (Shepherd *et al*, 1985).

Based on molecular bases the DNA content of the *Candida albicans* are ranging from 0.1% 5-methylcytosine at the time of their growth in yeast shape while decrease subsequently if the growth phase converts to mycelial type (Russell *et al*, 1987). Reassociation kinetics (19.6 and 14.8 fg by different methods) are the methods used for the calculation of the total sizes of the genomes of *Candida albicans* well with the DNA-per-cell values if one assumes a diploid genome (Riggsby WS *et al*, 1982).

1.2.1. Nosocomial Infections

All infections contracted between 48 hours and 3 days after hospital admission are considered nosocomial infections. One in ten patients are affected hospital-wide, and they cost the National

Health Service a lot (around £1 billion). The four most prevalent infection types are urinary catheter-related infection, ventilator-associated pneumonia (VAP), central line-associated blood stream infection (CLABSI), and surgical site infection (Edwardson S *et al*, 2019). Nosocomial infections with mostly all the fungal species with relative frequencies are related to the strength of immunosuppression essential to incline them (Stolpman M *et al*, 2002).

Alterations in the immune system as the suppression and immunocompromising are the vital conditions to pose a patient to a life threaten bloodstream infection with *Candida albicans* and other *Candida* species. When such infections ascend in hospitalization time, they are called to as nosocomial infections. But species of *Candida* or *Candida albicans*, are accountable for most of the nosocomial fungal infections (Pfaller MA, 1996), exist in the natural microflora of a greatly of immune-competent persons as relatively benign commensal organisms (Soll DR *et al*, 1991).

Among all the nosocomial infection related to *Candida albicans* or other *Candida species*, the Urinary tract infections and nosocomial pneumonia are the primary two (Crump JA and Collignon PJ, 2000). Although they are less frequent in the community setting, candida UTIs are more frequently encountered in hospitalized patients. In patients with candiduria, colonisation or contamination is typically the most common cause (Odabasi, Z & Mert, A 2020).

Based on the ranking of the nosocomial infections, the *Candida albicans* is the fourth known major cause of all nosocomial infection isolated from hospitalized patients blood samples after *Enterococcus*, *Staphylococcus epidermidis* and *Staphylococcus aureus* (Jose A *et al*, 1993).

Since blood culture positive defines bloodstream infection, these datasets are constrained. Based on older autopsy & clinical data, it is known that 30 to 50% of people of interest had widespread candidiasis (Kami M *et al*, 2002).

1.3. TREATMENT OF CANDIDA INFECTIONS

There are many types of antifungal drugs, but the most common on clinical level are ketoconazole, amphotericin B, fluconazole, flucytosine and itraconazole. This research investigation has found that these drugs were less active against *Candida albicans* in biofilm

form compared to planktonic cell from. *Candida tropicalis* and *Candida parapsilosis* also resistant to the above-mentioned antifungal agents (Hawser SP and Douglas LJ, 1995).

Triazole i.e., fluconazole and itraconazole with low side effects and very efficient comparatively become first choice drugs and influence against *Candida albicans* (Liu X *et al*, 2011).

According to Kuhn DM *et al*. (2002), the new triazoles like ravuconazole and voriconazole also showed no effect against the biofilms of *Candida parapsilosis* and *Candida albicans*, however, the amphotericin B associated with the lipid ligands and two other echinocandins (caspofungin and micafungin) indicated some good antifungal effect (Kuhn DM *et al*, 2002).

The association of lipid ligands with the amphotericin B has found to be very effective against some infection diseased conditions like thrombophlebitis, abdominal abscesses based on the clinical experience of the clinicians and experts (Alexander BD *et al*, 2013).

Fluconazole taken intravenous or by mouth, is most common antifungal drugs for life threatening infections with *Candida albicans*, if not subsequently treated with fluconazole, the health practitioner may suggest other antifungal drugs, but treatment of esophagus infection is mostly treated with fluconazole according to (Cdc. Gov), but for the candidiasis of the mucosal linings the itraconazole is recommended, and the itraconazole is also recommended for the treatment of invasive form of candidiasis as well (Saag MS *et al*, 1999).

The oral and parenteral preparation of new azole like voriconazole is also effective for the oesophagus as the CDC stated before, and this drug is fortunately available parenteral and oral form (Ally R *et al*, 2001). The *Aspergillus* species and *candida* are also treated successfully with caspofungin and the echinocandin antifungal drugs (Kurtz MB and Rex JH, 2001).

Fluconazole and amphotericin B deoxycholate both can be considered as of choice for oesophageal and oropharyngeal candidiasis treatment (Sable CA *et al*, 2002). Though amphotericin B deoxycholate earlier used as ordinary antifungal drug for the choice therapy of invasive candidiasis, whose toxicity is highly valued. The amphotericin associated with lipid ligands were initially considered drug of choice for the amphotericin intolerant patients or those patients who have an infection problems to the deoxycholate type of amphotericin (Bates DW

et al, 2001), while amphotericin B deoxycholate seems to have parallel kinetics in adult and neonate patients (Van den Anker, 1995).

The solution of itraconazole cyclodextrin (5 mg/kg per day) given to children and infants indicated successful treatment and the choice level of concentrate in their plasma (Leclerc JM *et al*, 1998).

Amphotericin B is the reliable and accepted prescription and treatment option for the *Candida* infection, but the caspofungin given intravenous, oral, or intravenous fluconazole or amphotericin B and fluconazole combined with amphotericin B administered for the first 5–6 days only) is also the therapy of choice. On behalf of many health practitioners, amphotericin B combined with flucytosine is also effective in case of life-threatening severe type of *Candida* infections (Sobel JD, 2000).

1.3.1 Traditional Treatment of *Candida* and *Candida albicans* Infections

The infection with fungi especially *Cryptococcus neoformans* and *Candida* has posed a major public health concern in the human community from last two decades (Pfaller and Diekema, 2004). Treatment of fungal infection is possible with antifungal drugs but also may be possible with several medicinal plant extracts which are traditionally used to treat life threatening fungal infections (Obi *et al*, 2003).

According to a research study, in the Coast, Morogoro and Tanga locations of Tanzania, different plant species are identified with having potential of medicinal and pharmaceutical effect over the treatment of multiple *candida* and *candida albicans* infections (Runyoro DKB, 2006).

A research investigation conducted by (Steenkamp *et al* 2007) have shown the effect of multiple plant extracts over the *Candida albicans*. They had used *P. africanum*, *B. micrantha*; *C. transvaalensis*; *Ficus sycomorus* and *S. decussate* plant methanolic extracts while another study conducted by (Samie A *et al*, 2010), acetone and hexane were manipulated though having different polarity.

The potential of activity of the solvent are determined by difference in the polarities of the solvents (Samie A *et al*, 2010), conversely methanolic and water extracts of medicinal plants naming *C. transvaalensis* have not shown any fruitful activity against the clinical isolates of the *Candida albicans* while the extracts with hexane shown a highest level of antifungal activity against *Candida albicans* and many more clinical fungal isolates as well (Steenkamp *et al*, 2007).

The *W.salutaris* plant bark is used for cough, cold problems because it is used as expectorant, it can also be used as anti-inflammatory solution. Not only the bark, the leaves of this plant also have potential antifungal activities because it contains drimane sesquiterpenoids including waburganal and polygodial, and they are known to kill multiple *Candida* species and especially *Candida albicans* (Rabe & Van Staden, 2000).

Root extracts with acetone from the plant *T. sericea* can suppress the visible growth of *C. krusei* and *C. albicans* efficiently (Motlhanka *et al*, 2008). *T. sericea* extracts were also the likely effective among the 6 *Terminalia* species that were checked against *C. albicans* (Masoko *et al*, 2005).

A research investigation by Okemo *et al*, (2001) reported that neem plant crude extracts *Azadirachta indica* discarded the 8mg/ml *Candida albicans* in 24 hours, while Patel and Coogan (2008) reported killing of *Candida albicans* strains being treated with *Dodonaea viscosa* extracts for 30 minutes. Ethnos medical data from traditional healers offers a solid lead towards expansion of innovative medications discarding the idea of random screening. Now it is required to screen the extracts for implementing a bioassay-guided fractionation of the energetic extracts to isolate the active materials (Runyoro DKB, 2006).

As the traditional treatment or home remedy for the treatment of fungal species like *Candida albicans* and other *Candida* in the case of oral candidiasis, *Tulbaghia violacea* and *Allium sativum* plant bulb extracts are respectively used to be more effective with an MIC range of 0.56 to 3.25. As these plants showed good results so the scientists focused on *Glycyrrhiza glabra* and *Polygala myrtifolia* to investigate for use as an oral mouthwash in clinics and households (Motsei ML *et al*, 2003).

In traditional Chinese medicine, roundabout three hundred herbs have been identified with having “pesticidal” activities, some were also used as antifungal agents in hospital managements. *Galangin*, *magnolol*, *pseudolaric acid A*, *hnolkiol*, *Palmatine*, *Berberina* and *Allicin* have shown potential anti-Candidal activities (Liu X *et al*, 2011).

1.3.1.1. Orange peel and extract for fungal infections

The effect of secondary metabolites synthesis by plants has raised from last 50 years while using for different infectious diseases. The secondary metabolites of plants are usually synthesized by plants for their adaptation to a suitable environment but have a potential role in pharmacology as well (Bourgaud F *et al*, 2001).

Scientists have found different molecular pathways for secondary metabolites where the alkaloids are known to be more effective agent in pharmaceuticals (Yun DJ *et al*, 1992). Not only higher plants, but the fruits as well contain different types of active agents for killing or stating the growth of microorganism like the peel of oranges.

Microorganisms are dramatically increasing resistance to different types of antibiotics therefore the scientists now have a great focus upon the peel of oranges due to its antifungal properties because it contains natural antioxidants (Ruhnke M, 2006), while the peel of the sweet oranges compose of antiseptic, analgesic, and anti-inflammatory properties (Muhammad G *et al*, 2008).

According to study, peel and seed extraction agents with ethanol grade were observed to be active against *Aspergillus flavus* and *Candida albicans*, but the extraction of the agent from the peel in aqueous grade showed higher antifungal activity against both the fungi rather than seed (Anthony C *et al*, 2016).

Not only the peel, but other leaves essential oils of the *Citrus sinensis* and *Citrus maxima* (Burm) are also effective against multiple fungal species due to their antioxygenic, antifungal and antioxidant properties (Prajapati *et al*, 2003).

DL-limonene was tried in contradiction of fungal isolates by using poisoned food assay technique which yields to stop the growth of fungal hyphae within 0 to 10 days of incubation (Chang *et al*, 2008). Using Tukey's test and ANOVA comparison, the antifungal assay, all the concentrations of EOs (give the range of the concentration) were found significantly effective over control from day-2 to day-10 (Priyanka Singh *et al*, 2010).

According to a study conducted by (Yamina Ben *et al*, 2018), seven days test for the antifungal activities of the *C. limon* and *C. sinensis* essential oils were studied. As the concentration of the oil was increased from so the growth was significantly decreased ($p < 0.05$). Thus, it was reported that there is a dose dependent activity of the essential oil such as the concentration of oil found inversely proportion to the fungal growth graph (Yamina Ben *et al*, 2018).

1.4 COMPOSITION OF ORANGE PEEL

Retinol, Ascorbic acid, Niacin, Riboflavin, Thiamine, Calcium, Magnesium, Sodium, the most common was the potassium. The orange peels and seeds were enriched for vitamin A followed by ascorbic acid. Usually, the minerals (mg/100 g) were highly enriched in peels than in seeds. The peels were enriched with Calcium, followed by magnesium, sodium, and potassium (Anthony C *et al*, 2016).

The GC–MS profile showed that DL-limonene (31.83%), E-citral (17.75%), 1-hexene-4- methyl (15.22%) and Z-citral (13.38%) were the main agents in *C. maxima* oil whereas, DL-limonene represented 90.66% in *C. sinensis* followed by linalyl acetate (2.80) (Priyanka Singh *et al*, 2010).

Another study revealed the percent composition of the orange peel, moisture 40%, ash 8%, fat 3%, pectin 7%, lignin 6%, crude fiber 9%, total sugar 14%, reducing sugar 10% and non-reducing sugar 3 % (Ishtiaq Ahmed *et al*, 2015).

1.5 DETERMINATION OF THE ANTIFUNGAL EFFECTS OF MEDICINAL PLANTS AGAINST CANDIDA

From last two decades, *Candida sp.* and *C. neoformans* infections are fatal to human population (Pfaller MA *et al.*, 2004). There are very examples and studies regarding plants antifungal

treatment against *Candida* and major microbiological methods of detecting it (Obi CL et al., 2004). Here we will focus on them.

Common Microbiological Methods

Clinical microbiology is an emerging area of research. Among many others, the efforts of Pasteur and Koch (Morency *et al.*, 2017) that resulted in clinical improvements i.e., treatment and prevention of infectious diseases along with related modifications in human condition until 19th century (Isenberg 2003). Van Leeuwenhoek, widely recognized as the "father of microbiology," penned his first letters on microscopy research in the seventeenth century (Leeuwenhoek 2013). These intentions, however, are challenged. Antibiotic resistance, including potentially spreadable genetic components in the main human bacterial pathogens which provide resistance to the majority of antimicrobials already still being used, has hinted to the potential resurgence of serious untreatable infections (McGann *et al.*, 2016). This is predominantly due to the unnecessary excessive use of antibiotics both within and outside of hospitals, which is considered to account for 30 to 50% of all prescriptions (Pollack *et al.*, 2014). Various scientific trials on peel extract can be utilized to find out activities against bacteria in vitro. Antibiotic diffusion methods can be considered as fundamental methods. For testing antifungal agents, other methods are used, such as the technique of poisoned food. Time-kill test as well as flow cytofluorometric techniques have been recommended for more detailed examination of an agent's antimicrobial effect, bioavailability and cell toxicity of, the test organism experiences (Balouri *et al.*, 2016). In vitro antimicrobial procedures are discussed in the following.

Agar Disk Diffusion Method

It is an antibiotic susceptibility test that has been developed in the 1940s (Heatley 1944). Several recognized and validated standards for bacterial and fungi are available acc to the Clinical and Laboratory Standards Institute (CLSI) (Wayne 2004). This technique in an accurate manner is not actually applied on some fastidious microbes such as *streptococci*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*. This test

credibility is linked to growth in specific media, followed by providing certain incubation criteria and interpretation of inhibitory zones (Kulkani *et al.*, 2015).

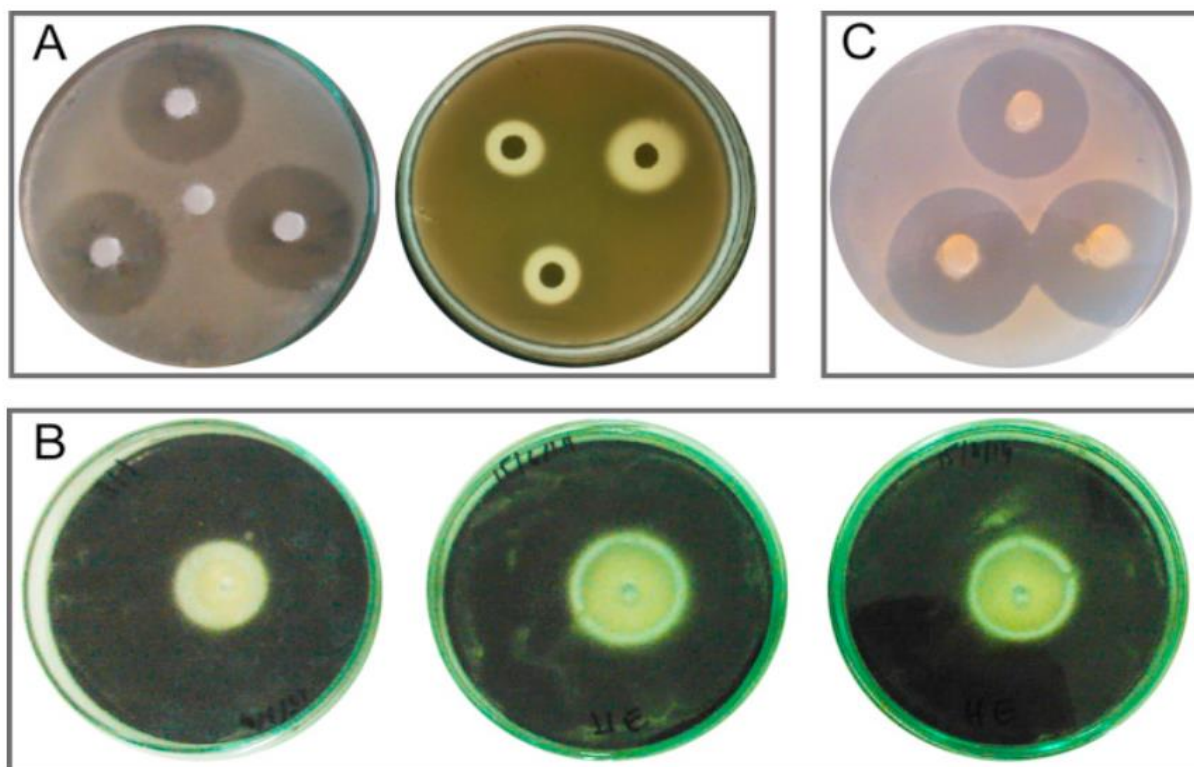


Figure 1.1: Examples of agar diffusion techniques include (A) the disk-diffusion method as a standard using test microorganism i.e., *C. albicans* (B) the agar well diffusion technique for essential oil using *Aspergillus niger* as the test microorganism, and (C) the agar plug diffusion technique using *Bacillus* sp. as the test microorganisms.

This well-known method involves inoculating agar plates with a standard CFU of test microbe. The ingredient is deposited on 6 mm-diameter filter paper discs at the desired concentration before being applied to the agar surface. At the ideal temperature, it is then incubated. The effectiveness of antimicrobial drug is then reported in terms of the inhibition zones diameter or radius suppressing microbial growth. (Fig. 1A). Bacteria are categorized by Antibigram as being susceptible, intermediate, or resistant to delivering qualitative results. Additionally, the outcome of this technique helps the clinicians of selecting the antibiotics and its resistance profiles keeping in mind the patient condition (Reller *et al.*, 2009). Since having no bacterial colonies appeared next day does not always imply bactericidal effect, this technique is unable to differentiate between bactericidal as well as bacteriostatic effects. (Caron 2012).

Antimicrobial Gradient Method (Etest)

This approach, which incorporates the dilution procedure and diffusion concepts, is used to compute the MIC value. (Hausdorfer *et al.*, 1998). The potential for establishing a gradient in the bactericidal or static concentration under investigation in the agar medium. Using this technique, the MIC of antibiotics, antifungals, and anti *mycobacterials* can be determined (Reller *et al.*, 2009). Similarity among MIC values calculated by Etest along with the one acquired by following the dilution mechanism has been demonstrated in a few previous research projects (Berghaus *et al.*, 2015). The technique can be applied to compare effectivity of drugs using for the said purpose (White *et al.*, 1996). To investigate the synergy of two antibiotics, an Etest strip loaded with a first antibiotic is kept on test organism lawn on agar plate. The old strip is replaced with a new one that has been treated with a different antibiotic after an hour. The Etest strips can also be put on the agar medium for the same purpose in a cross formation with a 90° angle (Gülmez *et al.*, 2010). The fractional inhibitory concentration index (FICI) can then be determined upon incubation utilizing the formula below.

$$\sum FICI = FIC(A) + FIC(B)$$

$$\text{where } FIC(A) = \frac{\text{MIC(A) in combination}}{\text{MIC(A) alone}} \text{ and}$$
$$FIC(B) = \frac{\text{MIC(B) in combination}}{\text{MIC(B) alone}}$$

Antagonism was determined by FICI >4 and synergy by FICI 0.5. Between 0.5 and 1, the FICI was regarded as addition, while between 1 and 4, it was interpreted as indifference.

Agar Well Diffusion Method

This method is usually utilized to estimate antibacterial activity of plants or microbial extracts (Magaldi, *et al.*, 2004 and Valgas *et al.*, 2007). The microbial lawn is made on agar surface and wells are made in the media. The drug or extract in solution form is added to the well volume (20–100 mL) of the required concentration has been aseptically inserted into a 3-4mm radius hole and is incubated accordingly. As seen in fig. 1 B, the antibiotic component diffuses

throughout the agar media as well as preventing the growth of the tested microbiological strain. (Valgas *et al.*, 2007).

Agar Plug Diffusion Method

The disk-diffusion method's procedure is comparable to the agar plug diffusion method, which is regularly utilized to highlight the interaction among microorganisms (Jiménez-Esquilín & Roane 2005 and Elleuch *et al.*, 2010). Making an agar culture of the target strain using tight streaks on the plate surface requires using the strain's proper culture media. As microbial cells grow, chemicals are released, which spread throughout the agar medium. Once the test microorganism has been incubated, an agar-plug or cylinder is sliced aseptically using a sterile cork borer and placed on the agar surface of another plate. The chemicals spread out into the agar media from the plug. The appearance of the inhibition zone around the agar plug serves as a further indicator of the antibacterial activity of the chemicals released by the microbes (Elleuch *et al.*, 2010).

Cross Streak Method

To quickly check for antagonism, bacteria are examined using the cross-streak method (Lertcanawanichakul & Sawangnop 2008). A single smear amid the agar plate seeds the target microbial strain. After an incubation period dependent on the microbial strain, the plate is seeded with the microorganisms under investigation via a single streak perpendicular to the central streak. By measuring the size of the inhibitory zone after subsequent incubation, the antimicrobial interactions are explored.

Dilutions Methods

The best techniques for determining MIC values involve dilution since they give the option of estimating the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macrodilution or microdilution). Either the broth method or the agar dilution method can be utilized to quantitatively test the in vitro antibacterial activity against bacteria and fungi.

The MIC value, which is typically reported in mg/mL or mg/L, is defined as the lowest concentration of the assayed antimicrobial agent that prevents the observable growth of the microorganism tested (Pfaller *et al.*, 2004).). There are various accepted guidelines for the dilution antimicrobial susceptibility testing of fastidious or non-fastidious bacteria, yeast, and filamentous fungi. The most commonly acknowledged standards are provided by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These recommendations offer a standardized testing process that is doable in many clinical microbiology laboratories. The creation of such methodological guidelines does not ensure that the testing is clinically relevant. However, it does enable the bioassay to be carried out in a consistent manner in order to assess the clinical applicability of results (Pfaller *et al.*, 2004).

1.6. NOVEL METHODS FOR THE DETECTION OF ANTI-FUNGAL ACTIVITIES OF MEDICINAL PLANTS AGAINST CANDIDA

In recent years, novel approaches have been developed for this purpose in addition to the standard microbiological techniques. The "General Information" section will go into further detail on the uses of FTIR and ATR-FTIR spectroscopy among these techniques.

As a quick, direct, non-destructive, and bioanalytical method, SR-FTIR micro spectroscopy based on synchrotron radiation has been created. This method can examine the molecular chemistry within the microstructures of a biological tissue at ultra-spatial resolutions within cellular dimensions without destroying inherent structures by utilizing synchrotron light brightness and a small effective source size (Yu P *et al.*, 2004; Gurbanov et al 2018). Innovative Methods for Real-Time Simultaneous Monitoring of the Curing Reaction and Diffusion of the Curing Agent at Rubber Using In-Situ ATR-FTIR Spectroscopy and Spectroscopic Imaging.

A novel attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopic technique called "nanocomposite surface" allows for real-time monitoring of both the curing reaction and the diffusion behaviour of curing chemicals at the surface of rubber (Shun M *et al.*, 2021).

We have established a general strategy for optimizing structural information gleaned from a combination of protein vibrational and electronic circular dichroism (VCD, ECD), and Fourier transform infrared (FTIR) spectra, as opposed to the conventional interpretation of these spectra in terms of the fractional composition (FC) of various secondary structure types. In order to make the most accurate prediction of the protein secondary structure composition using any of these methods, we found that it was necessary to significantly reduce the amount of raw spectral data (Pancoska P *et al*, 2014).

Studying the Crystalline and Non-Crystalline Structure of Cellulose Using FTIR Spectroscopy. This work introduces two highly effective techniques for probing the super molecular structures of cellulose that combine FTIR. In one, a microscopic accessory is used, and in the other, a specific reaction cell is used for deuteration. In order to identify the crystalline structures during the production of the cell walls of coniferous wood, the earlier method relies on the interpretation of changes in the distinctive IR bands for the cellulose I and I crystalline phases (Kondo T *et al*, 1998).

A brand-new method for microbial source monitoring in the food business based on high throughput cultivation in Bio Screen and fingerprinting by FTIR spectroscopy. This method involves comparing the FTIR spectra of a product and microorganisms that were isolated along the production line (Shapaval V, *et al*, 2016).

Modifications to the data and multiplicative signal correction for a more precise FTIR spectrum analysis. The OPUS program developed by Bruker Optik GmbH and primarily designed for analyzing bacterial infrared spectra is now widely utilized as a quality control measure. In this study, the OPUS quality check is applied to a wide range of microbial spectra and hyperspectral pictures, including those of bacteria, yeasts, and molds. The authors propose a new approach to spectral quality testing using extended multiplicative signal correction (EMSC) and provide a way for raising the minimum acceptable values of the OPUS quality test's parameters (Valeria T *et al*, 2019).

The broad field of X-ray spectroscopy has undergone a revolution with the introduction of semiconductor detectors. Originally created for particle physics, semiconductor detectors are

now extensively utilised for X-ray spectroscopy in a wide range of disciplines, including X-ray fluorescence studies, X-ray astronomy, and diagnostic medicine (Shatendra K *et al*, 2012).

An objective physical technique for examining the vibrations of matter is infrared spectroscopy. The interferometer, developed by Michelson during the end of the 19th century, is still in use for IR spectroscopy today. All these advancements have benefited in the study of biological systems, especially complex systems like membranes, proteins, nucleic acids, and biomacromolecules (Gal LE *et al*, 1991).

Transform of Fourier can use infrared spectroscopy as a flexible tool for a variety of tasks, including characterizing drug formulations and understanding the kinetics of drug distribution. Drug release in semisolid formulations, drug penetration, the influence of penetration modifiers, and in vivo studies are all studied using the ATR-FTIR approach (Dole, *et al*, 2011).

Fourier transformed mid-infrared (FT-MIR) spectroscopy, which provides fast processing speeds and necessitates little to no sample preparation, has recently gained popularity as a technique for identifying the components of food. Numerous food samples, such as fruits, vegetables, or beverages, have been examined using FT-MIR spectroscopy, frequently in conjunction with chemometrics, to examine various quality features. One such example is the apple epicuticular wax. For the investigation of must and wine, FT-MIR spectroscopy has been frequently utilized (Sylvie B *et al*, 2009).

It is also commonly used in medical studies including the characterization of microorganisms (Garip *et al.*, 2009; 2010a; 2010b, Solis N *et al*, 2022), detection of anti-fungal ((Dole, *et al*, 2011), anti-bacterial (Mural *et al*, 2021) and anti-cancer effects (Machna S *et al*, 2016) of different chemicals/agents or drugs.

FTIR imaging has been effectively used to examine human colorectal adenocarcinoma. Other examples of medical applications include the investigation of compositional variations in genetically modified bovine chondrocytes, the protein along with lipid ratio in the cerebella and epidermis, and the imaging of bone. FTIR spectroscopic imaging was used to measure the - amyloid protein deposit in a slice of human brain tissue containing the Alzheimer's illnesses.

For the purpose of diagnosing diseases, spectral signatures from saliva, blood, and urine are collected using ATR-FTIR spectroscopy. Although distinct sample processing methods and different chemometric analysis algorithms were used, there is a significant degree of commonality in the peak definitions from the same regions of the ATR-FTIR spectrum. While Fourier transform infrared (FTIR) spectroscopy measures the amount of light absorbed by the sample by flashing polychromatic light to it. (Jože G *et al*, 2002).

Total reflectance attenuated A multivariate statistical model has been used with Fourier-transform infrared (ATR-FTIR) spectroscopy, a quick chemical testing technique, to classify illnesses. The potential of ATR-FTIR as a quick screening test for COVID-19 utilizing plasma or nasopharyngeal swab was recently revealed by two investigations (Kazarian SG *et al*, 2006 and Sylvie B *et al*, 2009).

Given the reported blood biochemistry changes in severe COVID-19 disease (Arghya B *et al*, 2021). When a tumoral T cell line is exposed to a UV source that can cause both apoptosis and necrosis, FTIR spectroscopy in attenuated total reflection can be utilized to distinguish between necrotic and apoptotic cell death. Significant spectrum variations in the irradiated cells vs. time were seen in the lipid-proteins ratio absorbance band at $1,397\text{ cm}^{-1}$ and in the lactic acid IR band at $1,122\text{ cm}^{-1}$, using Jurkat cells as the model system (Lucia DG *et al*, 2011). Vibrational modes linked to proteins' absorption intensities and shifts changed, indicating that the secondary conformation of the tumor tissue had changed (Cássio AL *et al*, 2015).

1.7 ADVANTAGES OF FTIR SPECTROSCOPY

The identification of viral particles and composition using ATR-FTIR spectroscopy, which operates in the mid-infrared range, is a particularly effective application of this technology. The $4000\text{ to }400\text{ cm}^{-1}$ range of the electromagnetic spectrum is included in this region. The region between $1800\text{ and }900\text{ cm}^{-1}$ in biological samples is referred to as the "bio fingerprint region" because it contains a lot of data on significant biomolecules (Marfran CD *et al*, 2020).

Various times, ATR-FTIR sampling mode is used because of its many benefits, including its simplicity in sample preparation, and minimized light scattering. It has many uses, including screening of high-risk individuals, disease diagnosis, subtype classification, and surgical margin

determination. It has been extensively employed in biomedical studies (Paraskevaidi M *et al*, 2018).

ATR-FTIR is a sensitive technique that measures the vibration of molecules with applications of electromagnetic radiations at infrared wavelength. It examines sample at molecular level without damaging biological system to get information about all biomolecules in the system at the same time. ATR-FTIR technique is used to get spectrum without requiring sample preparation and work with small amount of sample as well. This technique is user independent and low cost (Marfran CD *et al*, 2020),

1.8 PURPOSE OF THE STUDY

Infection with candidiasis is linked to significant morbidity and death. The demand for a naturally effective substitute such as medicinal plant or fruit extracts increased due to the fungicidal resistance of several currently available fungicides. There are several studies investigated the antifungal effects of orange extracts and/or essential oils (*Citrus sinensis*) against *Candida albicans*, the major source of candidiasis. In those studies, traditional techniques with several technical limitations and disadvantages were used to detect the antifungal effects of *citrus sinensis*.

There is no study which examines the antifungal effects of orange peel extracts by comparison with different dose percentage values, and also investigates the mechanism by which extracts interact with *Candida* fungus and suggests a new method that detects the dose-dependent antifungal effects quickly, inexpensively, sensitively, and user-independently. With the current research, we have considered infrared spectroscopy as a novel technique that can describe and monitor the dose-dependent antifungal effect at a detailed molecular level.

2. GENERAL INFORMATION

For the creation of safe and useful nanomaterials, quick and precise analytical methods to analyse the interactions between microorganisms are crucial. The unintended negative impacts that nanomaterials might have on ecosystems and public health might also be mitigated with the aid of these techniques. Infrared (IR) spectroscopy-based methods for broad spectrum molecular analysis were first developed around 1950 (Kenner *et al.*, 1958; Levine *et al.*, 1953 and Riddle *et al.*, 1956). Scientists in the 1970s created Fourier transform IR (FTIR) spectroscopy based on a novel computational methodology, following various advancements to IR equipment which explains that (Helm *et al.*, 1991 and Helm *et al.*, 1991). Because energy is absorbed in the infrared range, FTIR may detect molecular markers in the makeup of bacteria. Based on the degree of molecular rotation and the type of movement, such as stretching, biochemical linkages can be identified (Nydegger *et al.*, 2011), bending, scissoring (Podstawka- Proniewicz *et al.*, 2011), or twisting (Kanne *et al.*, 1997).

Whittaker *et al.*, (2003) and Mariey *et al.*, (2001) employed FTIR methods to identify bacteria and categorise bacterial strains using spectrum libraries for each species of bacterium (Mauer *et al.*, 2008). Through several scientific firms, a package of these libraries is offered (Faghihzadeh *et al.*, 2016). Additionally, numerous studies have demonstrated that under stress, FTIR can distinguish between healthy and damaged cells (Liu *et al.*, 2011). From a biochemical standpoint, it's critical to identify molecular interactions since they may reveal essential details about the systemic effects that are being noticed. The growing need for quick and accurate toxicity screening in the realm of nanotoxicology could be satisfied by FTIR spectroscopy because of technological advancements, ease of sample preparation, and high analytical speed. ATR-FTIR spectroscopy is a potent molecular spectroscopy method, as well as various publications have been discussed its benefits (Lindon *et al.*, 2016; Smith 2011 and Chalmers *et al.*, 2012). Fig. 1 indicates the relationship between ATR-FTIR spectroscopy and other closely related techniques, which are all referred to as vibrational spectroscopy collectively. Theoretically, the interaction between IR light penetrating a small/thin layer of sample surfaces along with the chemical makeup of the samples led to the ATR-FTIR spectrum. Detailed discussion of IR spectroscopy theory can be found in the study of Stuart published at 2004).

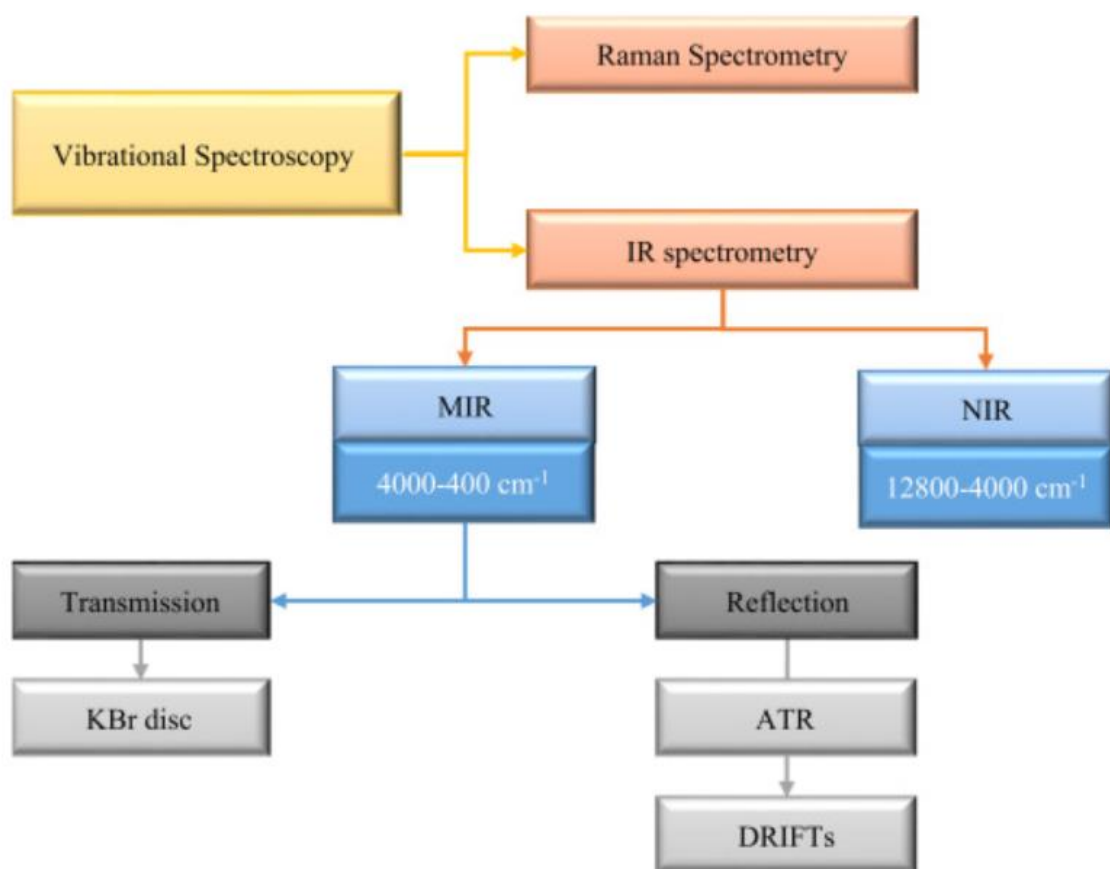


Figure 2.1: The position of ATR-FTIR spectroscopy and the divisions of vibrational spectroscopy

ATR-FTIR's non-destructive nature helps preserving sample integrity besides, containing both informative (i.e., signals of analyte of interest) along with uninformative (i.e., signals of analyte of no interest) regions despite being non-destructive. Various multivariate methods geared toward exploratory as well as classification goals may perform less well due to noise and associated wavenumbers. Such restriction could be overcome by either providing only the most informative wave number subsets or by using appropriate DP techniques to exclude extraneous signals (Rinnan *et al.*, 2009 and Anzanello *et al.*, 2014).

This vibrational spectroscopy method has been used in an exponentially growing number of studies published recently to assess the structural alterations in food-associated bacteria resulting from stress situations, additionally to the substantial scientific work put forth for the cellular characterisation as well as differentiation of several microbes that are contaminating food at the species and subspecies level utilizing FT-IR spectroscopy (Lam *et al.*, 2022;

Mohammad et al.,2016). The implementation of more recent, ambitious studies that showed the potential application of this method to identify the presence and quantity of injured vegetative cells in food products and to test the effectiveness of food processing treatments was made possible by the successful application of FT-IR spectroscopy in this field. The effectiveness of FT-IR spectroscopy as a technique to identify the cellular target of various antimicrobial substances and food processing methods has been demonstrated by several writers (Alvarez-Ordóez, *et al.*, 2010). DNA is repaired enzymatically, and the DNA substrate and its distinctive IR assignment bands may be identified. Thus, by catalysing photolyase, the production of thymidine dimers by UV light and the light-driven DNA repair processes in *E. coli* (Schleicher *et al.*, 2005). Additionally, FT-IR spectroscopy has been effectively utilised to examine how inorganic toxins interact with active bacterial cells (Feo *et al.*, 2004). These latter authors demonstrated that the IR spectra of *E. coli* were affected by the presence of selenium species at varied concentrations in the culture medium, which mostly differed in the spectral characteristics typical of the outer membrane components. In order to link Fourier, transform infrared (FTIR) spectral data with beef deterioration during aerobic storage at cool and abuse temperatures, a multilayer perceptron (MLP) neural network technique was used (Argyri *et al.*, 2010). Three-dimensional fluorescence spectroscopy (3D-EEM) and Fourier transform infrared spectroscopy (FTIR) technologies were used to analyse the main components of sludge EPS during aerobic sludge granulation. The findings demonstrated that during aerobic sludge granulation, the components of sludge EPS tended to be stable. The peak F (Ex/Em = 230/308.5) from 3D-EEM and the dominant spectral band at about 1517 cm⁻¹ from the FTIR spectra of the matured granular sludge suggested the significance of aromatic protein-like compounds collectively, particularly tyrosine, in preserving the stable structure of the granular sludge (Zhu *et al.*, 2012).

To provide patients with the best care possible and to stop the spread of antifungal resistance, quick identification of yeasts is essential. The last ten years have seen a revolution in microbial identification thanks to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), which is simpler, faster, and more accurate at identifying microorganisms to the species level than traditional biochemical methods (Gutam et al., 2017

& Jeffery et al., 2018). However, this technique is high-cost one with expenses of reagents and disposable target plates besides the need for additional pre-treatment steps and sample preparation. Thus, its use in clinical setting is limited to midsized laboratories (Seng et al., 2010).

Fourier transform infrared (FTIR) spectroscopy has been widely used in the field of microbiology to study cell metabolism, identify microorganisms, and type strains with whole organism fingerprinting (Lam et al., 2019 and Novais et al., 2019). Spectral database evaluation studies as well as standards, however, are lacking in the clinical setting. With the addition of the attenuated total reflectance (ATR) mode of spectral capture, FTIR spectroscopy is transformed into a quick, reagent-free, as well as low-maintenance method that does not call for sample preparation following culture or downtime due to heat, drying, or vacuum. Since *Candida* spp. are the third or fourth leading reason of nosocomial bloodstream infections and 40% of those infections are caused by *Candida albicans*, studies for identifying and characterization of those yeast species have great importance for diagnosis and prognosis of related diseases (Lam et al., 2022; Pappas et al., 2018)

In previous studies, “Spectro typing” technique which includes the process of defining the variations of different spectra belonged to microorganisms due to the absence or presence of IR bands or changes in their intensities was used for providing a yeast spectral library (Lam et al., 2019). For that reason, Lam et al. (2019), recorded ATR-FTIR spectra of 65 yeast species from 23 genera with a multitier classification strategy for identifying and classifying an unknown yeast sample. In this study, ATR-FTIR spectroscopy was suggested as a valuable in-vitro diagnostic technique for the routine identification of yeast and bacterial species in clinics.

An infection brought on by a type of *Candida*; vaginal candidiasis was also investigated using differential scanning calorimetry along with FTIR spectroscopy analysis in the previous studies (Hsin et al., 2022). Toubas et al. (2007) successfully discriminated 5 *Candida* species including *C. albicans*, *C. glabrata*, *C. krusei*, *C. glabrata* and *C. parapsilosis* which are highly pathogenic for human.

The largest concern to world health today is antimicrobial resistance, which affects individuals of all ages. The number of casualties has increased as a result of numerous illnesses becoming

more resistant to standard medications (Jaffar & Zailan 2021). For that reason, treatment of yeast infections with medicinal plants, antioxidants, vitamins and several plant-synthesized nanoparticles is currently a popular field in research. In previous studies, antifungal effects of some medicinal plants against *Candida* species including *Candida albicans* were studied in literature (Al Obaiti et al., 2022; Shaheen et al., 2022; Singh and Pulikkal 2022; Arasu et al., 202; More et al., 2021., Lewtag et al., 2019; Arulmozhi et al., 2018). The effectiveness of green manufacture of selenium nanoparticles against oral *Candida albicans* infection was assessed using FTIR analysis (Singh & Pulikkal 2022). Antifungal effects of zinc oxide nanoparticles against *Candida albicans* were also reported by Grewal et al. (2021). The other nanoparticles used in the studies include phyto-fabricated silver nanoparticles (Zianab et al., 2022; Naik et al., 2021). FTIR analysis was also used to determine the antifungal activity of several *Vitex agnus-castus* extracts against various species of *Candida* (Al Obiti *et al.*, 2022). There are various medicinal plants studied for the antifungal effects against *Candida albicans*. The most common ones are *Citrus* species, *Dalbergia sissoo*, *Momordica dioica*, *Sida hermaphrodita*, *Vachellia* species, *Trachyspermum ammi*, *Peganum harmala*, *Capparis zeylanica*, *Streblus asper* and *Tribulus terrestris* (Shaheen et al., 2022; Arasu et al., 2021; More et al., 2021; Lewtag et al., 2019; Arulmozhi et al., 2018).

There are fewer studies which investigate the antifungal effects of *Citrus sinensis* against *Candida albicans* in the literature. The first study investigated the antimycotic activity of aqueous, ethanolic and petroleum ether extracts of *Citrus sinensis* L. (Osbeck) (Trovato et al., 2000). *Candida albicans* samples were isolated from acute vaginitis patients from hospitals in the study. Irkin and Korukoglu (2009) studied the antifungal effects of orange essential oil besides garlic, bay, black pepper, organum, thyme, tea tree, mint, clove, and cumin essential oils on *Candida albicans* and found the most inhibitory effect in orange, thyme, organum and clove essential oils (Irkin and Korukoglu, 2009). A current study reported the main components with highest proven antifungal effects like catechin, gallic acid, thymol as a flavonoid as well as tannins, terpenoids and saponins as polyphenols (Hsu et al., 2020).

In all those studies antifungal properties of *Citrus sinensis* were studied by micro dilution technique along with agar diffusion technique, toxicity by flow cytometry and genotoxic

evaluation by Ames test (Mirzai et al., 2021; Alex et al., 2020; Oikeh et al., 2020; Ruiz-Perez et al., 2016). Although those techniques are commonly applied, they have several limits including being time-consuming, not very accurate, expensive besides technical limits due to protocol complexities. There is no study in the literature which studies dose-dependent antifungal effects of *Citrus sinensis* (orange extract) on *Candida albicans* by investigating the molecular changes on the yeast due to the extract exposure.



3. METHODOLOGY

3.1. MATERIALS AND EQUIPMENTS

Gloves, SDA, Slides, Sterile cotton swabs, Saline solution (0.009 NaCl), Glass liner, Lead pencil.

Inoculation Equipment

Inoculation loop, Micro incinerator, Incubator.

Microscope and equipment

Microscope, Immersion oil, Lens paper and lens cleaner.

Staining Equipment

Slide drying rack, Wash bottle, Staining trays, Forceps, Bibulous paper.

Disc Diffusion Method

Mueller Hinton agar, Antibiotic disc, *Mueller Hinton Broth*, Cotton swab, Petri Plates, 0.5 MacFarland Turbidity standard, Inoculum, Forceps, Metric ruler, or calliper.

ATR-FTIR Spectroscopy

Attenuated Total Reflectance Fourier Transform Infrared Spectrophotometer, Liquid nitrogen gas, vortex, and computer system.

3.2. ORANGE FRUIT EXTRACTION

Orange (*Citrus sinensis*) fruits are washed under distilled water. Washed fruits are dried with drying paper. Dried fruits are carefully separated from their shell with the help of sterilized knives or graters. The resulting fruit peels are dried in shade on drying paper. Dried fruit peels are powdered in the plant grinding mill. The fruit peels, which are ground and powdered, are macerated with hexane, ethyl acetate and methanol solvents 3 recurrent, respectively.

We took orange peel extract in solid form and added 70% ethanol as a solvent for homogenous structure. We took 10 ml of ethanol for 1 mg of orange peel extract. We added 100 ml of absolute ethanol to the orange peel extract vortex in order to mix it and carried out filtration process with

the help of filter paper. During this process the supernatant were discarded and used the filtered solution for further process.

After the maceration process, the liquid part is filtered with filter paper and the solvents are flown in the evaporator to obtain raw extracts. Extracts are stored in the refrigerator at 4°C until the day of operation (Mehmood et al., 2015).

Using different organic solvents on a project basis, extracts with different polarities have been obtained and the efficiency of the project has been worked with the highest level.

3.3. OBTAINING FUNGAL STRAIN

Candida albicans ATCC 10231 strain have been acquired from Sakarya Educational Research Hospital Microbiology Laboratory. This strain of fungi has been tested and evaluated in the microbiology laboratory and have reached the processing stage. Samples from these strains have been transferred to our microbiology research laboratory.

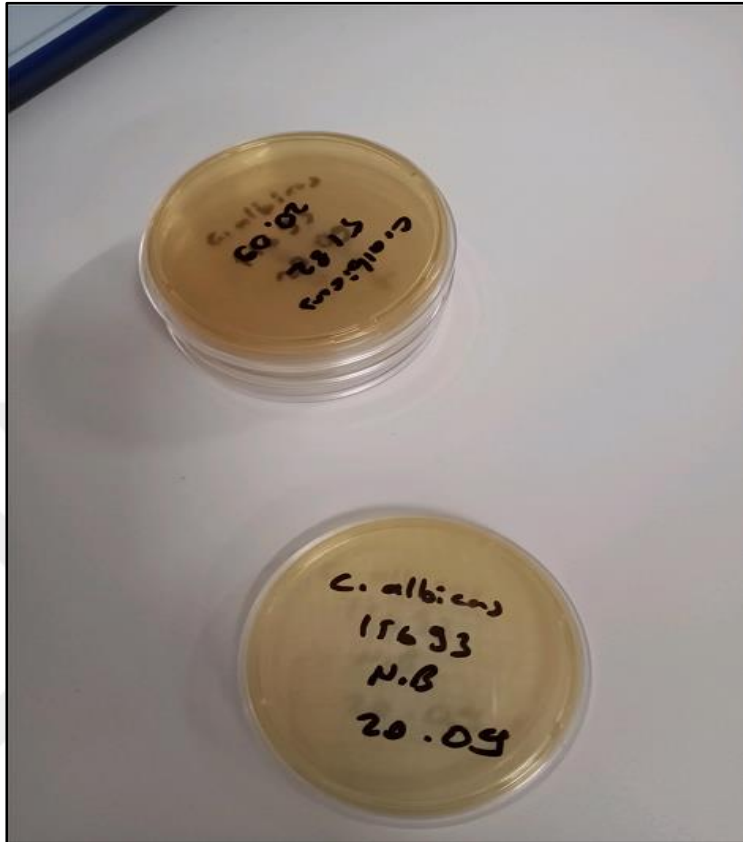


Figure 3.1: *Candida albicans* ATCC 10231 strains that assign to our microbiology research laboratory.

3.4. MEDIA PREPARATION AND SENSITIVITY TEST

Before carrying out the Antibiotic sensitivity testing or Kirby Bauer Antibiotic Sensitivity we have prepared the standard inoculum of *Candida Albicans*. We took 2 ml of saline in the tube and added *Candida albicans* colonies that have been grown on SDA media using disposable loop in order to adjust the turbidity of this suspension to a 0.5 McFarland standard and used that suspension.

Later, we performed the disc diffusion and tube dilution methods with that standard.

At first, we have prepared the dilutions we will use for Kirby Bauer Antibiotic sensitivity testing. The Cryotubes were labbled as 1-6. We poured 200 µl of extract using micropipette and vortex it that is the 100% concentration of the extract. In order to get the 50% concentration, we took 200 µl from the 1 tube and added 200 µl solvent and vortex it. We poured 200ul solvent in the 3rd tubes along with 200 µl from the 2nd tube and made it vortex to get 25% of concentration. In the 4th tube 200 µl of solvent was poured with the help of micropipette and 200 µl of solvent was added from the 3rd tube and vortex it to obtain 12.5% concentration. For 6.25% concentration 200 µl has been taken from the 4th tube as well as 200 µl added from the solvent and vortex it. In the cryotube No.6 we have been poured 200 µl of solvent an added it with the solvent taken from the tube No. 5 in order to get 3.13% of the concentration. Solvent was used as negative control.

Disc Diffusion Assay

Disk-diffusion testing, which was established in the 1940s, is the accepted method for conventional antibiotic susceptibility testing in many clinical microbiology laboratories (Heatley 1944). This test is mostly used to evaluate how susceptible or resistant aerobes or facultatively anaerobes are to various antibiotic classes. During this technique plates are inoculated using a standardized inoculum of the microbes (Caron 2012). Antibigram classifies bacteria as susceptible, intermediate, or resistant to deliver qualitative results. Additionally, the outcomes of this typing tool, that are based on the resistance phenotype of the tested microbial strain, assist clinicians in selecting the ideal initial empiric treatments and antibiotics to use for individual patients in particular situations. (Reller *et al.*, 2009)

We put the standard inoculum to the *Mueller Hinton Agar* (MHA) and spread evenly with the help of swab. We put the blank antibiotic discs on plates and label by names of concentration along with one control on each petri plate.

We put/poured 15 µl of orange extract on each disc according to the concentration we have been selected that start from the 100% concentration until the last one which was 6%. we poured 70 % ethanol for the control and waited for 15-20 minutes to make it dry. We kept the petri plates in the incubator for 24 hours.



Figure 3.1: 0.5 McFarland turbidity standard

Tube Dilution Assay

The reference methods for antimicrobial susceptibility testing, that are used to figure out the minimum inhibitory concentrations (MICs) of antimicrobial drugs (Pfaller *et al.*, 2004). MIC is the lowest concentration of drug that inhibits the growth of microbes. This procedure can be carried out using an agar or liquid medium. With a broth dilution procedure, serial antibiotic dilutions have been added to the broth as part of the standard technique to calculate the MIC (Gurbanov, et al 2018). The test bacteria are infected with a fixed quantity in each tube, which also includes a varying concentration of the antimicrobial agent. Following incubation, the MIC is determined as the lowest dose at which no growth is observed. This is a quantitative test, and the outcomes are given in $\mu\text{g/ml}$ (Schwalbe, et al., 2007).

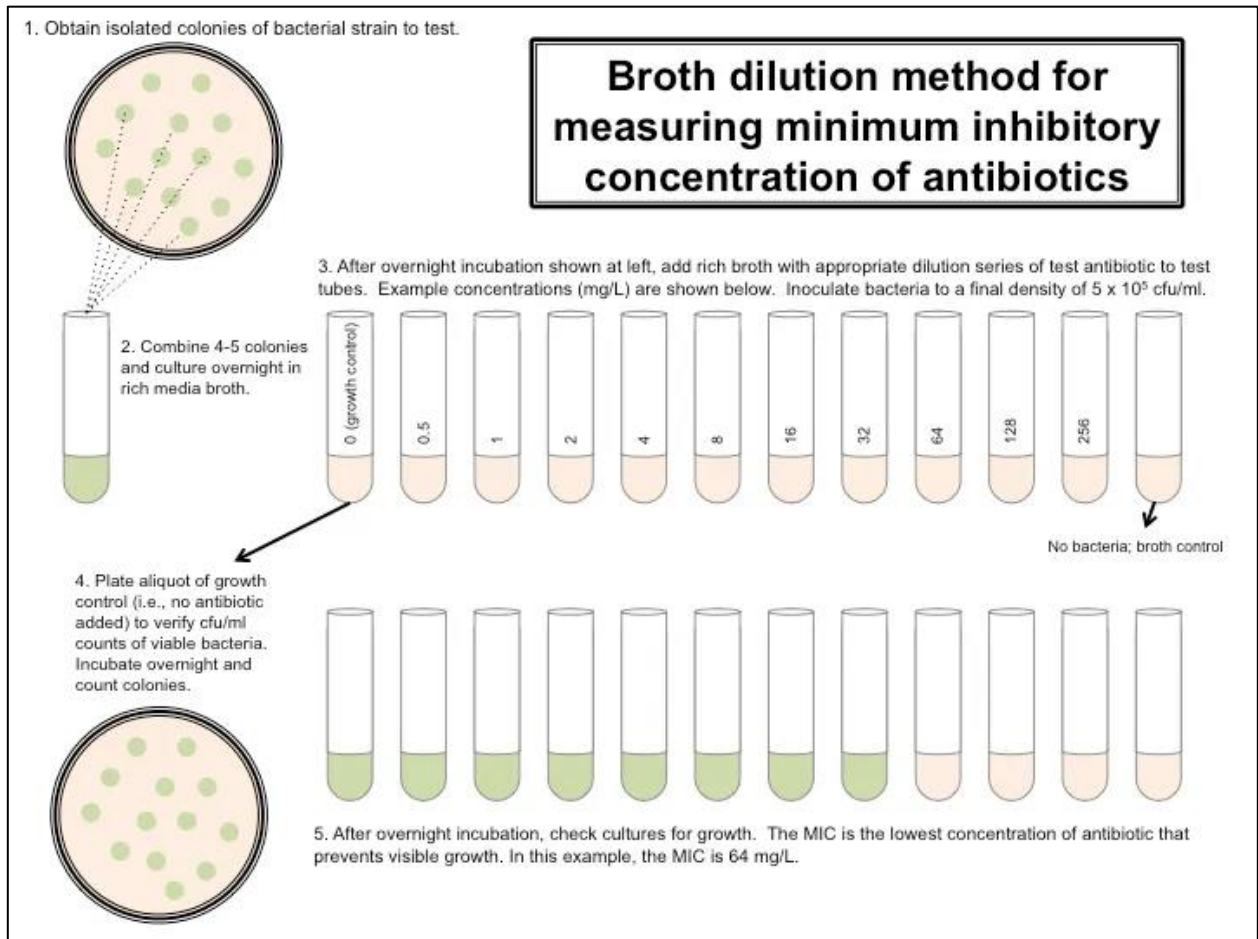


Figure 3.3: Method of determining the minimum inhibitory concentration of antibiotics via broth dilution. (Photo credit: labome.com).

We also performed microdilution method to figure out MIC And MFC. Minimal inhibition concentration (MIC) is the lowest concentration of drug that inhibits the growth of microorganisms. Minimum Fungicidal Concentration (MFC) is the drug concentration that actually kills the fungi. Dilution experiments have performed on experiment tubes with the same concentrations. At first, we added 500µl of medium added to all tubes. Then orange extracts(500µl) and Candida standard (10 µl) was also added.

There were two other groups called *Candida* and control groups. The control contained alcohol and orange extract of the same amount. The *Candida* control carried fungal colonies along with *Mueller Hinton Broth* (MHB) media of the same amount. All tubes are then incubated for 24hours. Then tubes turbidity was observed to figure out MIC.

The MIC from tube dilution assay was found to be OE12.5. There was no turbidity in OE50%, OE25%, as well as OE12.5%. There was turbidity in positive control, but negative control showed no turbidity. After finding out the MIC MFC also has been determined which was found to be 50%.

Based on the results of disc diffusion and microtubule dilution tests, we determined the concentration of the orange extract that we use for IR spectrum analysis which are OE50%, OE25%, OE12.5% and OE6%.

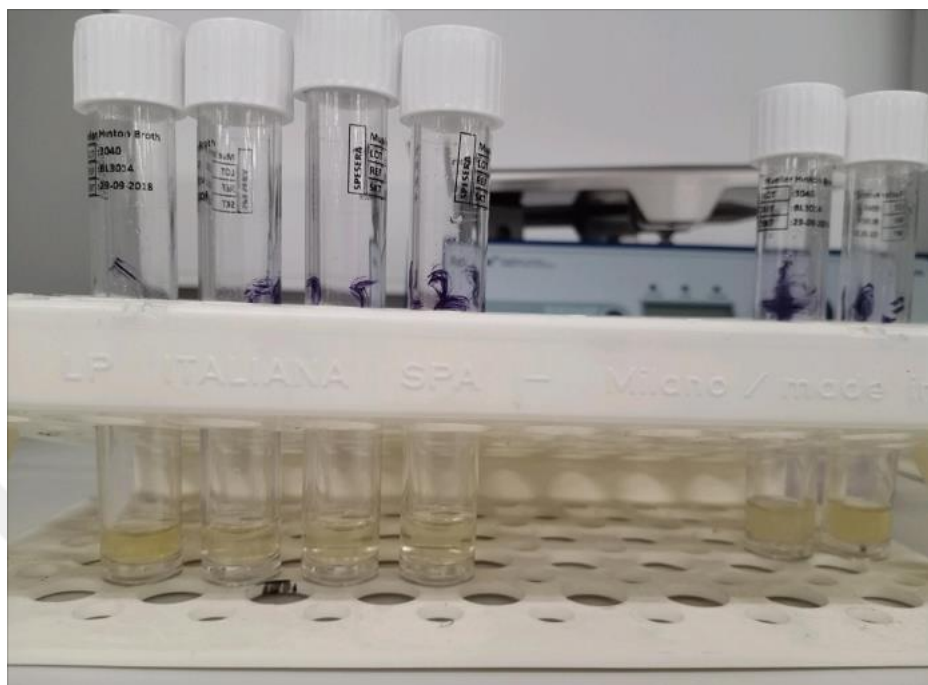


Figure 3.4: Different groups of orange extracts (OE50%, OE25%, OE12.5%, OE6%, Candida (CA) and controls groups) labeled based on concentrations using FTIR for further investigation.

3.5. PREPARATION OF SAMPLES FOR ATR-FTIR SPECTROSCOPY STUDIES

As we conducted microbiological dilution experiments and determined doses of fungicide and fungistatic (suppressing fungal growth). Different doses have been determined for these two effects. The names of these methods are 'disc diffusion' and 'tube dilution'.

In line with the results obtained by sensitivity tests, IR analysis and MIC and MFC densities have gained at more precise values in line with IR analysis results. - The duration of the treatment of "fungal active substance" has investigated for the fungicidal effect to occur. With the upper density of the active ingredient MFC, the McFarland 0.5 densities of the *Candida albicans* are first treated for 1 hour, in line with the IR results to be taken. At the end of these studies, the specified fungicide and fungistatic optimum dose have been utilized for detailed infrared studies.

3.6. OBTAINING ATR-FTIR SPECTRUM FROM SAMPLES

The samples were combined using a vortex after the concentration was adjusted to OD 0.5 with McFarland. After cleaning the crystal unit with 70% isopropanol and distilled water, the samples were scanned on the Diamond/ZnSe (Di/ZnSe) crystal plate of the Universal ATR utilizing the one-bounce ATR mode of the Bruker Alpha II 100 FTIR spectrometer (Bruker, Berlin, Germany).

CO₂ along with water vapor in the atmosphere created interference spectra, which the system removed automatically by taking background spectrum. After the background collection, for obtaining the sample spectra, 1 µl sample was taken and placed directly on the ATR crystal. The sample was exposed to nitrogen gas treatment for a light drying performed with nitrogen gas for 4 minutes. The reason for light drying with nitrogen gas is the removal of free water in the feeder environment. The chosen time for drying process was decided after the optimization studies including different drying minutes. It was noticed that after 4-minute drying, the obtained spectrum was not changed with further drying. Thus 4 minutes drying time was used for all samples during ATR-FTIR studies. Following the drying process, the spectrum was started to be recorded in the range of 4000-850 cm⁻¹ wave count, 4 cm⁻¹ resolution, with 128 scan number.

Opus Software was used for the recording and analysis of the spectrum data (version 8.5, Bruker Optic, GmbH). Each sample was taken three times, each time taking a 1 µl sample from the initial sample suspension. The molecular characteristics of the samples were then studied using the average of these three spectra.

3.7. ANALYSIS OF FTIR SPECTROSCOPIC DATA

The spectrum analysis began with a thorough spectral baseline correction, that is often utilized to get rid of a sloping or curved baseline prior to proceeding with the study. Band positions (wavenumber values) have been determined using the middle 80% of peak heights in the spectral data. After integration of the band area values by checking the area under the target band or measuring the intensity of the band by using vector normalization, ratios of band areas or band intensities were also calculated. When determining the half bandwidth for the CH₂-antisym band, we also considered the halfway point of the band's wavenumber.

All results reported here are the average of three duplicates of each sample. In order to visually demonstrate the differences between the groups, the average spectra were normalized with respect to the indicated bands in various figures spread throughout the thesis.

3.8. STATISTICAL ANALYSIS

The coefficient of variation (SD/mean) for each indication in each group concentration was calculated, and the mean and SD for each group were used to summarize the findings. The statistical analysis has been carried out utilizing GraphPad (version 9.0). (GraphPad Software Inc., San Diego, USA). The spectral values for each group were compared using the ordinary one-way ANOVA with Tukey's multiple comparisons tests, with a single pooled variance test (parametric) to prove significant differences among the groups after checking for normal distribution and homogeneity of variance with Kolmogorov-Smirnov normality tests.

The one-way ANOVA was used to assess band area/intensity ratios in accordance with the results of the normality test, A mean, and standard deviation were used to show the data (SD).

P values of less than or equal to 0.05 used to demonstrate statistical significance “(*, + $p \leq 0.05$; **, ++ $p \leq 0.01$; ***, +++ $p \leq 0.001$; ****, ++++ $p \leq 0.0001$)”.

4. RESULTS

In this research, the antifungal effects of orange extracts on *Candida albicans* in different dose ranges were studied and the optimal dose was determined by traditional microbiological methods and FTIR spectroscopy.

4.1. RESULTS OF MICROBIOLOGICAL STUDIES

During microbiological studies we grow the *Candida albicans* utilizing the blood agar media. We put the standard inoculum to the *Mueller Hinton Agar* (MHA) and spread evenly with the help of swab. We put the blank antibiotic discs on plates and label by names of concentration along with one control on each petri plate.

Results of Disc Diffusion Technique

We put/poured 15 µl of orange extract on each disc according to the concentration we have been selected that start from the 100% concentration until the last one which was 6%. we poured 70% ethanol for the control and waited for 15-20 minutes to make it dry. The petri plates were incubated in the incubator for 24 hours.

On the second day we measured the zones. There were no zones on 3.25%, 6.25%, 12.5% and 25% of concentrations. The zones which were measured for 100 % was 11mm and for 50% of concentration was 8.5mm respectively.

Table 4.1: Inhibition zones with different dose concentrations for discs diffusion assay

Dose Concentration	Inhibition Zone
100%	11 mm
50%	8.5 mm
25%	No Zone
12.5%	No Zone
6.25%	No Zone
3.35	No Zone

Results of Tube Dilution

The MIC from tube Dilution assay was found to be 12.5%. There was no turbidity in 50%, 25% as well as 12.5%. There was turbidity in *Candida*, but control showed no turbidity. After finding out the MIC, MFC also has been determined which was found to be 50%.

Based on the results of disc diffusion and microtube dilution tests, we determined the concentration of the orange extract that we use for IR spectrum analysis which are control (C), *Candida* (CA), OE6%, OE12.5%, OE25%, OE50%,



Figure 4.1: Zones of the orange extract for different concentrations

4.2. RESULTS OF FTIR SPECTROSCOPIC STUDIES

Fig 4.2 the average spectra of *Candida albicans* in the medium. The multiple bands here are the result of the various functional groups, which include nucleic acids, phospholipids, symmetric and antisymmetric CH_3 and CH_2 lipids, Amide I along with Amide II, and CH_2 -bending lipids. Table 4.2 shows the spectra for these bands along with the main bands labelled. The carbonyl ester triglyceride as well as olefinic bands can be only seen in the second derivatives spectra. Area and the intensity ratios of the studies bands were calculated to get information about DNA

concentration, aliphatic chain length, protein structural and conformational changes, protein concentration, saturated along with unsaturated lipid concentration besides carbonyl, carbohydrate, as well as nucleic acid contents. The olefinic, saturated lipid, carbonyl ester and carbohydrate contents were calculated using band intensity ratios while the other parameters were measured band area ratios.

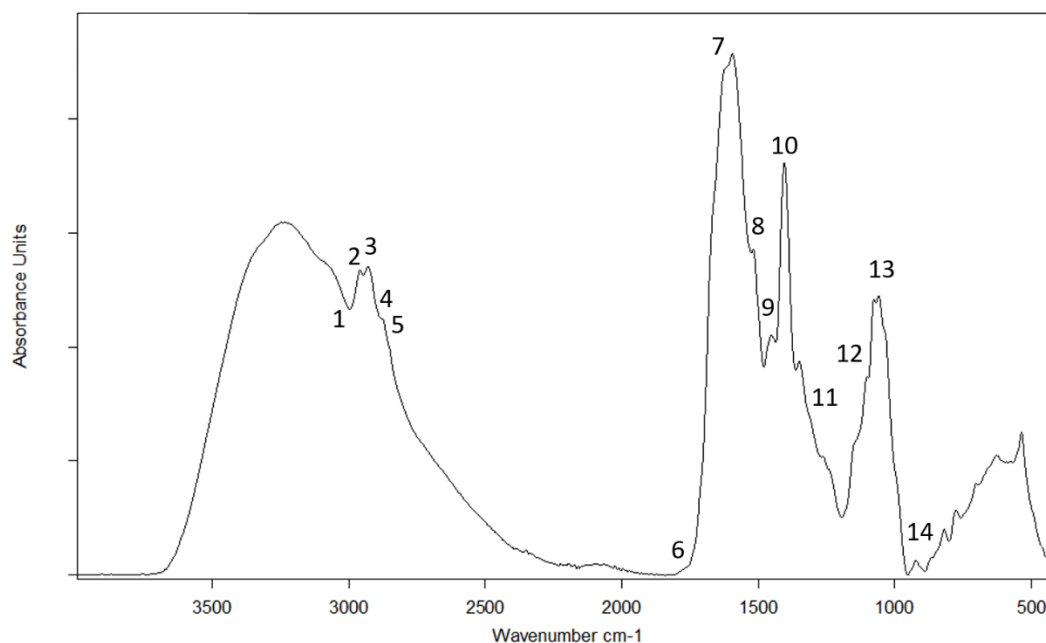


Figure 4.2. The representative spectrum of *Candida albicans* between 4000-400 cm^{-1} wavenumber region

Spectral parameters, such as transformation in peak position, changes in bandwidths, and band area/intensity values of the infrared bands can be utilized to get significant structural and functional information. ATR-FTIR spectroscopy has been used to compare to control, candida and different orange extract concentration groups based on their spectral variations in 3000-2800 and 1800-750 cm^{-1} spectral region.

Fig. 4.2 shows the band area/intensity ratio value, and Table 4.3 provides the band area/intensity ratios as well as the band wavenumber values for certain bands.

Table 4.2: The band assignment of an IR spectrum between 3000-750 cm^{-1} wavenumber region. (Algrubi et al., 2022; Garip et al., 2007; 2009; Ustaoglu et al., 2018). Dogan et al., 2021)

Band Number	Wavenumber (cm^{-1})	Band assignment
1	3015	“Olefinic HC=CH stretching (unsaturated lipids)”
2	2957	“CH ₃ antisymmetric stretching (protein and lipid)”
3	2934	“CH ₂ antisymmetric stretching (mainly lipid)”
4	2878	“CH ₃ symmetric stretching (mainly protein)”
5	2850	“CH ₂ symmetric stretching (mainly lipid)”
6	1740	“Ester C=O stretching (triglyceride, cholesterol esters)”
7	1584	“Amide I band mainly due to C=O stretching vibration of amide groups: protein”
8	1515	“Amide II band due to N-H bending strongly coupled to C-N stretching vibration of amide groups: protein”
9	1454	“CH ₂ bending (lipid)”
10	1398	“Fatty acids and amino acids”

Table 4.2: The band assignment of an IR spectrum between 3000-750 cm⁻¹ wavenumber region. (Algrubi et al., 2022; Garip et al., 2007; 2009; Ustaoglu et al., 2018). Dogan et al., 2021) 'table continued'

11	1237	“PO ₂ ⁻ asymmetric stretching; mainly nucleic acid with a little contribution of phospholipids”
12	1076	“PO ₂ ⁻ symmetric stretching; phospholipids”
13	1030	“Carbohydrate”
14	933	“Z- form DNA”
15	835	“A- form and B-form helix conformation of DNA”

The band area/intensity ratios of a few distinct lipid functional groups in the C-H stretching region (3015-2800 cm⁻¹), as well as the C=O stretching vibrations of carbonyl ester groups, were used to analyze the changes in lipid composition and structure. Following are the calculated ratios: Olefinic HC=CH stretching / (CH₂ antisym stretching + CH₂ sym stretching); Ester C=O stretching/ (CH₂ antisym stretching + CH₂ sym stretching); (CH₂ antisym stretching/ CH₂ antisym stretching + CH₂ sym stretching) and CH₂ antisymmetric stretching/ CH₃ antisym stretching .

Table 4.3: The band area and intensity ratios, wavenumber, and band half-width values of control (C), *Candida* (CA), OE6%, OE12.5%, OE25%, OE50% groups. “(*, +, $p \leq 0.05$; **, ++, $p \leq 0.01$; ***, +++, $p \leq 0.001$; ****, +++++, $p \leq 0.0001$)”.

Band area/intensity ratio	Control (C)	Candida (CA)	Orange Extract (OE) 6%	Orange Extract (OE)12.5%	Orange Extract (OE)25%	Orange Extract (OE) 50%
Olefinic HC=CH stretching / (CH ₂ antisym stretching + CH ₂ sym stretching)	0.088±0.003	0.063±0.05	0.108±0.003 **** ++++	0.112±0.05 **** ++++	0.146±0.014 **** ++++	0.061±0.004
CH ₂ antisymmetric stretching/CH ₃ antisymmetric stretching	0.634±0.032	0.257±0.045 ****	0.090±0.027 **** ++++	0.551±0.018 ** ++++	0.645±0.021 ++++	1.342±0.015 **** ++++
CH ₂ s v / (CH ₂ as v + CH ₂ s v)	0.098±0.008	0.503±0.063 ****	0.146±0.010 ++++	0.046±0.008 ++++	0.053±0.011 ++++	0.301±0.018 **** ++++
Ester C=O stretching / (CH ₂ antisym stretching + CH ₂ sym stretching)	1.770±0.122	0.651±0.095 ****	1.494±0.092 *** ++++	1.164±0.059 **** ++++	0.841±0.039 **** ++	0.185±0.006 **** ++++
Amide I/Amide II	139.397±4.659	93.847±3.760 ****	36.962±1.562 **** ++++	27.664±0.394 **** ++++	112.270±1.227 **** ++++	102.309±1.906 **** +++

Table 4.3: The band area and intensity ratios, wavenumber, and band half-width values of control (C), Candida (CA), OE6%, OE12.5%, OE25%, OE50% groups. “(*, +, $p \leq 0.05$; **, ++, $p \leq 0.01$; ***, +++, $p \leq 0.001$; ****, +++++, $p \leq 0.0001$)”. “table continued”

Amide I/Amide I + Amide II	0.993±0.000	0.989±0.000 ****	0.974±0.001 **** ++++	0.965±0.000 **** ++++	0.991±0.000 *** +++	0.990±0.000 ****
(CH ₂ as ν + CH ₂ s ν) / (amide I+amide II)2nd	0.202±0.014	0.394±0.063 ****	0.132±0.009 ** ++++	0.132±0.006 ** ++++	0.182±0.004 ++++	0.359±0.010 ****
(PO ₂ - s ν + PO ₂ - as ν) / (amide I+amide II)	0.018±0.001	0.010±0.001 **	0.031±0.000 **** ++++	0.014±0.000 *** ++++	0.018±0.000	0.015±0.000 ** ++++
A835 / (PO ₂ - s ν + PO ₂ - as ν)	0.506±0.029	0.382±0.024 ****	0.152±0.005 **** ++++	0.488±0.011 **** ++++	0.419±0.022	0.469±0.017 ++++
C—O ν / (amide I+amide II) 2nd	0.387±0.036	0.159±0.052 ****	0.031±0.002 **** ++++	0.316±0.004 ** ++++	0.581±0.010 **** ++++	0.0790± 0.012 **** ++++
Wave number	Control (C)	Candida (CA)	Orange Extract (OE) 6%	Orange Extract (OE)12.5%	Orange Extract (OE)25%	Orange Extract (OE) 50%
CH ₃ antisymmetric stretching	2957.503± 0.728	2958.668± 0.555	2956.729± 0.721 ++	2957.395± 0.500	2957.960± 1.028	2957.081± 0.592 +
CH ₂ Antisymmetric Stretching	2934.076± 0.595	2934.372± 0.789	2934.045± 0.635	2934.259± 0.326	2934.182± 0.338	2931.258± 0.404 **** ++++

Table 4.3: The band area and intensity ratios, wavenumber, and band half-width values of control (C), Candida (CA), OE6%, OE12.5%, OE25%, OE50% groups. “(*, +, $p \leq 0.05$; **, ++, $p \leq 0.01$; ***, +++, $p \leq 0.001$; ****, +++++, $p \leq 0.0001$)”. “table continued”

CH3 Symmetric Stretching	2876.303± 0.576	2875.852± 0.582	2876.429± 0.509	2878.406± 0.798 ** ++++	2878.383± 1.111 ** +++	2878.577± 0.443 *** ++++
CH2 Symmetric Stretching	2851.353± 0.627	2852.752± 0.173 ****	2850.799± 0.074 ++++	2850.830± 0.112 ++++	2848.813± 0.110 **** ++++	2850.661± 0.423 * ++++
Amide I	1575.797± 1.173	1581.742± 0.444 ****	1585.361± 0.991 **** ++++	1584.649± 0.783 **** +++	1586.716± 0.932 **** ++++	1592.581± 0.919 **** ++++
Amide II	1516.942± 0.680	1516.706± 0.547	1517.667± 0.630	1516.878± 0.390	1518.638± 0.684 ** +++	1517.471± 0.709
PO ₂ ⁻ Antisymmetric Stretching	1233.223± 0.597	1234.037± 0.597	1230.027± 1.051 **** ++++	1236.739± 0.617 **** ++++	1236.662± 0.823 **** ++++	1235.687± 0.442 *** +
PO ₂ ⁻ Symmetric Stretching	1077.213± 0.324	1076.392± 0.649	1078.032± 0.616 ++	1076.799 ± 0.926	1077.681± 0.361 +	1075.743± 0.826 *

The total saturated lipid content was determined by taking the band intensity ratios of CH₂ antisymmetric stretching to CH₂ antisym stretching + CH₂ sym stretching bands (Ustaoglu et al., 2021). As can be seen from the figure 4.3 comparing the Candida group to the control, the total saturated lipid content was significantly higher in the Candida group. Although all the OE groups decreased the total saturated lipid content of yeast, both low and medium doses of orange extract (OE6%, OE12.5%, OE25%) lowered this value close to the control and significantly decreased when compared to Candida group. Although high dose orange extract (OE50%) also decreased the total saturated lipid content, the antifungal effect was not enough to take the values close to control group as the doses did.

The olefinic (unsaturated lipid) content was calculated by taking the band intensity ratio of olefinic HC=CH stretching band at 3015 cm⁻¹ to the sum of CH₂ antisym stretching bands (2934 cm⁻¹) and CH₂ (2850 cm⁻¹). Unsaturated lipid content has been significantly increased in Candida group compared to the control, while significantly increased in both low and medium doses of orange extract (OE6%, OE12.5%, OE25%) as compared to control and Candida groups. However, the high dose orange extract (OE50%) has been lowered the total olefinic content and showed an opposite effect with low and medium doses of OE. However, the antifungal effect is not that much enough to take the value close to control but instead close to Candida group.

The triglyceride content of groups was calculated by taking the ratio of the intensity values of C=O stretching band at 1740 cm⁻¹ to the sum of CH₂ antisym stretching (2934 cm⁻¹) and CH₂ sym stretching bands (2850 cm⁻¹). The triglyceride content was significantly decreased in Candida group compared to the control while all the doses of orange extracts significantly increased this value again to higher values. However, none of the antifungal effects of the OE doses could take the triglyceride value close to the control group with a non-significant result.

Changes in the structure of aliphatic chains on the cell wall of yeast affects the yeast cell shape and can alter the cell fusion during mating of yeast cells (Aguilar et al., 2010). The aliphatic chain length values of groups were calculated by taking the band area ratio of CH₂ antisym stretching band to CH₃ antisym stretching band. This ratio shows significantly lower values in *Candida* group compared to the control. Although all the OE doses significantly increased this

value compared to the candida group, just 25% OE group could take this value closest to control group (non-significant change compared to the control) implying a better antifungal effect among all doses.

The optimal antifungal effects of medium doses of OE on lipid structure and composition of *Candida albicans* can also be seen on Figure 4.4 which shows the spectra of all groups in CH region (3000-2800 cm^{-1}). The spectra of medium doses of OE (12,5 % and 25 %) were close to control group while the spectra of low dose (6%) and high dose (50%) of OE groups were quite different than the rest (Figure 4.4).

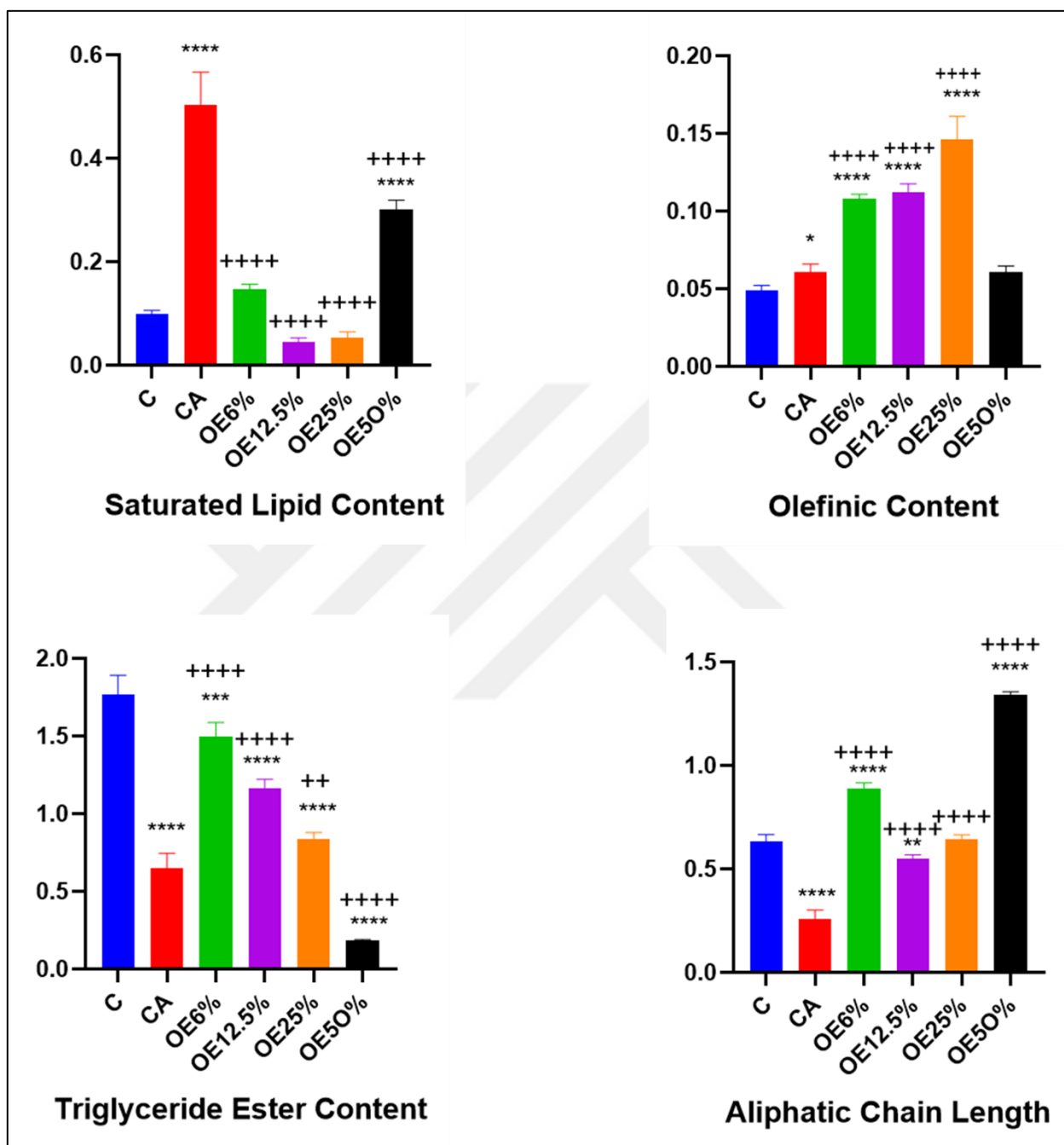


Figure 4.3: The band area/intensity ratio of various total lipid (saturated lipid content, olefinic content, triglyceride content and aliphatic chain length) for control (C), *Candida* (CA), OE6%, OE12.5%, OE25%, OE50% groups. “(*, ++, p ≤ 0.01; p ≤ 0.001; ****, +++++, p ≤ 0.0001)”. The significance compared to the *Candida* represents by (+) symbol, whilst significance between the control (C) represents by (*) symbol.

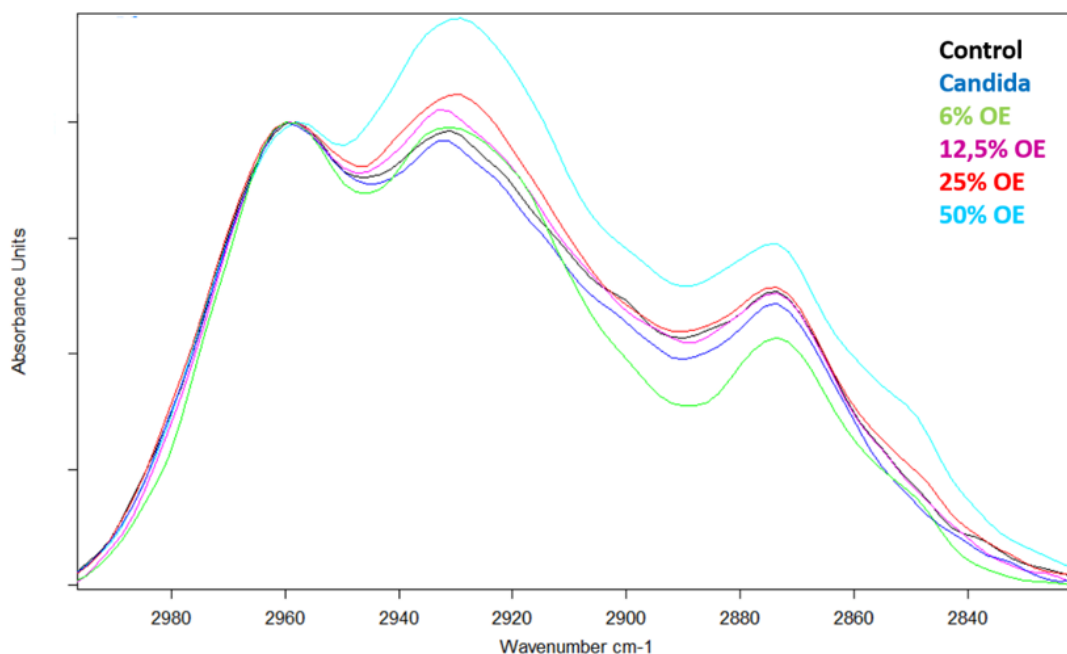


Figure 4.4: The average spectra of Control (C), Candida (CA), (OE6%, OE12.5%, OE25%, OE50%) groups in the wavenumber region 3000-2800 cm^{-1} (The spectra were normalized to CH_3 antisymmetric stretching band).

Total protein concentration was calculated by taking the ratio of the band area of Amide I (1575 cm^{-1}) to Amide I + Amide II (1516 cm^{-1}) bands. As seen from Fig. 4.5 along with Table 4.3, the protein content was significantly decreased in Candida group compared to the control. And increased the protein content significantly compared to Candida group while took the value closest to the control group (non-significant compared to the control). Moreover, lipid to protein ratio which was calculated by taking the band area ratio of (CH_2 antisym + CH_2 sym stretch.) bands to (Amide I + Amide II) bands, also showed similar results as seen in total saturated lipid and total protein contents with a closest value to control group in 25% OE medium dose (Figure 4.5 and Table 4.3).

Protein structural and conformational changes in the *Candida* species due to different doses of OE exposure can be investigated by checking any possible shift on the wavenumber values of Amide I band or taking the ratio of the band areas of Amide I band to Amide II band. In the current study, both of them were considered and gave similar results. As seen in Table 4.3 and Fig. 4.5, although OE exposure caused significant changes on the structure and composition of yeast proteins, none of the OE doses could take this value close to the control group implying a lack of optimal antifungal effects on the protein structure of *Candida albicans*. Figure 4.6B also shows the shifting of Amide I band position in *Candida* and OE groups. As seen from the figure, there is a dramatic change in the shape as well as peak position of the Amide I band of OE groups compared to both *Candida* and control.

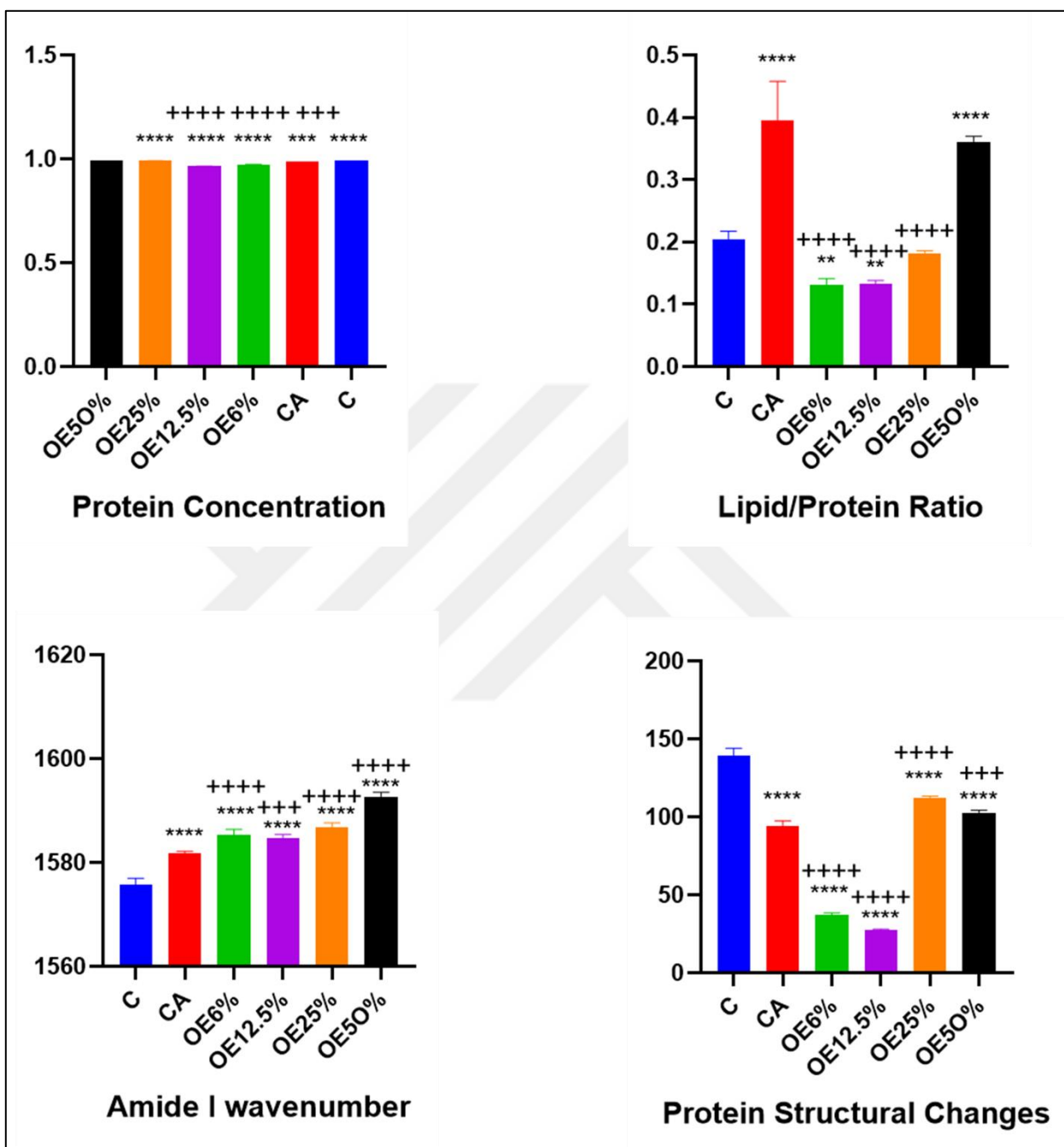


Figure 4.5: The band area/intensity ratio of total protein (protein concentration, lipid/protein content, amide I wavenumber, Protein structural and conformational changes,) for control (C), *Candida* (CA), OE6%, OE12.5%, OE25%, OE50% groups. “(**, ++, $p \leq 0.01$; $p \leq 0.001$; ****, +++, $p \leq 0.0001$) The significance compared to the *Candida* represents by (+) symbol, whilst significance between the control (C) represents by (*) symbol.

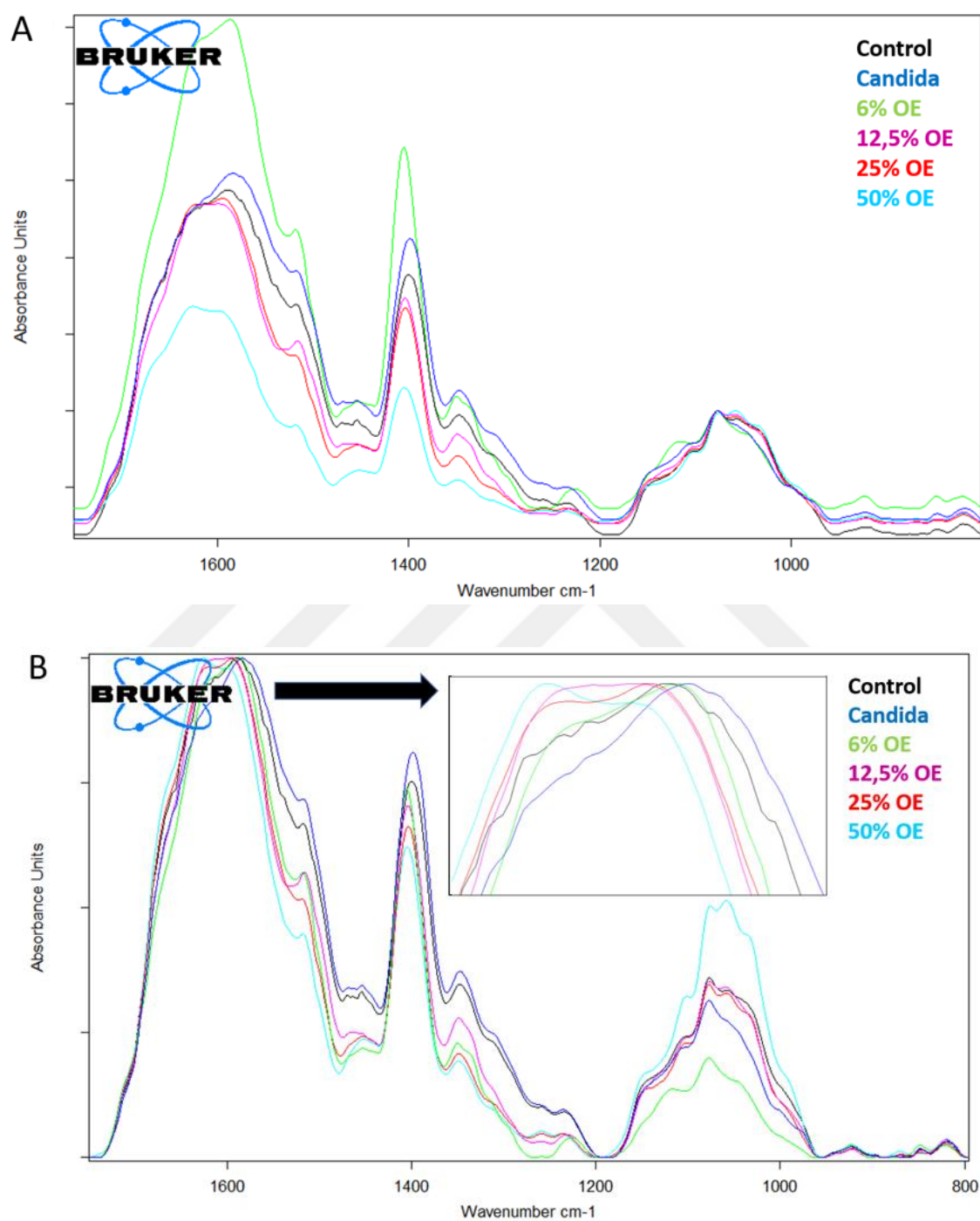


Figure 4.6. The average spectra of control (C), Candida (CA), OE6%, OE12.5%, OE25%, OE50% groups in the wavenumber region of 1800-800 cm⁻¹. The spectra were normalized to (A) PO₂-symmetric stretching band and (B) Amide I band. The small square in section B shows the shifting of the peak position of Amide I band in all groups.

The nucleic acid content was calculated by taking the band area ratio of the sum of PO₂-symmetric (1076 cm⁻¹) and antisymmetric (1237 cm⁻¹) stretching bands to the sum of Amide I + Amide II bands. The nucleic acid content of Candida group was dramatically higher than the control group. Although all the medium and high doses of OE groups decreased this value compared to the Candida group, the optimal antifungal effect was seen in 25% OE medium dose group since it is the only group with non-significant relation to control group (Fig. 4.7 along with Table 4.3). Moreover, there is an opposite effect of low dose OE (6% OE) on Candida group for this parameter when compared with the other OE groups.

Comparing the Candida group to the control, there was a significant decrease in the content of total carbohydrates. Fig. 4.7 and Table 4.3 demonstrate that all the doses of OE except the low dose (6%), increased the carbohydrate content significantly when compared to the Candida group. However, the changes were also significantly different than the control group implying a lack of optimal antifungal effect on carbohydrate concentration of *Candida albicans*. The low dose orange extract significantly decreased this value compared to both control and Candida groups by showing opposite effects with the medium and high dose.

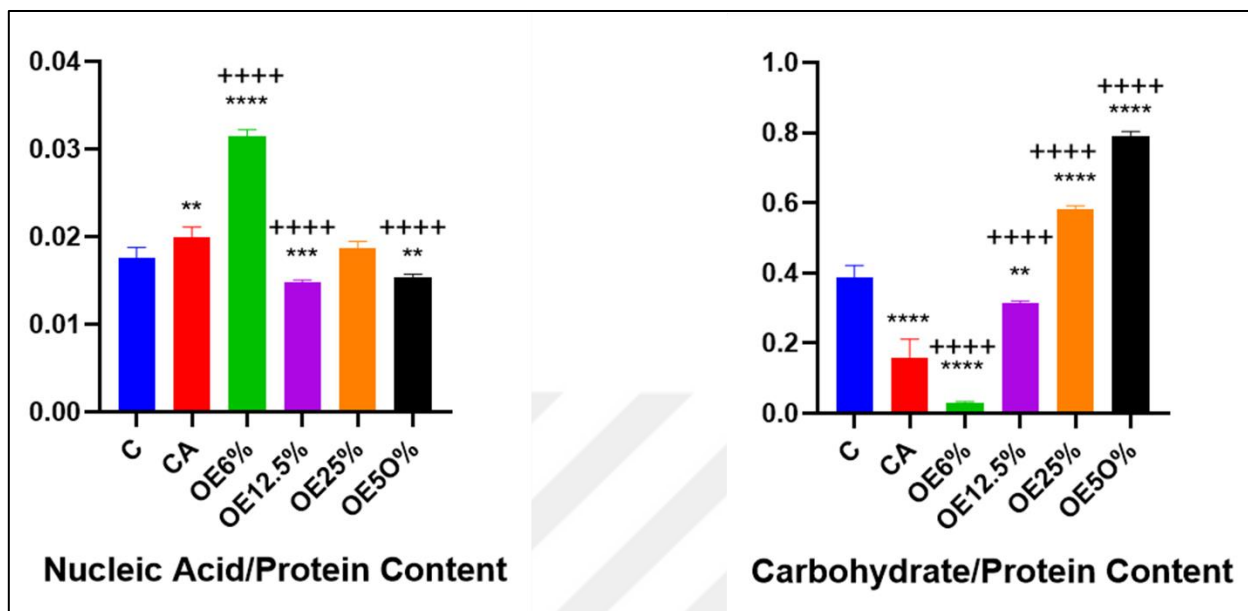


Figure 4.7: The band area/intensity ratio of (Nucleic acid/protein content, and carbohydrate/protein contents) for control (C), *Candida* (CA), OE6%, OE12.5%, OE25%, OE50% groups. “(*, ++, $p \leq 0.01$; $p \leq 0.001$; ****, +++++, $p \leq 0.0001$)”. The significance compared to the *Candida* represents by (+) symbol, whilst significance between the control (C) represents by (*) symbol.

As a summary of results, different orange extract doses can show antifungal effects on *Candida albicans* by effecting the molecular structure and composition of the components of yeast. The most effected components were seen as saturated and unsaturated lipids with triglycerides. In contrast, the protein components of yeast seem to be not affected significantly from the antifungal effects of OE. There is a dose difference in the antifungal effects of orange extract on *Candida albicans* samples. The best doses for the antifungal activity were determined as medium doses (25 % and 12,5 %) especially 25% OE dose. The highest (50%) and lowest (6%) doses show opposite effects on yeast when compared to the medium doses.

5. DISCUSSION AND CONCLUSION

Candida albicans are part of our natural microflora which is responsible for mucosal and systematic infections that can lead to death in individuals with malnutrition, diabetes, HIV positive or immune compromised. They are a member of gastrointestinal tract and the most common species to cause nosocomial diseases (Acarkan et al., 2014; Jabra-Rizk et al., 2016).

Candida albicans are capable of developing resistance to antifungal antibiotics that lead to serious issues. The mechanism of action of antifungal agents that has been utilized against fungal infections are different from antibacterial drugs as fungi are eukaryotes and bacteria are prokaryotes. (Bilgehan., 2009).

There are several studies with microorganisms, animals as well as patients to investigate the antifungal effect of orange peel extract and mostly the essential oil on different yeast species including *Candida albicans* (Mirzaii et al., 2021; Alexa et al., 2020; Hsu et al., 2020; Oikeh et al., 2020; Ruiz-Pérez et al., 2016; Travato et al., 2000). Some of them supported the antifungal effect of *Citrus sinensis* essential oil/extract on the growth of *Candida albicans* (Mirzaii et al., 2021; Irkin and Korukoglu, 2009), while the others showed no inhibitory effect of it when used alone (Alexa et al., 2020).

Results of the current research has indicated that the spectral parameters exhibit numerous, significant variations of orange extracts on *Candida albicans* in different dose ranges. Particularly the variance in the spectral bands created by the functional units of proteins, lipids, carbohydrates, as well as nucleic acids. The results of this study also showed that ATR-FTIR spectroscopy has the capacity to distinguish the antifungal effects of different doses of orange extract samples on the composition of *Candida albicans* species.

The saturated and unsaturated lipid content of *Candida albicans* were reported to affect the function and activity of the fungi (Goyal and Khuller, 1994). Thus, any antimycotic effect which alters the composition as well as structure of lipids in yeast cell wall, can inactivate the yeast or inhibit its growth. The increased total saturated lipid content in *Candida* group compared to the control was decreased by all doses of OE. However, both low and medium doses of orange

extract (OE6%, OE12.5%, OE25%) lowered this value close to the control group by a significant effect on *Candida* group. Different than the saturated lipid content, increased unsaturated lipid content in *Candida* group compared to the control, significantly reached more higher values by the effect of both low and medium doses of orange extract (OE6%, OE12.5%, OE25%) as compared to control and *Candida* groups. The high dose orange extract (OE50%) did not show any significant effect on the unsaturated content of *Candida albicans* different than the low and medium doses of OE. Besides those results, although all the OE doses increased the low triglyceride content of *Candida albicans* significantly, none of the antifungal effects of the OE doses could take the triglyceride value close to the control group with a non-significant result.

Candida albicans produce saturated long chain fatty acids (C14, C16, C18) at the end of exponential growth and produce high level of saturated lipid. Analyses of fatty acid composition revealed myristic acid as the highest levels of fatty acids (40%) with high levels of saturated lipids in *Candida albicans* (Goyal and Khuller, 1994). Variations in the concentration of saturated to unsaturated lipids has been reported to play a vital role in various cellular functions such as the permeability of the cell membrane and transport of nutrients from it besides the regulation of enzyme activities. Previous studies also reported that Phosphatidylethanolamine (PE) which is more closely packed than Phosphatidylcholine (PC) was found to be higher in yeast form of *Candida albicans* (Goyal and Khuller, 1994). Studies with different antioxidant properties of plant extracts, it was shown that flavonoids and polyphenols which have the highest antioxidant components in the extracts, have varying inhibitory effects on oxidation of PC liposomes and their aggregation (Omar, 2007). This can explain the opposite effect of OE on saturated and unsaturated lipid contents of *Candida albicans*. While decreasing the saturated lipid content of *Candida albicans*, especially the medium doses of OE protect the oxidation of unsaturated phosphorylated lipids as PC on the structure of *C. albicans*. Previous clinical studies did not show any significant effect of orange extract on the triglyceride's levels of the hypertensive patients (Valls et al., 2021; Cesar et al., 2010) which can support our results with lack of any effect on triglycerides levels of *C. albicans* by all OE doses.

The findings of Martinez et al. (2002) lead them to hypothesize that isoespintanol's (ISO) antifungal activity is caused, at least in part, by the fact that this molecule is capable of disrupting

the integrity of the fungal plasma membrane, resulting in the alteration of the membrane's permeability and the loss of intracellular material as a result. Consequently, ISO is a naturally occurring molecule with antifungal characteristics (Martinez O.C et al., 2022).

According to Miri et al., (2018) the citrus EOs yield, and chemical composition depend on the kind and age of the plant, the environment, the time of harvest, and the extraction process. As the concentration of EOs from *C. limon* and *C. sinensis* increased, the fungal growth of *A. flavus* considerably decreased, indicating dose-dependent action. At day 7, the level of fungal growth inhibition was calculated. Compared to the control, fungal growth was 75.14% inhibited by *C. limon* EO at 1.5 mg/mL. *C. limon* EO indicated absolute inhibition of *A. flavus* at 1.75 mg/mL and *C. sinensis* EO provided 68.24 % inhibition at the same concentration compared with the control (Miri Y.B et al., 2018).

The major components of *C. sinensis* determined by GC-MS technique were d-limonene (96%) and α -myrcene (2.79%) besides the flavonoids, carotenoids and vitamin C that are present at high concentrations in the *Citrus* genus (Ruiz-Pérez et al., 2016). A research study by Ruiz-Pérez et al. (2016), *C. sinensis* showed less evident growth inhibition of *C. Albicans* when compared with amphotericin B (antimycotic agent) effect. Moreover, *C. sinensis* showed the best inhibitory effect against *C. glabrata*. The authors suggested that the antimycotic activity of essential oils or extracts are limited due to the mixture of several compounds resulting in less intensity than their sole effects. The dose dependent antifungal effect of *C. sinensis* can be explained by the higher concentration of limonene, β -pinene and myrcene contents which are implicated on growth stimulation of other species of fungi besides vitamin C and flavonoid content (Ruiz-Pérez et al., 2016). Those contents can cause lower antifungal activity of the extract in high doses.

A research study by Oikeh et al. (2020), describes that *Citrus sinensis* seeds showed higher antifungal activities than the peel extract or EO since it contains phytoconstituents like flavonoids, saponins, tannins, cardiac glycoside alkaloids, and steroids which are known to have huge antifungal features (Oikeh et al., 2020).

Comparing the *Candida* group to the control, there was a significant decrease in the concentration of total protein. The medium doses of orange extract (OE12.5% and OE25%) increased the protein content significantly compared to *Candida* group while took the value closest to the control group. Moreover, OE exposure caused significant changes on the structure and composition of yeast proteins, However, none of the OE doses could take this value close to the control group implying a lack of optimal antifungal effects on the protein structure of *Candida albicans*. Those changes in protein concentration and structure can be explained by the higher synthesis of some enzymes related to the lipid metabolism due to OE exposure in *Candida albicans*. *Candida albicans* expresses multiple fatty acid desaturases including $\Delta 9$, $\Delta 12$, and $\Delta 15$ enzymes which is responsible for the production of polyunsaturated species 9Z,12Z-18:2 and 9Z,12Z,15Z-18:3 (Oh et al., 2006).

That enzymes are membrane-bound proteins located on endoplasmic reticulum of the fungi. Moreover, for the desaturation reactions there are regulatory proteins such as scSpt23p/scMga2p proteins to control the expression of the products of desaturases (Oh et al., 2006). Since there is an elevation in the production of unsaturated lipids due to OE exposure, the significant changes in protein concentration and structure of *C. albicans* upon to all OE doses can be clearly explained by the activation of the synthesis of specific enzymes and their regulatory proteins in unsaturated lipid.

In the nucleic acid content, medium dose (25%) of OE showed optimal antifungal effect by decreasing the nucleic acid content of *C. albicans* species. Moreover, all OE doses increased the carbohydrate content of *C. albicans* except low dose OE (6% OE).

Comparing the *Candida* group to the control, the total carbohydrate content was considerably lower in the *Candida* group. Fig. 4.7 along with Table 4.2 show the exception of the low dose (6%), all OE doses significantly increased the carbohydrate content as compared to the *Candida* group. By exhibiting opposing effects with the medium and high doses, the low dose orange extract dramatically reduced this value in comparison to both the control and *Candida* groups.

An investigation by Fiolka MJ, 2019 where the ATR-FTIR revealed that the AAF spectrum and the spectrum of egg white lysozyme were highly comparable (82%); nevertheless, a comparison

of these spectra reveals that the variances between these spectra were primarily caused by vibrations in the areas typical for polysaccharides. The GC-MS study revealed substances including inositol, glucose, galactose, and N-acetylglucosamine in the AAF, while the NMR analysis revealed the presence of sugars.

They discovered a protein-carbohydrate component in the coelomic fluid of the earthworm *D. veneta* that efficiently attacked *C. albicans* and *C. krusei* cells. The AAF is a promising compound with antifungal activity due to its efficient action against *Candida* in the absence of endotoxicity and cytotoxicity against normal skin fibroblasts. Further investigation with an eye toward potential medical applications should be conducted given that the AAF demonstrated strong efficacy against the clinical *C. albicans* isolate (Fiołka MJ., 2019).

According to a study by (Staniszewska M., et al 2013), variations in carbohydrate content may be caused by the kind of shape created by various environmental factors. Among the examined carbohydrates, glucan proved to be present in both tested morphological fractions in noticeably higher concentrations. When compared to hyphae, the blastoconidial cell wall's ultrastructure showed startling variances, pointing to changes in the amount of carbohydrates used for wall building during hyphal growth at 37 degrees and an alkaline pH. The study gave proof of the connection between morphogenesis, serum-induced cell-cell adhesion, and variations in the amount of carbohydrates content.

There is an opposite effect on both nucleic acid and carbohydrate content of *C. albicans* when exposed to low dose OE.

In conclusion, the optimal antifungal effect was seen with medium OE doses (12,5% and 25%) by affecting the saturated and unsaturated lipid concentration and composition of *C. albicans*. The protein changes were related to the changes of lipid metabolism of yeast related to the enzymes and regulatory proteins of that metabolism. There was no dose effect in the changes of nucleic acid and carbohydrate contents except low dose of OE (6%) which shows opposite effect for those parameters when compared to the other doses of OE.

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