

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**STRUCTURE ACTIVITY RELATIONSHIP FOR
ANTIFUNGAL ACTIVITY CHALCONE DERIVATIVES**



SENA NUR RAKİPOĞLU

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOENGINEERING**

ADVISOR: ASSOC. PROF. BENGÜ ERGÜDEN

FEBRUARY 2024

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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ**

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SENA NUR RAKİPOĞLU

**YÜKSEK LİSANS TEZİ
BİYOMÜHENDİSLİK ANABİLİM DALI**

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Dedication / İthaf

To my Family

ABSTRACT

Fungal infections are infections caused by pathogenic fungal cells or fungi. These infections may be life-threatening in severe cases. For this reason, the need to develop new antifungal agents with new mechanisms of actions has raised. In addition, the frequent use of the existing antifungal drugs and agents, has given the fungal cells resistance against them. Chalcones are phenolic organic substances that exist naturally in plants. These substances can be extracted from fruits and vegetables to be used in pharmaceutical drugs industry as they have properties such as having good anticancer, antibacterial, anti-inflammatory features. Chalcones participate in the biosynthesis of flavanones, which give them biological properties similar to the chalcones. The studies on the link between chalcone derivatives with different chemical structures and the fungal cell viability are very few. Thus, in this study, we aim to clarify the relationship between chalcones and its derivatives on yeast cells *Saccharomyces cerevisiae*. We have determined the antifungal activity of various chalcone derivatives against *S. cerevisiae* investigated the exact effect of these substances on yeast cells via extracellular conductivity measurement, UV and FTIR spectra measurements. Most of the existing antifungal agents target the cellular membrane of the fungal cell. Cell membrane acts as the external barrier that controls substances entering or leaving the cell. When cell membrane is disrupted by such agents the cell loses its viability. Our results also showed that chalcones disrupt cell membrane integrity, cause leakage of ions out from the cell and cause metabolic distortions. Our study will take part in the development of the current knowledge regarding antifungal agents, and it will help in the discovery and of new and novel antifungal agents.

Keywords: Antifungal Agents, Fungi, Chalcones, Cell Membrane, *S. cerevisiae*, Yeast.

ÖZET

Mantar enfeksiyonları patojenik mantar hücrelerinin veya mantarların neden olduğu enfeksiyonlardır. Bu enfeksiyonlar ciddi vakalarda hayatı tehdit edici olabilir. Bu nedenle yeni etki mekanizmalarına sahip yeni antifungal ajanların geliştirilmesi ihtiyacı ortaya çıkmıştır. Ayrıca mevcut antifungal ilaç ve ajanların sıklıkla kullanılması mantar hücrelerine bunlara karşı direnç kazandırmıştır. Kalkonlar bitkilerde doğal olarak bulunan fenolik organik maddelerdir. Bu maddeler iyi antikanser, antibakteriyel, antiinflamatuvar özelliklere sahip olmaları nedeniyle ilaç endüstrisinde kullanılmak üzere meyve ve sebzelerden ekstrakte edilebilir. Kalkonlar, flavanonların biyosentezine katılarak onlara kalkonlara benzer biyolojik özellikler kazandırır. Literatürde farklı kimyasal yapıya sahip kalkon türevleri ile mantar hücresi canlılığı arasındaki bağlantıya ilişkin çalışmalar oldukça azdır. Bu nedenle, bu çalışmada, *Saccharomyces cerevisiae* maya hücrelerinde bulunan kalkonlar ve türevleri arasındaki ilişkiyi aydınlatmayı amaçladık. Çeşitli kalkon türevlerinin *S. cerevisiae*'ye karşı antifungal aktivitesini belirledik ve bu maddelerin maya hücreleri üzerindeki etkisini hücre dışı iletkenlik ölçümü, UV ve FTIR spektrum ölçümleri yoluyla araştırdık. Mevcut antifungal ajanların çoğu, mantar hücresinin hücre zarını hedef alır. Hücre zarı, hücreye giren ve çıkan maddeleri kontrol eden dış bariyer görevi görür. Hücre zarı bu tür ajanlar tarafından bozulduğunda hücre canlılığını kaybeder. Benzer şekilde bu çalışmamız da kalkonların hücre zarı bütünlüğünü bozduğunu ve iyonların hücre dışına sızmasına neden olduğunu, ayrıca metabolik bozulmalara neden olduğunu göstermiştir. Çalışmamız antifungal ajanlarla ilgili güncel bilgilerin geliştirilmesine katkı sağlayacak, ve yeni antifungal ajanların keşfedilmesine ve geliştirilmesine yardımcı olacaktır.

Anahtar Kelimeler: Antifungal Ajanlar, Mantarlar, Kalkonlar, Hücre Membranı, *S. cerevisiae*, Maya.

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February 2024.



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LIST OF SYMBOLS AND ABBREVIATIONS

<i>S. cerevisiae</i>	:	<i>Saccharomyces cerevisiae</i>
<i>C. albicans</i>	:	<i>Candida albicans</i>
GI	:	Gastrointestinal
AIDS	:	Acquired Immune Deficiency Syndrome
PMFs:	:	Polymethoxylated flavones
<i>E. coli</i> :	:	<i>Escherichia coli</i>
<i>M. Tuberculosis</i>	:	<i>Mycobacterium tuberculosis</i>
MIC	:	Minimum Inhibitory Concentration
MFC	:	Minimum Fungicidal Concentration
<i>S. aureus</i>	:	<i>Staphylococcus aureus</i>
<i>P.aeruginosa</i>	:	<i>Pseudomonas aeruginosa</i>
<i>S. enteritidis</i>	:	<i>Salmonella enteritidis</i>
UV	:	Ultraviolet
FTIR	:	Fourier Transform Infrared
YPD	:	Yeast extract Peptone Dextrose
DMSO	:	Dimethylsulfoxide

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1. INTRODUCTION

Yeasts are part of the fungi kingdom, which includes eukaryotic organisms such as molds, and mushrooms. As other eukaryotic organisms yeast cells contain real nucleus which enclose the genetic material DNA, and real organelles with endomembrane system as any typical eukaryotic cell. Yeasts are unicellular, heterotrophic. Heterotrophic organisms rely on other animals or plants to get their nutrient in order to stay alive, rather than making their own food by themselves. Yeasts mostly reproduce asexually in a process called budding [1]. Budding is a route of asexual reproduction in which a bud or a projection is developed by mitosis from the mother's body then separates to develop into a new yeast cell. One of the most remarkable yeast species is *Saccharomyces cerevisiae* which feeds on the fermentation of glucose. It converts glucose into ethanol and carbon dioxide to get its nutrients [2]. In the ancient time people were using *Saccharomyces cerevisiae* and the process of fermentation it carries out to produce products such as, bread, yogurt, cheese, and beer (Figure 1.1).



Figure 1.1: Yeast used in bread making.

1.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a eukaryotic unicellular organism that has been used as a model in many studies. It is commonly known as baker's yeast. *Saccharomyces cerevisiae* has been utilized in many scientific fields such as biology, food and medicine industry, and biotechnology. *Saccharomyces cerevisiae* belongs to the kingdom fungi, and the kingdom *Saccharomycetes*, yeast. Yeast exists naturally on the fruit's skins, and on tree barks. In addition, it has been sold in markets for domestic uses, such as bread, and bakery.

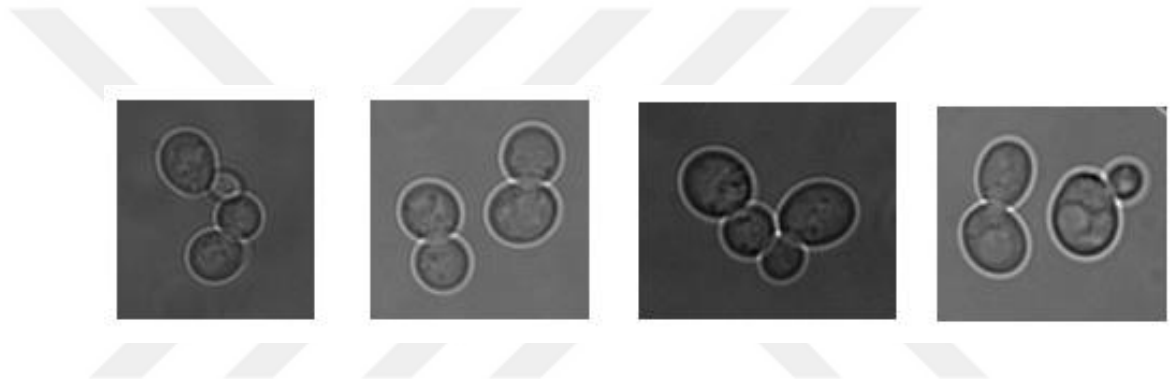


Figure 1.2: Microscopic view for *Saccharomyces cerevisiae*.

S. cerevisiae cells have the cell wall as the most outer layer (Figure 1.3). Cell wall of yeast cells has attracted the attention of scientists, especially in the last few decades. Cell wall plays important roles, such as, providing mechanical attention to yeast cells, plus the participation in many vital events within the cells, like budding, mating, or sporulation, stabilization of the osmotic conditions, and offering protection against the mechanical damage. Cells wall also keeps the specific shape of yeast cell, which is usually elongated shape.

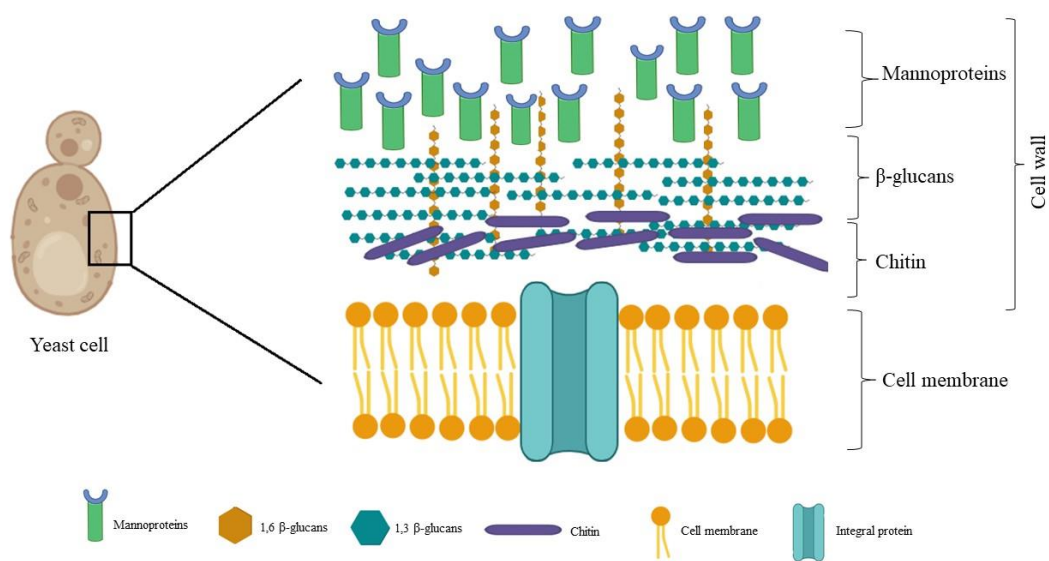


Figure 1.3: Cell wall and cell membrane structure

S. cerevisiae cell walls consists mainly of carbohydrates, with a percentage range from 80% – 85%, in addition to proteins that ranges from 15% - 20%. The carbohydrates that make up the cell wall of yeasts are glucan, mannan, and chitin. The cell wall may vary among *S. cerevisiae* according to the surrounding environment depending on several factors, such as, pH, temperature, osmolarity, and oxygen levels. The cell wall of yeasts consists of two layers, outer layer that is 200 nm thick, which contains high amounts of glycosylated mannoproteins, and an inner layer that gives mainly the mechanical strength.

Saccharomyces cerevisiae's cell membrane consists of phospholipid bilayer, which consists of two layers of phospholipids with hydrophilic heads, and hydrophilic tails. In addition to main component in the cell membrane called ergosterol. Ergosterol is the essential sterol exists in the fungal cells' membranes. These compounds play an important role in maintenance of the integrity, permeability, and fluidity of the cell membrane. The process of ergosterol biosynthesis is considered as complex process that requires high amount of energy, and specific enzymes. Any problem in the process of ergosterol biosynthesis within the *S. cerevisiae* cell membranes affects the cellular proliferation, and adaptation mechanisms. Ergosterol biosynthesis is affected by the external conditions of the *S. cerevisiae* cells, like the existence of some metabolites

such as, sterols, oxygen, and iron. Ergosterol has been the target for many antifungal drugs such as azole, and polyenes, or targeting the enzymes involved in the biosynthesis of ergosterol. These drugs contribute to the disruption of the cellular membrane, and eventually cell death.

The cell membrane of *Saccharomyces cerevisiae* is involved in many functions such as endocytosis, and exocytosis. Endocytosis is the process in which cells uptake nutrients from the external environment, while the exocytosis is when cells release vesicles to the outer environment. These vesicles contain content that need to leave yeast cells. Beside this function, cell membrane is critical for the process of budding, or forming buds, which is the route in which *Saccharomyces cerevisiae* reproduce. Receptors reside in the plasma membrane of yeasts help them to notice the environment they live in.

Saccharomyces cerevisiae has been a simple model organism to be used in the scientific research and studies, due to several reasons, such as, the simplicity of its genome. The *Saccharomyces cerevisiae* genome contains approximately 12 million base pairs, so it is smaller than higher eukaryotic genomes such as humans. In addition, yeast cells are easy to grow and cultivate in the lab conditions, as these organisms are unicellular. Moreover, a wide variety of mechanisms such as gene knock outs, gene overexpression, and conditional mutants, are all happen and available in yeast cells. Also, many other mechanisms that happen in higher eukaryotes such as humans are happening in yeast cells which gives them advantage in comparison to other microorganisms. Yeast cells have been used in the field of human disease testing, such as neurodegenerative diseases, like Alzheimer's, and Parkinson diseases.

S. cerevisiae has been used in the field of biotechnology. Yeasts have been used in the process of production of recombinant enzymes and antibodies. In addition, several vaccines have been produced using *S. cerevisiae* yeast strain. *S. cerevisiae* has been used also in pharmaceuticals industry, especially in drug discovery and drug metabolism. Yeast has been used in many screening assays in drugs discovery process. These organisms are also have been utilized in much environmental research, such as using them in the process of bioremediation.

S. cerevisiae has been used widely in the industrial field, especially in bread, wine, yogurt, and beer manufacturing. Ancient Egyptians were the first people to use

Saccharomyces cerevisiae or simply the yeast in the process of bread making. *Saccharomyces cerevisiae* is used also in chocolate production. Theobroma cacao plant is the basis in chocolate manufacturing, but this plant needs to be fermented before starting the process. The addition of *S. cerevisiae* to Theobroma cacao helps in the releasing of different aromas and flavors. Moreover, *Saccharomyces cerevisiae* has been used in the process of biofuels production, such as ethanol, which is a widely used biofuel. The process of biofuel production has been started since 1826. *S. cerevisiae* is added to the sugar in corn or sugarcane so it can ferment it and convert it into ethanol through a process known as alcoholic fermentation. Yeasts consume sugars glucose and fructose in corn or sugarcane converting them into carbon dioxide and alcohol. Specific enzymes are involved in the process of alcoholic fermentation. Nowadays, researchers are working on the development of yeast strains that are designed especially to give high yield in the process of alcoholic fermentation. *S. cerevisiae* is also involved in the production of eco-friendly biofuel which is manufactured from agricultural residues and forests wastes.

1.2. Flavonoids as Antifungal Agents

Yeasts can be beneficial and consumed safely by humans such as *Saccharomyces cerevisiae*, but many yeast species are harmful or pathogenic. Pathogenic types of yeast can contribute to serious or even fatal infections for plants, animals, humans, or for other types of fungi. Pathogenic yeasts that infect plants play an important role in the damage of crops, decreasing the crop yields, and increase the chances of desertification [3]. Pathogenic yeasts also infect human beings. One of the most infectious species among yeasts is *Candida albicans*. *C. albicans* is a yeast species that have the ability to infect many areas within the human body, starting from the oral cavity to the skin and nails, until gastrointestinal (GI) and reproductive tracts [4]. Under the normal conditions the human body has the ability to fight and destroy such pathogens, but when the immune system fails in destroying these infectious entities yeast infections develops. This means that people with AIDS or those who receive immunosuppressive medications are more likely to develop such infections.

As the yeast infections increased remarkably in the last decades, it's important to use antifungal agents or medications to eliminate and get rid of these infections. The

misuse of such agents led to the development of a condition called antifungal resistance [5]. In which the antifungal agent is no longer effective in destroying pathogenic yeasts or infections. For this reason, the need to develop new and novel antifungal agents against these infectious yeasts has increased.

It has been demonstrated that natural products have an excellent potency to give promising results in the mechanism of destroying microbes and bacteria [6]. Among these promising natural products flavonoids. Flavonoids are compounds that exist naturally in plants such as, fruits, vegetables, flowers, and in some drinks like tea and wine. Flavonoids in plants participate in vital processes such as photosynthesis, defense against pathogens that may infect the plant, energy transfer, and the shaping of the plant or morphogenesis, etc [7]. It's important to have a good amount of these compounds in the diet to maintain a good health, and to promote the defense mechanism against pathogens. This amount is suggested to be between 500–1000 mg [8]. The basic structure for flavonoid compounds consists of two benzene rings that are connected by heterocyclic pyrane ring. Recent studies have approved that flavonoids show anti-inflammatory, antioxidant, and antiallergic activities beside its antimicrobial activity [9]. Studies regarding flavonoids structure and its interaction with other molecules in terms of some enzymes inhibition mechanism are growing in number day by day. There is big evidence that this inhibition happens due to the interaction of parts of flavonoids structure like the benzene ring for example and macromolecules such as carbohydrates [10]. Flavonoids were prescribed in the old ages to treat diseases such as infections, sores and ulcers. As an example, *Scutellaria baicalensis* which is a type of herbs that contains a high level of different flavonoid types, was used in China to treat periodontal abscesses and oral infections. Also, *Tagetes minuta* was used in Argentina to treat various infectious diseases [11].

Flavonoids are classified in terms of their structure into, flavanones, flavanonols, flavones flavonols, isoflavones, chalcones, catechins, and anthocyanidins, flavonoids general structure is represented (Figure 1.4).

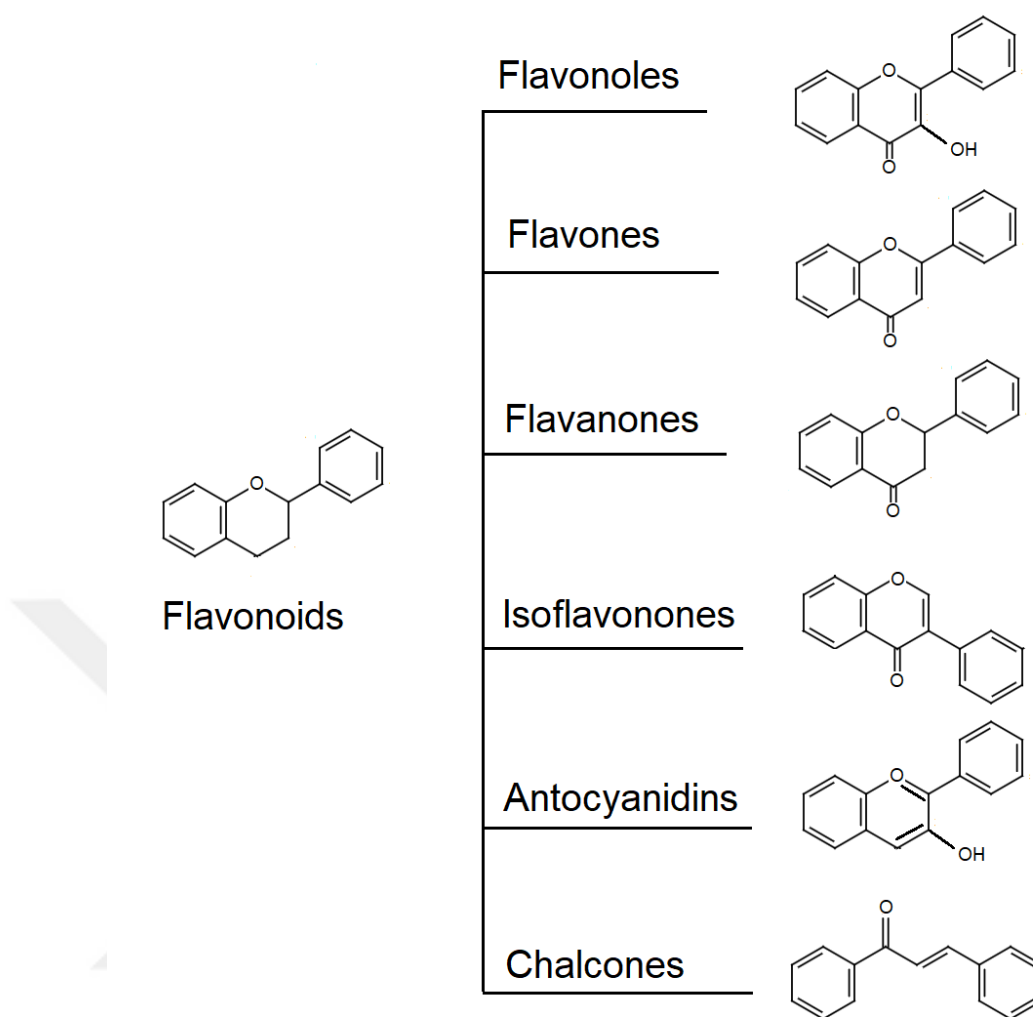


Figure 1.4: Flavonoid families.

Flavonoids classes vary in their antimicrobial activity. In a recent work [12], eight flavonoids have been tested to compare their antibacterial activity (Five flavones and three flavanones), the results have shown that flavones were more active in the gram-positive bacteria inhibition than flavanones. For this reason, it can be said that the C2=C3 double bond is crucial for the antibacterial activity. In addition to the previous conclusion, it was also proved that the number of hydroxyl groups is not critical for the antibacterial, unlike the position of two hydroxyl groups in the flavanones, that have shown to be important for the antibacterial activity.

Chalcones are a class of flavonoids that has shown tendency to be a good anti-inflammatory, antiinvasive, anti-cancer, and antifungal activity. Chalcones consists of two benzene rings connected with α , β -unsaturated carbonyl chain [13].

Chalcones are different in their antimicrobial activity. The activity may change according to the structure of the chalcones [6]. An example for this the chalcone that have 4-alkylthio- or 4-alkoxy in their structure exhibit weaker antibacterial activity. By contrast, chalcones that have piperazine or 2,5- dichlorothiophene on the basic skeleton, have shown better antibacterial activity [14]. Studies before have concluded that the chalcones' activity is due to the existence of phenolic hydroxyl groups, which sticks to the proteins, changing their 3D shape, so the microbial or bacterial inhibition takes place. According to Avila et al, it was demonstrated that hydroxylation of all positions in ring A especially for 2' position, carboxylation of 4' position, and lipophilicity of the ring in chalcones that contain prenyl or geranyl at 3' position, increase the antibacterial activity. In contrast, the acetoxylation or methoxylation of 2' position, and fluorination at 3' and 5' positions on ring A, decrease the antibacterial activity. Regarding the ring B of chalcones, the same study has shown that any substitutions in the 4th position have said to be improving the antibacterial activity. Also, lipophilic substituents trifluoromethyl, and bromo in the position 3 and 5 of the ring boost the antibacterial properties of the chalcone.

1.3. Possible Target Site and Mode of Action of Antifungal Agents

Last few decades the resistance to antibacterial and anti-fungal agents has increased noticeably [15]. This increase put the societies under severe risks such as increasing of morality, in addition to the pressure and costs that will be on the health care facilities and hospitals to reduce the negative effects and consequences that may lead with society to a pandemic. That's why a better understanding of antifungal agents and their mechanism of action is becoming necessary day by day. Most of the studies that emphasize the importance of finding new and novel antimicrobial agents have focused on antibacterial agents [5], the most obvious reason for this is that most of infection cases in hospitals are bacterial infections. Anti-fungal agents mechanisms studies has lagged behind the antibacterial agents studies because diseases caused by fungi have not been categorized to be serious until a recent time [5]. An example for this is andidiasis, which is a fungal infection caused by a specific type of yeasts called *Candida*. Candidiasis cases were stable between 1950 and 1970. By 1970 the cases started to increase significantly, due to the wide use of drugs that suppress immune

system, the change in infections treatment strategies, and the spread of immune system viral infections such as AIDS. Thus, the scientists decided to develop new safer antifungal agents. By early 1990s imidazoles and triazoles were approved to be used in the treatment of fungal infections due to their effectiveness and safety, so that fluconazole, which is a class of azoles, has saved the life of approximately 16 million patients in the United States only [16]. Mainly there are three major antifungal agents that are used clinically, these are, azoles, polyenes, and allylamine/thiocarbamates [15]. These three classes of antifungal agents are common in the mechanism of action, they interfere with the ergosterol biosynthesis. Ergosterol is a type of sterols that maintain the cell membrane fluidity and semi-permeability. Ergosterol exists only in fungal cells membranes, while it does not exist in human cellular membranes, which makes it a good target for a new antifungal agent designing [17].

Azoles are aromatic, two double bond containing compounds. Azoles are divided into two main classes, imidazoles, with two nitrogen atoms in the azole ring, and triazoles with three nitrogen atoms in the azole ring [18]. Azoles work with the same mechanism of action which is the inhibition of ergosterol synthesis. Ergosterol is the major component in the cell membrane of fungi and protozoa that maintain the fluidity and integrity [17]. The exact mechanism starts with the inhibition of fungal cytochrome P450. This inhibition prevents the conversion of lanosterol into ergosterol, so the membrane integrity and fluidity change [19]. As a result, the fungal growth and replication stops, Moreover, toxic effects on the membrane phospholipids, less fungal adhesion, and morphological changes were observed.

Other type of antifungal agents are polyenes. Polyenes were one of the first antifungal drugs discovered that helped in the treatments of fungal infections [20]. There are three polyene types that are used clinically today, these are: amphotericin B, nystatin, and natamycin.

Although polyenes were among the first antifungal agents discovered, they are still in use nowadays due to their high efficiency in the treatment of fungal infections. Nevertheless, many studies suggested that amphotericin B is not that efficient in the process of fungal inhibition in some species like, *Aspergillus terreus*, *Scedosporium spp.* and *Candida auris*, due to the resistance to that drug [21].

The mechanism of action in polyenes is a bit different from azoles. Many research studies have proposed many modes of action. Currently, there are many accepted modes of antifungal mechanism for polyenes. The first is pore forming model, which suggests that polyenes interact with ergosterol molecules in the fungus membrane, causing pores that leak the cellular content to the surrounding, which ends up with cell mortality. In addition to the previous mode of action, there are, surface adsorption and sterol sponge models, these models work in the same mode of action against fungi [15]. These two mechanisms hypothesize that the extraction of ergosterol from the fungus membrane will alter the membrane properties like fluidity, so many vital mechanisms such as exocytosis, or endocytosis, will be inhibited [21].

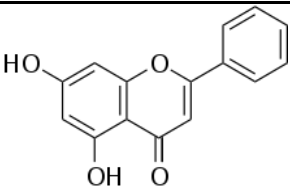
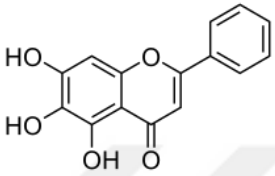
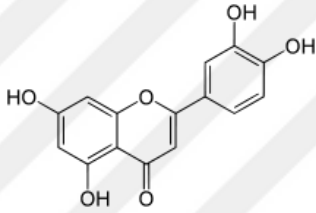
The other class of antifungal agents is allylamine. Allylamine blocks the biosynthesis of the ergosterol through the inhibition of the enzyme squalene epoxidase, which carry out the process of squalene conversion to lanosterol, that then is converted into ergosterol [22]. This leads eventually to the fungal cell death. Two examples for this class are, terbinafine and naftifine.

1.4. Review of Recent Structure Activity Relationship for Antibacterial Activity of Flavonoids

1.4.1. Flavones

Wu et al., examined the antibacterial effect of three polymethoxylated flavones (PMFs), and three flavones against the bacteria *E. coli* (Table 1.1) [23]. Tested agents were effective in DNA gyrase inhibition of *E. coli*. It has been proven that hydroxyl group existence in 6 position of ring A has adverse antibacterial effects. For PMFs the presence of methoxy group in the 6 or 8 position of ring A is said to be increasing the activity. While it's presence in 3' position of B ring decreased the activity. For flavones and PMFs the hydroxyl group in 5 position on ring A has promoted the bacterial inhibition. In inhibition, the presence of hydroxyl groups in 5, 6, and 7 positions has elevated the inhibitory features compared to these agents having methoxyl groups in the same position. Hydroxyl group in 4' position in ring B, and methoxyl group in positions 3, and 8 in ring A, are great for bacterial inhibition, while hydroxyl group in 6 position of A, 3' and 5' positions of B, and 3 in C ring reduced the antibacterial effects.

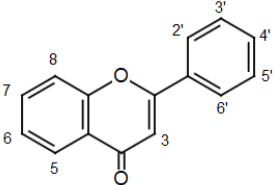
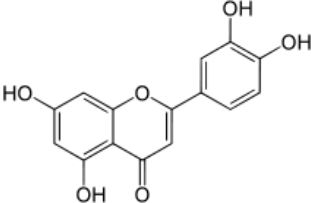
Table 1.1: MIC values of three flavones [23].

Flavones	MIC50 ($\mu\text{g}/\text{Ml}$)
 Chrysin	37
 Baicalein	71
 Luteolin	67

Xu et al., tested the antibacterial effects of two flavones (flavone, and luteolin) against methicillin-resistant *Staphylococcus aureus* (MRSA) [24]. Flavone does not include hydroxyl groups while luteolin does (Table 1.2). Luteolin showed activity because of the existence of keto group in position 4 and hydroxyl groups in 3, 5, and 7 positions. Plus, having at least one hydroxyl group in ring B. Flavone is an exception that it does not contain any hydroxyl group, but still shows antibacterial inhibition properties. This study showed also that the more hydrophilic the flavone is the more antibacterial inhibition it has.

Table 1.2: Zone of inhibition on 50 $\mu\text{g}/\text{disc}$ of flavone and luteolin [24].

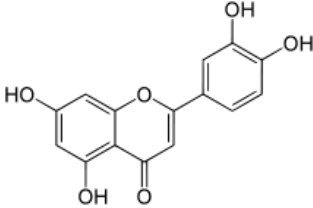
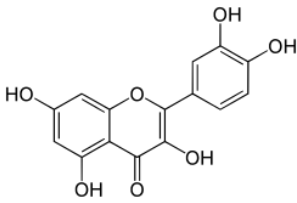
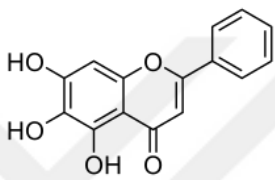
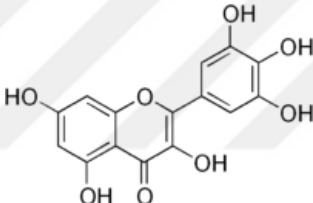
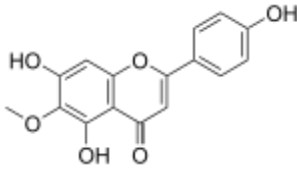
Flavones	MRSA5	MRSA
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 Flavone	9	9
 Luteolin	11.5	10.5

Yadav et al., examined five active flavonoids as promising antitubercular agents (Table 1.3) [25]. These five compounds are (baicalein, luteolin, quercetin, hispidulin, and myricetin). Structure activity relationships for these compounds were studied for baicalein, it has been demonstrated that the existence of hydroxyl groups at the 5, 6, and 7 positions is critical for inhibitory actions. For luteolin, quercetin, and myricetin hydroxyl groups in 3' and 4', and 4' position in hispidulin were found essential for antibacterial activity against *M. tuberculosis*. If hydroxyl group in previous position get methylated or glycosylated flavonoids become inactive against *M. tuberculosis*. The most active flavonoid among five tested compounds was luteolin.

Table 1.3: MIC values of 5 active flavones [25].

Flavonoid	MIC ($\mu\text{g/mL}$) at 100 $\mu\text{g/mL}$ concentration
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	25
Luteolin	
	50
Quercetin	
	50
Baicalein	
	50
Myricetin	
	100
Hispiludin	

In a recent work, authors aimed to investigate the antibacterial activity by adding or substituting different chemical groups to flavones, 4-thioflavones and 4-iminoflavones, against specific strains of bacteria *Escherichia coli*, *Bacillus subtilis*, *Shigella flexnari*, *Salmonella aureus*, *Salmonella typhi* and *Pseudomonas aeruginosa* [26]. It has been demonstrated that the presence of sulfur and nitrogen atoms instead of oxygen atom increased the antibacterial activity. Furthermore, the existence of

methoxy group in the 4th position in ring B enhanced the antibacterial activity of the compounds. While nitro group presence instead of methoxy decreased the antibacterial activity to some extent. On the other hand, methoxy groups in both 3' and 4' positions promoted the tested compounds antibacterial properties. The nitro group in 2' and 5' positions in ring B showed a decreased activity for tested flavones. In addition, having F atom in 4' position at ring B promoted the antibacterial features against all bacteria tested except *S. aureus*. Whereas compounds showed no antibacterial activity in the case of methyl group presence in the B ring. So, it can be concluded that electronegative groups are essential for antibacterial activity in tested flavones, especially if these groups exist in 4' position at B ring of flavones.

Echeverria et al. investigated the effect of oxygenated form of three flavanones and five flavones on four gram positive and four gram negative bacteria species (Table 1.4 and 1.5) [12]. The flavones tested were quercetin, galangin, 3-O-methylisorhamnetin, 3-O-methylgalangin, and 3-7-O-dimethylgalangin. Galangin and 3-O-Methylgalangin have the highest antibacterial activity among tested flavones. It has been proved that hydroxyl group number is related to antibacterial properties. For example, 3-7-O-dimethylgalangin with one hydroxyl group is less active than 3-O-methylgalangin with three hydroxyl groups, followed by the compound which has the least antibacterial activity quercetin. It was shown that the existence of a hydroxyl and a methoxy or two hydroxyl groups in ring B is said to be decreasing the antibacterial activity of tested flavones. Furthermore, the presence of two hydroxyl groups on ring A and absence of polar groups on ring B increased the activity significantly.

Table 1.4: MIC values ($\mu\text{g}/\mu\text{l}$) of five flavones against gram negative bacteria [12].

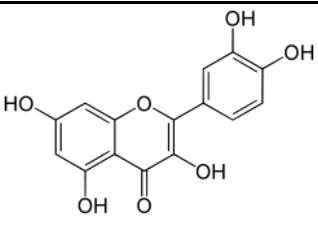
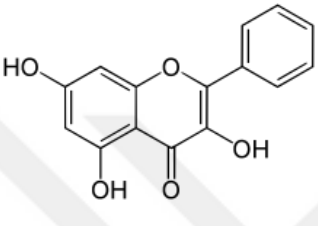
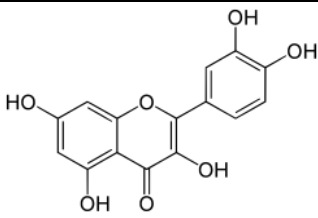
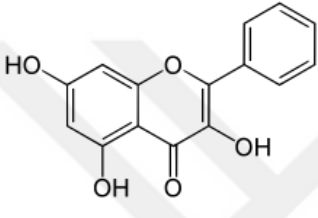
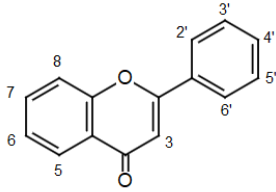
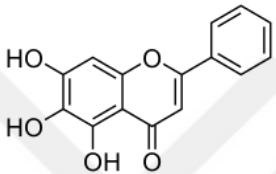
Flavone	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>
 Quercetin	>4.00	>4.00	0.50
 Galangin	1.00	0.50	0.25
3-O-Methylgalangin	0.50	0.50	0.25
3,7-O-Dimethylgalangin	>4.00	>4.00	>4.00

Table 1.5: MIC values ($\mu\text{g}/\mu\text{l}$) at 5 μl deposition of five flavones against gram positive bacteria at 5 μl deposition [12].

Flavone	<i>B. cereus</i>	<i>B. coagulans</i>	<i>S. aureus</i>
 Quercetin	2.00	2.00	2.00
 Galangin	0.25	0.25	0.25
3-O-Methylgalangin	2.00	1.00	>4.00
3,7-O-Dimethylgalangin	0.25	0.38	0.50
	>4.00	>4.00	>4.00

Xie et al., examined the antibacterial effect of thirty-eight polyphenol compounds which are well known to have antibacterial properties (Table 1.6) [27]. Among tested polyphenols ten were flavones. Baicalein was the most effective flavone among other ten flavones. The baicalein has a specific structure called pyrogallol on ring A, which means having three hydroxyl groups in 5, 6, and 7 positions. So, it has been proved that flavones with hydroxyl groups in 5 and 7 positions have potent antibacterial properties. Among tested flavones, Apigenin with C2=C3 double bond showed decreased antibacterial features, compared to narigenin which does not contain double bond. This may indicate that double bond existence decreases the antibacterial activity. Moreover, it has been demonstrated that glycosylation of flavones led to decreased activity. It can be concluded that hyperglycemic conditions have the potency to decrease antibacterial properties of tested flavones.

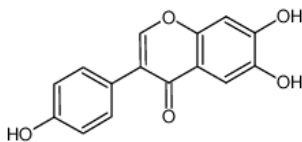
Table 1.6: Minimum inhibition concentrations (mmol/L) of flavone polyphenols on the tested bacteria [27].

Polypehol	<i>E. coli</i>	<i>S. aureus</i>
 Flavone	2	2
 Baicalein	0.5	1

1.4.2. Isoflavones

Hummelova et al., tested that antibacterial effects of fifteen different isoflavones against nine gram-positive and gram-negative bacteria strains. Among tested isoflavones demethyltexasin, hydroxydaidzein, biochanin A, demethylretusin, and genistein, showed the most significant antibacterial activity (Table 1.7) [28]. It has been demonstrated that the existence of hydroxyl group in C-5, 6 and 7 positions is essential for antibacterial activity. In addition, it has been shown that the presence of methoxy group in ring A decreases the bacterial inhibition significantly. An example of this is the tested compound 5,7-dihydroxy-4'-methoxyisoflavone, which was found to be inactive against tested bacteria strains. Demethyltexasin showed the most antibacterial activity especially against *Staphylococcus*, which makes this isoflavone a good candidate to create novel antibacterial agents.

Table 1.7: MIC ($\mu\text{g/mL}$) values of Demethyltexasin on bacteria [28].

Demethyltexasin	Tested bacteria	MIC value ($\mu\text{g/ml}$)
	<i>Bacillus cereus</i>	32
	<i>Enterococcus faecalis</i>	Inactive
	<i>Listeria monocytogenes</i>	128
	<i>Staphylococcus aureus</i>	16
	<i>Staphylococcus epidermidis</i>	32
	<i>Streptococcus pyogenes</i>	64

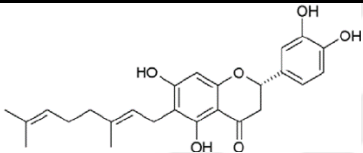
Sandgrove et al., investigated forty-seven natural Erythrina derived compounds against *Staphylococcus aureus* bacteria [29]. Among tested compounds it has been shown that the presence of prenyl groups in position 6 or 8 in ring A and 3' or 5' in ring B, and the existence of hydroxyl groups in 5 and 7 positions in ring A, are said to be important factors in increasing the bacterial inhibition. For example, Alpumisoflavone which lacks prenyl group is less active than other tested compounds. Moreover, the presence of methoxy group instead of hydroxyl group decreases the antibacterial activity, while the existence of methoxy group beside hydroxyl group does not decrease the antibacterial activity of the isoflavone.

1.4.3. Flavanones

Šmejkal et al., tested eight natural C-6-geranylflavonoids derived from *Paulownia tomentosa*, against selected gram-positive bacteria, gram-negative bacteria, and yeast species (Table 1.8) [30]. None of the eight compounds showed activity against gram negative bacteria or yeast. They showed activity against gram positive bacteria, with exception in tomentodiplacone B (C-6-geranylated diplacone). Tomentodiplacone showed antibacterial activity against four gram-positive bacteria out of six, while other compounds were said to be active against all tested gram-positive bacteria. It has been

demonstrated that 3'-O-methyl-5'-hydroxydiplacone, 3'-O-methyl-5'-O-methyldiplacone, and 3'-O-methyldiplacol, are the most active among tested compounds. The OH group in position 3 in 3'-O-methyldiplacol is said to be increasing the bacterial inhibition. While the presence of methoxy groups decreases the antibacterial activity. The presence of geranyl group in the tested compounds were found to be a reason for the weak activity against many tested species such as, *E. coli*, *P.aeruginosa*, and *S. enteritidis*.

Table 1.8: MIC ($\mu\text{g/mL}$) values of 6 flavonoids [30].

Compound	<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>	<i>Enterococcus faecalis</i>	<i>Staphylococcus aureus</i>
 Diplacone	4	4	4	4
3'-O-Methyl-5'-hydroxydiplacone	4	4	4	2
3'-O-Methyl-5'-O-methyldiplacone	4	4	4	4
3'-O-Methyldiplacol	2	4	4	2
3'-O-Methyldiplacone	4	8	8	8
Tomentodiplacone	16	-	-	16

Echeverria et al., investigated the effect of oxygenated form of three flavanones and five flavones on four gram positive and four gram negative bacteria species (Tables 1.9 and 1.10) [12]. The tested flavanones were pinocembrin, naringenin, and 7-O-methylepideriodictyol. It has been shown that flavanones are more active against the bacteria gram positive. The existence of more hydroxyl groups in the flavanones structure decreases the antibacterial activity. An example for this is Pinocembrin with

two hydroxyl groups, has higher inhibition rate than naringenin, and 7-O-methylepideriodictyol which have three hydroxyl groups. The presence of hydroxyl groups in 5th and 7th positions of the ring A is the main reason for tested flavanones inhibition activity.

Table 1.9: MIC values of three flavanones against gram negative bacteria [12].

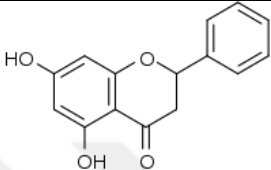
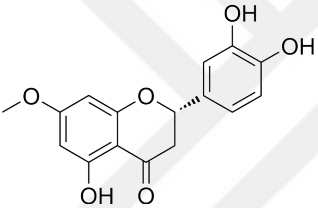
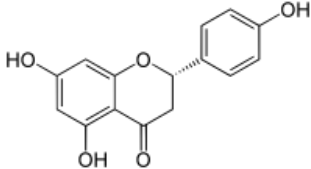
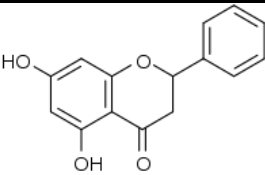
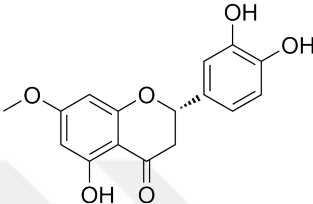
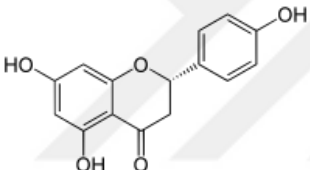
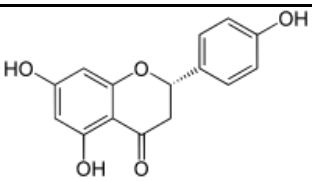
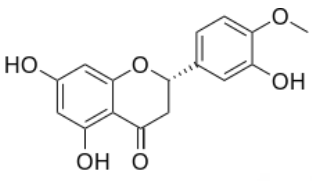
Flavanones	<i>E. cloacae</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>
 Pinocembrin	1.00	4.00	1.00	4.00
 7-O-Methylepideriodictyol	2.00	4.00	>4.00	0.50
 Naringenin	2.00	4.00	>4.00	2.00

Table 1.10: MIC values of three flavanones against gram positive bacteria [12].

Flavanones	<i>B. cereus</i>	<i>B. coagulans</i>	<i>B. subtilis</i>	<i>S. aureus</i>
 Pinocembrin	2.00	1.00	1.00	>4.00
 7-O-Methylepideriodictyol	2.00	1.00	2.00	4.00
 Naringenin	2.00	2.00	2.00	>4.00

Xie et al., investigated the antibacterial effect of thirty-eight polyphenol compounds which are well known to have antibacterial properties (Table 1.11) [27]. Among tested polyphenols nine were flavanones. These are, 6-hydroxyflavanone, 4'-hydroxyflavanone, 6-methoxyflavanone, naringenin, narigin, hesperitin, hesperidin, taxifolin, and neohesperidin-dihydrochalcone. It has been suggested that the existence of hydroxyl groups in the 5th and 7th positions of the ring A increases the bacterial inhibition. In addition, the saturation of the bond C₂=C₃ has also an effect on antibacterial activity. The overall results of the study showed that tested polyphenol compounds lose their antibacterial activity in the hyperglycemic medium.

Table 1.11: Minimum inhibition concentrations of two flavanones on the tested bacteria [27].

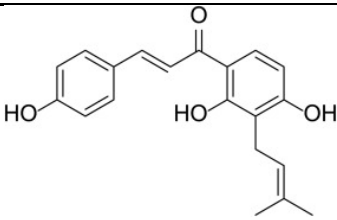
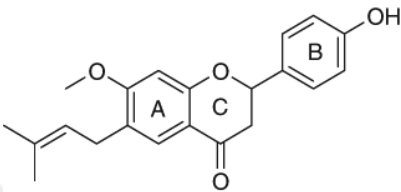
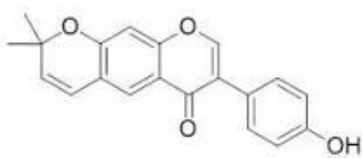
Flavanone	<i>E. coli</i>	<i>S. aureus</i>
 Narigenin	2	2
 Hesperitin	1	>2.5

1.4.4. Chalcones

Avila et al., studied the antibacterial activity of thirty-one chalcones against four different gram-positive bacteria. It was found that the most active compound is 4-Hydroxyderricin [31]. It has been found that 2' hydroxyl group is critical for the stabilization of the tested chalcones. Moreover, methylation or acetylation of 2' hydroxyl group leads to less inhibition. The presence of hydroxyl group in the 4th position of the ring B enhances the bacterial inhibition. Also, 4'-methoxyl group was found to be increasing the antibacterial features. In addition, the presence of prenyl or geranyl groups in the 3rd position of ring A elevates the activity. While the presence of isoprenyl group on the B ring, does not affect the activity.

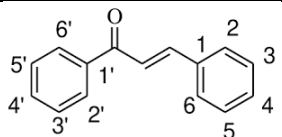
Yin et al., studied sixteen prenylflavonoids that were isolated from the plant species *Psoralea corylifolia* (Table 1.12) [32]. Compounds were tested against two-gram positive bacteria strain, *S. aureus*, and *S. epidermidis*. Compounds with prenyl group in the ring A showed significant activity, when this group is further oxygenated the compound loses its antibacterial activity. The existence of hydroxyl group in the 3' position enhances bacterial inhibition. It has been demonstrated that methoxy group in the 7th position led to stronger antibacterial properties. Among the strongest tested compounds were isobavachalcone, bavachinin, and erythrinin A.

Table 1.12: MIC values of five compounds against tested bacteria strains [32].

Compound	<i>S. aureus</i>	<i>S. epidermidis</i>
 Isobavachalcone	0.018	0.009
 Bavachinin	0.018	0.018
 Erythrinin A	0.018	0.018

Alcaráz et al., investigated the antibacterial activity of eighteen different flavonoids against the bacteria strain *Staphylococcus aureus* (Table 1.13) [33]. Among tested flavonoids, chalcones have showed the highest activity against inhibitory properties. It has been demonstrated that hydroxyl group in the 2' position of chalcones is critical for the antibacterial activity. On the other hand, the existence of methoxy group in the structure of the chalcone hinders antibacterial activity. Halogens substituents does not affect the antibacterial features of the chalcone. As a result, 2'-OH-chalcone, 2',4'-(OH)₂-chalcone and 2',4'-(OH)₂-chalcone were determined to be the highest in antibacterial agents in the inhibition of *Staphylococcus aureus*.

Table 1.13: MIC ($\mu\text{g/mL}$) values of chalcones [33].

Chalcone	MIC($\mu\text{g/mL}$)
	chalcone 38.7
2'-(OH)-chalcone	16.3
2',4'-(OH) ₂ -chalcone	31.6
2',4-(OH) ₂ -chalcone	36.6

Feng et al., synthesized the antibacterial activity of fifty-eight olympicin A, 4-chromanone, and chalcone derivatives, that were tested against *Mycobacterium tuberculosis* and other different gram-positive and gram-negative bacteria strains [34]. It has been demonstrated that phenol hydroxy groups at the 5- and 7-positions of 4-chromanone structure are essential for bacterial inhibition. In addition, hydroxy groups at 2'- and 4'-positions, plus the 2-alkyloxy substitution on the aromatic ring, enhanced the inhibition of gram-positive bacteria. The removal of any hydroxyl group led to less antibacterial activity for tested compounds.

1.5. Review of Recent Structure Activity Relationship for Antifungal Activity of Flavonoids

1.5.1. Flavanones

Yang et al. investigated the antifungal activity of 23 different 5,7- dihydroxyflavonoids against *Penicillium italicum*. It has been demonstrated that the presence of hydroxyl group at 4' position improves the antifungal activity of many tested compounds, while decreasing the inhibition of others [35]. In addition, the introduction of hydroxyl group at position 3 decreases the antifungal properties.

In Tian et al. 36 natural flavonoids were tested against *Aspergillus flavus* [36]. The study has shown that hydroxyl or methoxy groups in position 6 at ring A, and 4' at ring B are essential to enhance antifungal activity. Baicalein, flavone, hispidulin, kaempferol, and liquiritigenin were found among the strongest antifungal agents tested.

Ibrahim et al. investigated the activity of nine different synthetic flavonoids against different gram-positive bacteria and two fungi species [37]. Only 2'-hydroxy-4-chlorochalcone and 2'-hydroxy-4-nitrochalcone exhibited moderate inhibition against gram-positive bacteria, the rest seven compounds didn't show significant activity. The study has shown that the presence of methoxy group in the compounds' structure led to activity loss. α , β -unsaturated carbonyl group is found to be crucial for antimicrobial activity. On the other hand, chlorine atom presence is said to decrease the microbial inhibition.

Bernini et al, has synthesized 29 halogenated flavonoids, with their antifungal activity evaluation [38]. The study has shown that 8-chloro-5,7,3',4'-tetramethoxyepicatechin, a catechin derivative, exhibits strong antifungal features against *Trichoderma koningii*, *Fusarium oxysporum* and *Cladosporium cladosporioides*, with low MIC values.

1.5.2. Chalcones

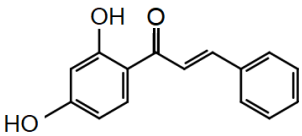
In Gupta et al, 10 chalcone derivatives were synthesized and their antifungal activity was examined against *Microsporum gypseum*, *Candida albicans*, and *Aspergillus niger* [39]. None of synthesized compounds showed activity against *C. albicans* and *A. niger*. However, they exhibited significant activity against *M. gypseum*. The study showed that the presence of Cl group in para position was found important for the antifungal activity as MIC was enhanced, while the presence of methoxy groups decreased the activity.

In a similar study Zheng et al, 30 chalcones have been synthesized and tested against several fungi, *Sclerotinia sclerotiorum*, *Helminthosporium maydis*, *Botrytis cinerea*, *Rhizoctonia solani* and *Gibberella zeae* [40]. Among tested compounds, pyridine containing compounds showed highest activity reaching 89.9% inhibition against *S. sclerotiorum* and 91.1% against *H. maydis*.

Andrade et al, studied the antifungal effect of 12 synthetic chalcone derivatives against *Candida albicans*. It was found that 2',4'-dihydroxychalcone has promising activity against tested *Candida*, with a good MIC value (Table 1.14) [41]. Although compound 2',4'-dihydroxychalcone inhibited the growth of *Candida albicans*, it has shown cytotoxicity in kidney cell lines. It has been observed that any substitution in the benzene ring in chalcone structure such as OMe, OEt, OBut, OHex and Cl groups, led

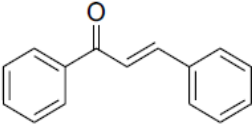
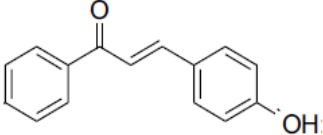
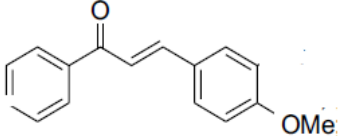
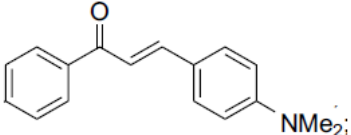
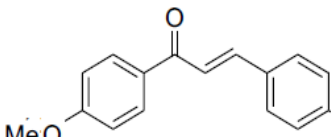
to decreased activity. In addition, the substitution of hydroxyl group by oxa-hexane also decreased the activity. Chalcone substituted with a morpholine group has presented good antifungal features against *C. krusei* and *C. tropicalis* but showed low activity against *C. albicans*. This activity is caused by the presence of morpholine group as a substitute.

Table 1.14: Minimum inhibitory concentration (MIC) of chalcones [41].

Compounds	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. crusei</i>	<i>C. tropicalis</i>
	15.6	15.6	15.6	15.6

Mellado et al, studied 15 chalcones that were synthesized and tested against the *B. cinerea* and *M. fructicola* phytopathogens[42]. The most active compounds are listed in Table 1.15. The study has shown that the presence of methoxy groups in ring A in compounds decreased the antifungal properties. For compounds that contain dihydrochromane part, the antifungal activity on *B. cinerea* dropped for all compounds, while for *M. fructicola* the effects did not change with the presence of this substitution. The presence of electroactive group such as Cl, F, or Br slightly increased the activity.

Table 1.15: IC₅₀ of chalcones [42].

Compound	<i>B. cinera</i>	<i>M. fructicola</i>
	43.9	48.5
	158.1	304.2
	45.5	135.6
	132.8	78.2
	76.2	51.8

2. PURPOSE OF THE STUDY

Chalcones are natural compounds shown to possess promising biological features such as working as a good anti-inflammatory, antibacterial, and antifungal agents. Yet, knowledge and research regarding the effect of these compounds on the yeast cells *Saccharomyces cerevisiae* are not sufficient, for this reason, we focused in this study on the influence of eight chalcone compounds on the yeast *Saccharomyces cerevisiae*. Our study aimed to unearth the effect of chemical configuration and different group substitutes on the antifungal activity of the tested compounds. First, the antifungal activity of the chalcone compounds were determined via minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) methods. Then we sought to determine the effect of the chalcone compounds on various cellular targets such as cell membrane, cell wall and cellular macromolecular structures by performing extracellular conductivity measurement, UV and FTIR spectra measurements. Overall, with our present study, we aimed to improve current knowledge regarding the relationship between chalcones as antifungal agents and fungal cells, in order to open up way for the development and discovery of new antifungal agents.

3. EXPERIMENT

3.1. Materials

3.1.1. Chemicals

Chalcones were purchased from abcr chemicals (www.abcr.com): Chalcone (AB113048), 4-nitrochalcone (AB178914), 4-methoxychalcone (AB177707), 4-hydroxychalcone (AB117243), 4'-nitrochalcone (AB242852), 4'-methoxychalcone (AB134078), 4'-hydroxychalcone (AB117242), 2',4'-dihydroxychalcone (AB151736).

3.1.2. Yeast Media and Plates

YPD Media	10 g Bacto-yeast extract (DIFCO)
	20 g Bacto-peptone (DIFCO)
	20 g Glucose
	to final volume 1 l dH ₂ O, autoclaved
YPD Plates	1 l YPD media
	15 g Bacto-agar (DIFCO), autoclaved

YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*) (Sikorski and Hieter 1989) was used as the *S. cerevisiae* strain in all experiments. Typically, cells were grown on solid yeast extract peptone glucose medium (YPD) at 25 °C and then precultured with an isolated colony in liquid YPD medium for 24 h. A small volume of inoculum was added to 10 mL fresh YPD medium, and the main culture was grown until it had reached the early exponential phase under aerobic conditions.

3.2. Methods

3.2.1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) Measurements

S. cerevisiae preculture was grown in YDP medium. YDP medium was used to grow *S. cerevisiae* in 25 °C, which is the optimum temperature to achieve the best growth for yeast cells. *S. cerevisiae* growth was under aerobic conditions, which means yeast cells are growing in the oxygen presence.

Chalcone stocks for the eight compounds were prepared according to specific calculations, and according to molecular weight of the compound. As an example, compound **1** stock calculation was done as follow:

The molecular weight for compound **1** is 208.26 g/mol. To prepare 1 ml of 50 mM stock:

$$\text{Molarity} = \frac{\text{Number of moles}}{\text{Volume in liters}}$$

$$0.050 \text{ Molar} = \frac{\text{Number of moles}}{0.001 \text{ liters}}$$

$$\text{Number of moles} = 0.00005 \text{ mole.}$$

$$\text{Number of moles} = \frac{\text{Mass}}{\text{Molecular weight}}$$

$$0.00005 \text{ mole} = \frac{\text{Mass}}{208.26 \text{ g/mol}}$$

$$\text{Mass} = 0.01 \text{ grams} = 10 \text{ mg}$$

The stock should be 10 mg/ml. To achieve 1 mM final concentration, 20 µl of 50 mM stock must be diluted to 1 ml.

Calculations are done for the other compounds in a similar way. After calculations are done, necessary amount of each of the eight compounds are weighed, then, compounds are dissolved in DMSO. Next, chalcone stock solutions are placed in 24 wells microtiter plate, in addition to fresh prepared YPD medium, and a specific volume of *S. cerevisiae* inoculum, with the attention to keep some wells without the addition of

chalcone stock as control wells. After this process, plates are incubated in the shaking incubator for 48 hours at 25°C. Finally, minimum inhibitory concentrations were determined. MIC is the minimum concentration of chalcones at which no microbial growth is observed. After the determination of MIC, an experiment for the determination of MFC was also done. For this experiment, YPD agar plate was prepared. YPD agar was prepared by following a specific recipe that contains three components, which are yeast extract, dextrose, peptone, and agar. Agar gives the medium its solid form. The process of MFC testing starts with the addition of 10 µL from the yeast culture that is used for the MIC experiment onto YPD agar plate. Plate is incubated for 24 hours at 25°C, finally, results are reported.

3.2.2. Sorbitol Protection Assay

This experiment is conducted to test the effect of sorbitol on the integrity of the yeasts cell wall integrity. For this experiment, microtiter plate needs to be prepared similar to the steps of MIC and MFC experiments. Yeasts culture, YPD fresh medium, and chalcone stocks are added to each well, plus the addition of sorbitol to each well, with the attention to leave some wells without sorbitol as control. After plate is prepared it was incubated for 48 hours at 25 °C. Finally, MIC values with the presence of sorbitol were compared to MIC values without sorbitol.

3.2.3. Extracellular Conductivity Measurement

As permeability of yeasts cellular membrane is identified and expressed through their electrical conductivity, extracellular membrane conductivity experiments need to be performed. For this experiment many preparations need to be prepared. *S. cerevisiae* was cultured in freshly prepared YPD medium at 25 °C for 48 hours, or until enough yeast growth is achieved. Next, is the preparation of chalcone stocks. Eight stocks of chalcone compounds need to be prepared by weighing specific amount of each chalcone compounds and dissolve them into DMSO solvent. Stocks were prepared according to the following calculations.

The molecular weight for compound **1** is 208.26 g/mol. To prepare 1 ml of 50 mM stock:

$$\text{Molarity} = \frac{\text{Number of moles}}{\text{Volume in liters}}$$

$$0.050 \text{ Molar} = \frac{\text{Number of moles}}{0.001 \text{ liters}}$$

$$\text{Number of moles} = 0.00005 \text{ mole.}$$

$$\text{Number of moles} = \frac{\text{Mass}}{\text{Molecular weight}}$$

$$0.00005 \text{ mole} = \frac{\text{Mass}}{208.26 \text{ g/mol}}$$

$$\text{Mass} = 0.01 \text{ grams} = 10 \text{ mg}$$

The stock should be 10 mg/ml. To achieve 1 mM final concentration, 200 µl of 50 mM stock must be diluted to 10 ml.

Calculations are done for the other compounds in a similar way. After the preparation of chalcone stocks, yeast cells need to be prepared for the experiment as well. Yeast cells were pelleted by centrifugation, divided according to the experiment, to have approximately 200 mg wet weight of yeast cells, and then washed for two times. The washing step needs to be with 10 mL of distilled water. After the preparation of yeast cells, chalcone stocks are added. For each compound, nine beakers with the addition of approximately 9-10 ml distilled water to them were prepared. After the addition of distilled water yeast cells were added, followed by the addition of chalcone stocks as follows; Compounds **1** and **7**: control, 0.5 mM and 1 mM; Compound **2**: control, 2 mM and 4 mM; Compound **3-6**: control, 1 mM and 2 mM; Compound **8**: control, 0.125 mM and 0.25 mM. Suspensions were shaken at 25 °C and extracellular conductivity was measured every 5 min with an AD 31 Waterproof EC/ TDS tester for 30 min.

Extracellular conductivity measurements were repeated at least three times. The slopes and the standard deviation of the slopes were calculated using Microsoft Excel LINEST tool. Student's t-test was used to assess whether slopes of the regression lines corresponding to the antifungal agent treated cells were different than the ones for untreated control cells.

3.2.4. Ergosterol Effect Assay

For this experiment *S. cerevisiae* was grown in freshly prepared YPD until sufficient growth was achieved. Chalcones were dissolved in DMSO after weighing them with different amounts according to each compound stock concentration, as shown in the calculations above. After the preparation of chalcone stocks, they were added to a 24-well microtiter plate, followed by the addition of yeast cells to each well, the volume was completed to 1 ml with the addition of fresh YPD. Plates finally were incubated at 25 °C, for 48 hours with constant shaking. MIC values observed in the presence of ergosterol were compared with the results obtained for the MIC study in its absence.

3.2.5. Release of Cellular Material Measurement

S. cerevisiae cells were grown in freshly prepared YPD medium at 25 °C under aerobic conditions and centrifuged to reach the pellet. The cells were washed twice with 10 mL of distilled water and for each experiment, approximately 200 mg wet weight of *S. cerevisiae* was re-suspended in 10 mL distilled water. Compounds **1** – **8** stocks were prepared according to specific concentrations, then they were added to yeast suspensions to give the same final concentrations as the extracellular conductivity measurement experiment. Suspensions were shaken at 25 °C for 30 min and centrifuged at 3200 rpm. The absorption spectra of the supernatants were measured using UV-1280 UV-VIS Spectrophotometer (Shimadzu) in the range of 190–400 nm. The absorbance of samples at 260 nm was determined and compared for 0. min and 30. min.

3.2.6. FTIR Measurement

S. cerevisiae cells were grown in fresh YPD medium at 25 °C then centrifuged to reach the pellet. Followed by washing step with distilled water twice. For each experiment, approximately 200 mg wet weight of yeast cells were resuspended in 10 mL distilled water and treated with the following amounts of chalcone stocks for 2 hours: Compounds **1** and **7**: 1 mM; Compound **2**: 4 mM; Compound **3** and **6**: 2 mM; Compound **8**: 0.25 mM. The cells were pelleted by centrifugation, washed twice with distilled water, and dried at 60 °C for 2 hours. Note that drying step is very crucial to get accurate spectroscopic measurements, otherwise the existence of water will lead to

wrong spectroscopic measurements. FTIR measurements were performed in transmission mode using Perkin Elmer Spectrum 100. All spectra were recorded in the range between 4000 and 900 cm^{-1} .

3.2.7. UV-Visible Spectroscopy

The chalcones **1 - 8** were dissolved in DMSO at a concentration of 50 μM . Calf thymus DNA (ctDNA) in Tris-buffer (pH 7.4) was added to the chalcones so that the final concentration of the chalcones were 10 μM , and that of the ctDNA varied from 0 to 20 μM . UV-vis measurements were performed at room temperature (Shimadzu UV-1800).

3.2.8. Statistical Analyses

All previous experiments performed MIC, extracellular conductivity, and release of cellular material measurements were repeated at least three times to decrease the chance of any experimental errors. Averages of all experimental groups were calculated, and standard deviations were determined using Microsoft Excel. All results were compared as the mean with the standard deviation.

4. RESULTS

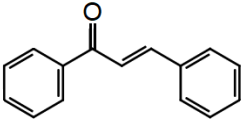
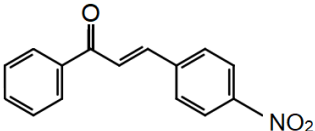
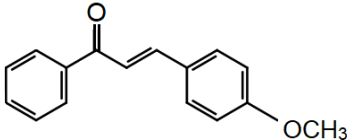
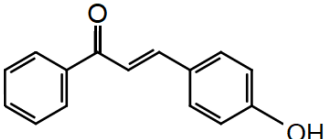
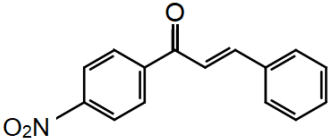
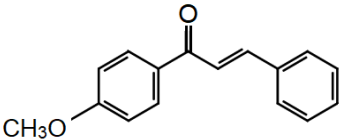
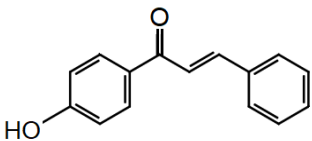
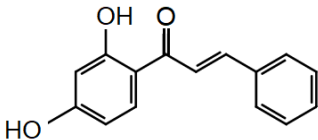
Chalcone derivatives play an important role as bioactive molecules that may participate in many biological properties. Our study focuses on the biological and structural effect of eight chalcone compounds on the yeast *Saccharomyces cerevisiae*. In addition, the effect of various chemical structures, and the substitution of different chemical groups in A and B rings that exist in chalcone structure were also investigated. Chalcone compounds effects on many vital cellular structures such as cell membrane, and cell wall are also examined.

4.1. 2',4'-Dihydroxychalcone Exhibited the Highest Antifungal Activity

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) experiments were performed in order to define the antifungal activity of the chalcone compounds (Table 1). It can be concluded from our experiments that the introduction of nitro group (-NO₂) to the para position of either ring A or B decrease the antifungal activity of compounds 2 and 5. On the other hand, hydroxyl group (-OH) presence in 4' position of compound 7 did not affect the antifungal activity of the chalcone. In addition, methoxylation of the same position decreased activity due to experiments performed on compound 6. The only compound that was superior to the parent chalcone in antifungal activity was compound 8 or 2',4'-dihydroxy derivative.

A similar observation was made for the growth dynamics of yeast cells (Figure 4.1). If compounds **1** and **7** were compared to compound **8**, it can be demonstrated that compound **8** was more effective in the yeasts' growth inhibition more than compounds **1** and **7**, as it reduced the growth rate of *S. cerevisiae* at 0.125 mM concentration, and fully stopped it at a concentration above 0.25 mM.

Table 4.1: Antifungal activities of chalcones **1-8** presented as MIC and MFC values.

#	Compound	MIC (mM)	MFC (mM)
1		0.5-1	0.5-1
2		2-4	2-4
3		1-2	1-2
4		1-2	1-2
5		1-2	1-2
6		1-2	1-2
7		0.5-1	0.5-1
8		0.125-0.25	0.125-0.25

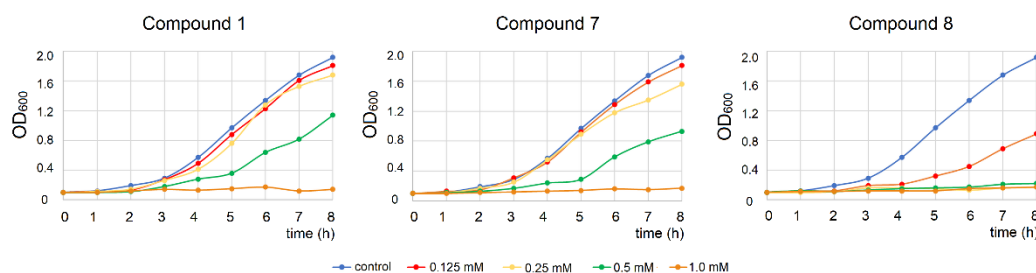


Figure 4.1. Effects of chalcones on the growth of *S. cerevisiae* cells.

The effect of sorbitol on antifungal activity of the tested eight chalcones was also investigated in our study. It has been concluded that presence of sorbitol did not affect the antifungal activity as it did not affect MIC values of the compounds (Table 4.2). Based on this result, we concluded that the yeasts cell wall is not affected by chalcones tested.

Table 4.2: Sorbitol test for chalcones 1-8

Compound	MIC (mM)	
	- sorbitol	+ sorbitol
1	0.5-1	0.5-1
2	2-4	2-4
3	1-2	1-2
4	1-2	1-2
5	1-2	1-2
6	1-2	1-2
7	0.5-1	0.5-1
8	0.125-0.25	0.125-0.25

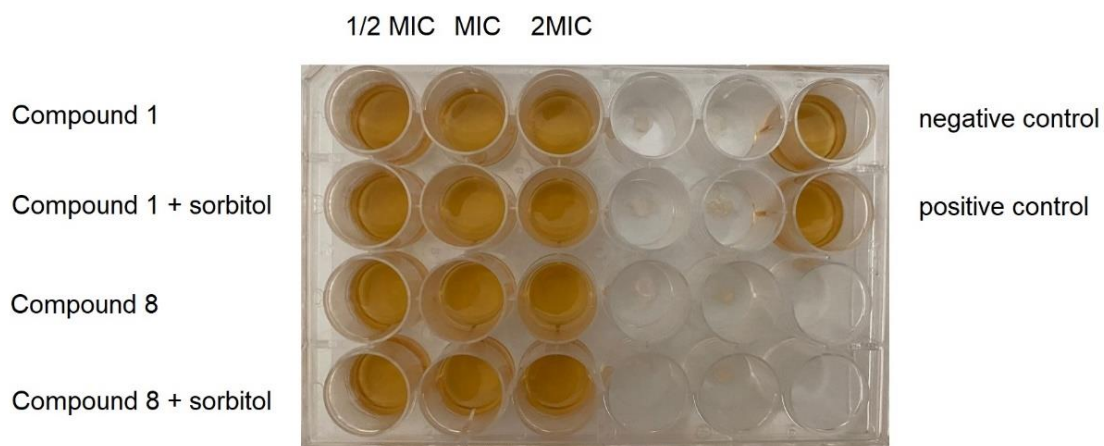


Figure 4.2: Sorbitol experiment MIC plates

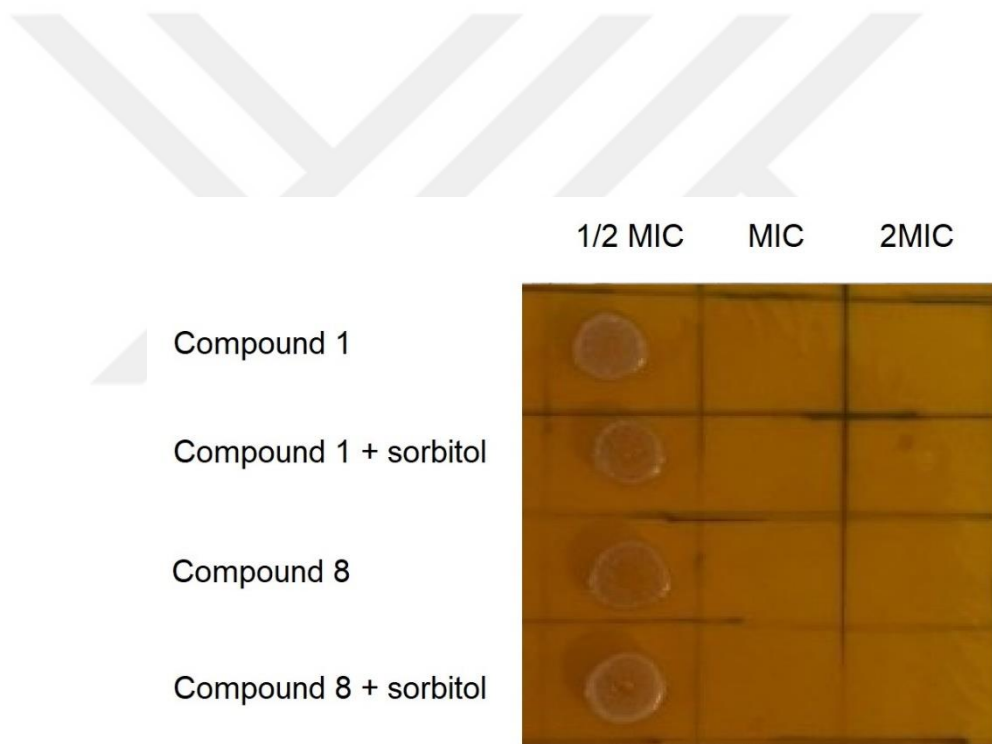


Figure 4.3: Sorbitol experiment MFC plates.

4.2. Some Chalcones Caused Ion Leakage from the Yeast Cell Membrane

Extracellular conductivity experiment was carried out to test the effect of chalcone compounds on yeast cells' cellular membrane upon treating them with the compounds (Figures 4.4 and 4.5). *S. cerevisiae* cells were treated with chalcone compounds with MIC and $\frac{1}{2}$ MIC previously determined amounts, and the increase in extracellular conductivity was followed and recorded over 30 mins. All experiments done were carried out along with having control cell suspensions without the addition of chalcone derivatives. Compound **1** or the parent chalcone, compound **2** with 4-nitro, and compound **4** with 4-hydroxy, did not caused ion leakage from yeast cells according to tests done. On the other hand, compound **5** with nitro group in the para position of the A ring, caused defect in the cell membrane of *S. cerevisiae*, leading to an increase in extracellular conductivity upon ion leakage. Similar to compound **4**, compound **7** did not cause any defect in yeast cell membrane, and further hydroxylation at the 2' position as in compound **8** or methylation of the 4'-hydroxy group as in compound **6**, was essential to disrupt *S. cerevisiae* cell membrane integrity.

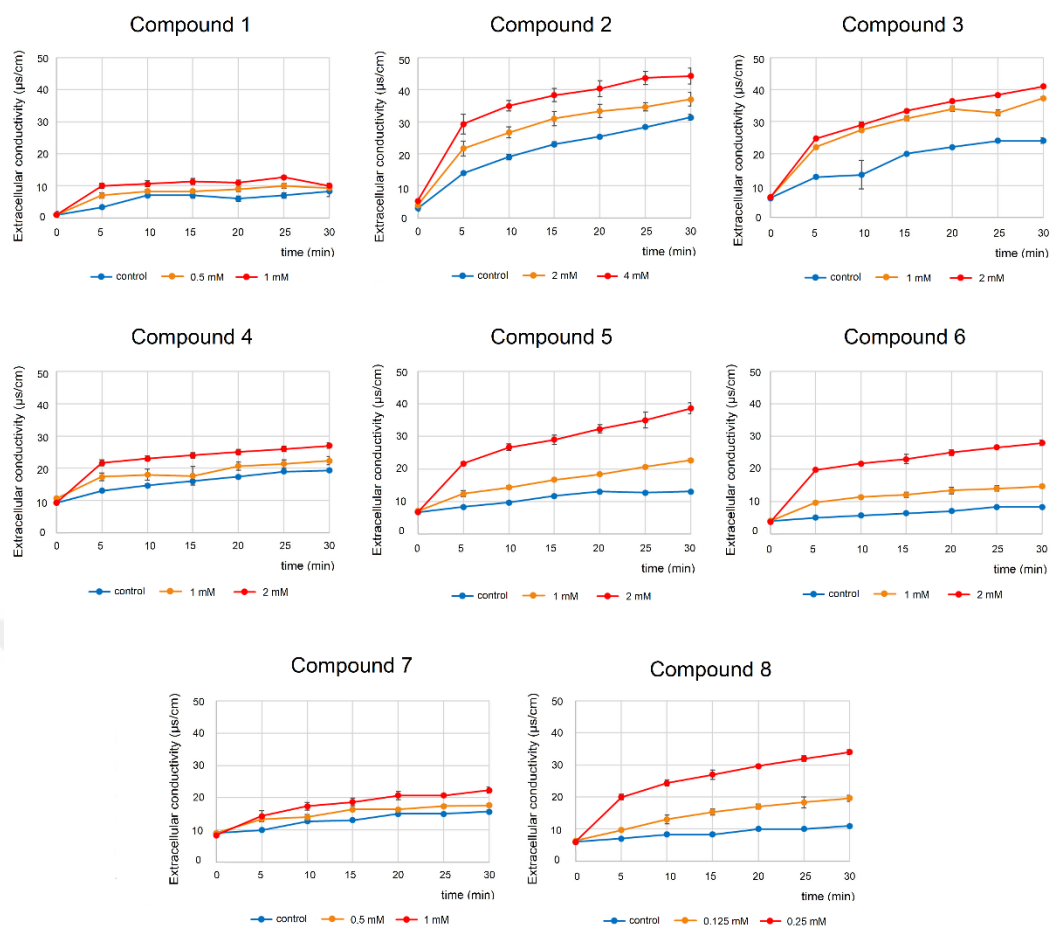


Figure 4.4: Effects of chalcones on the extracellular conductivity of *S. cerevisiae* cells.

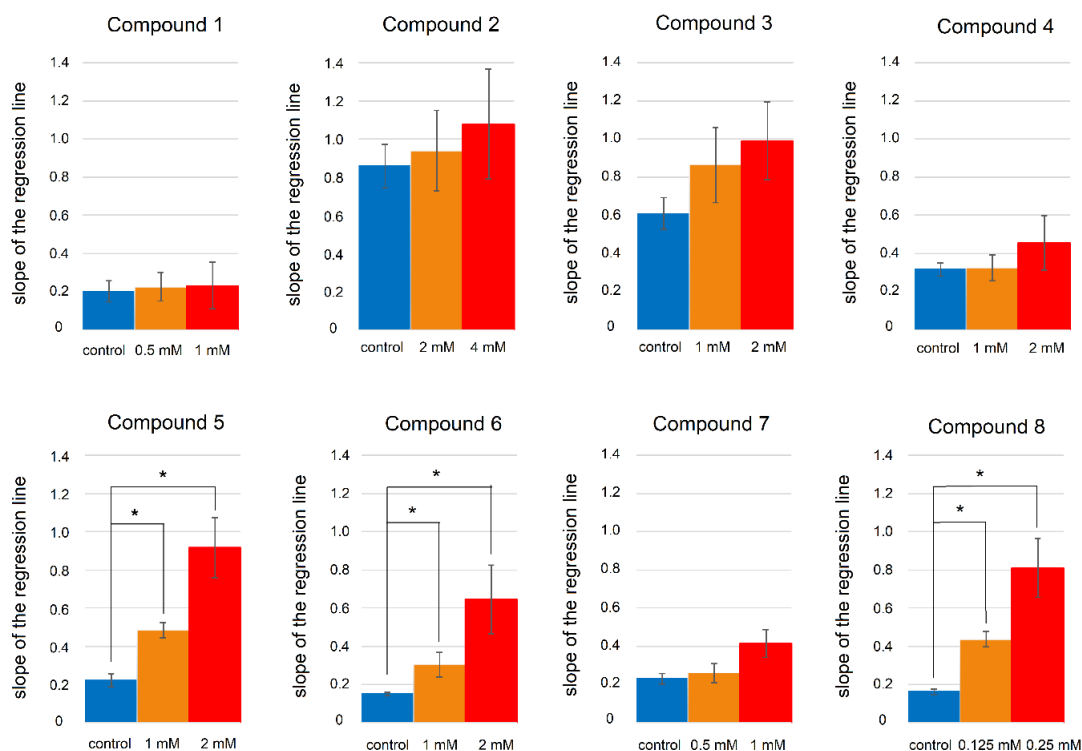


Figure 4.5: Effect of compounds 1-8 on the extracellular conductivity of *S. cerevisiae* cells: Slopes of regression lines.

Ergosterol effect on antifungal activity was investigated as well. Due to our results ergosterol did not affect MIC values (Table 4.3), so there was no effect on the antifungal activity of chalcone compounds.

Table 4.3: Ergosterol effect test for chalcones 1-8

Compound	MIC (mM)	
	- ergosterol	+ ergosterol
1	0.5-1	0.5-1
2	2-4	2-4
3	1-2	1-2
4	1-2	1-2
5	1-2	1-2
6	1-2	1-2
7	0.5-1	0.5-1
8	0.125-0.25	0.125-0.25

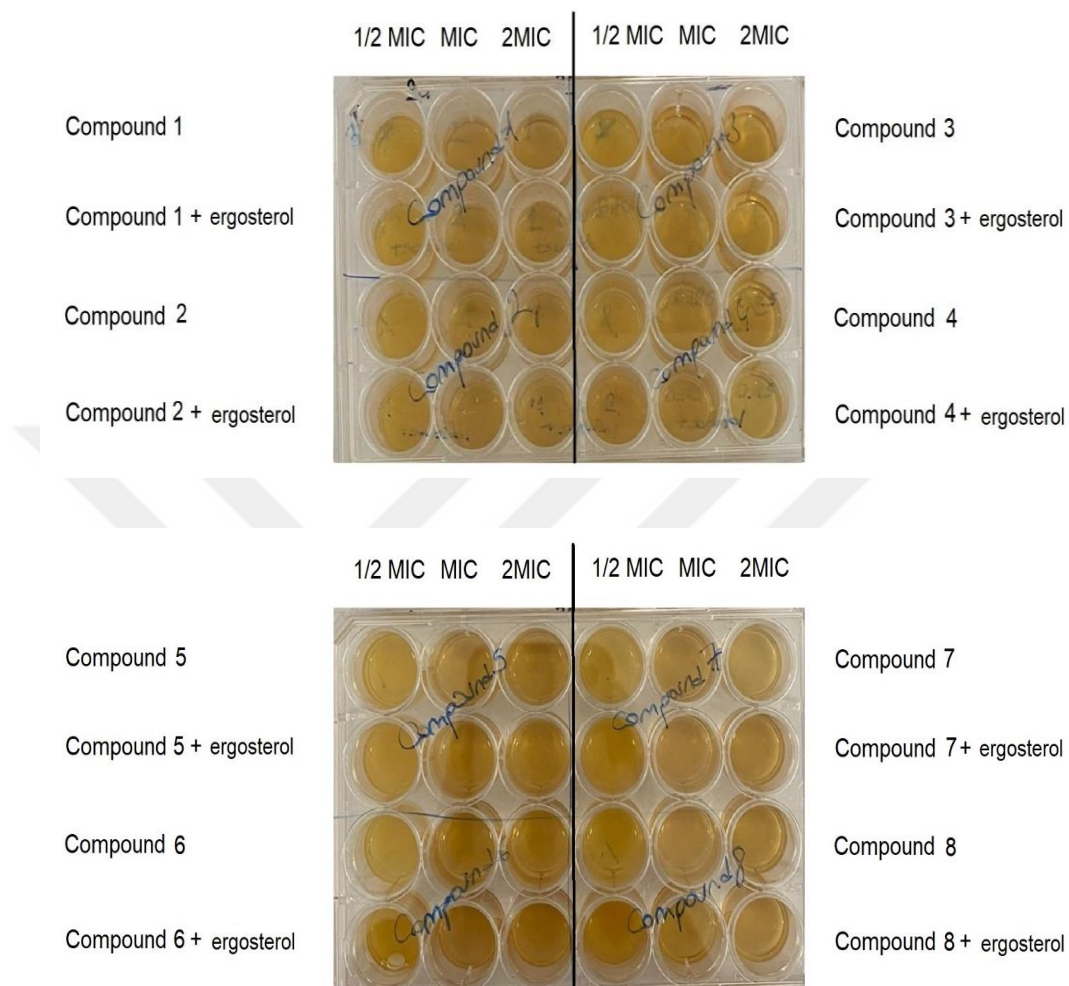


Figure 4.6: Ergosterol experiment for compounds 1-8, MIC plates.

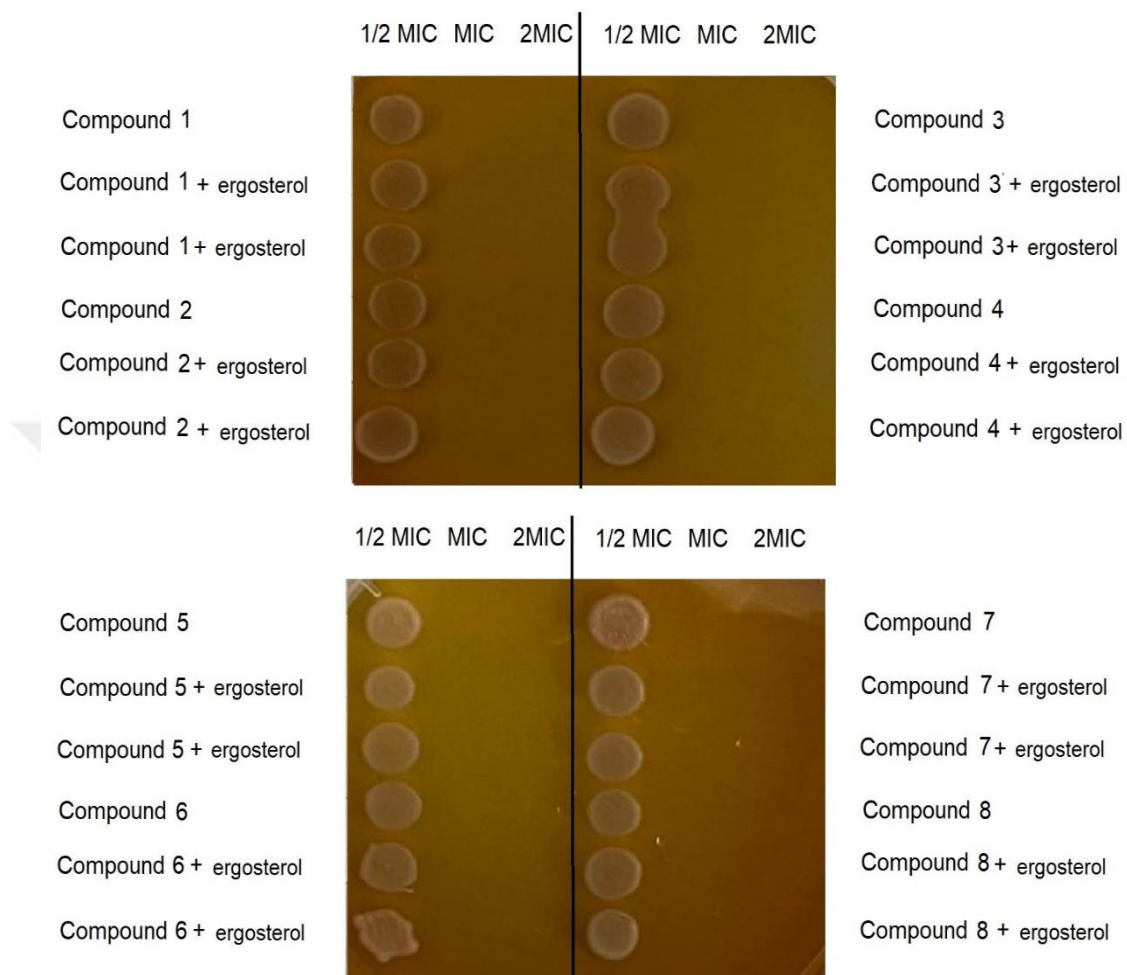


Figure 4.7: Ergosterol experiment for compounds 1-8, MFC plates.

Despite of some chalcones that caused defect in the cellular membrane of yeast cells, this defect or damage of the cell membrane was not sufficient to cause 260nm absorbing cellular content to leak out of the cells (Figure 4.8).

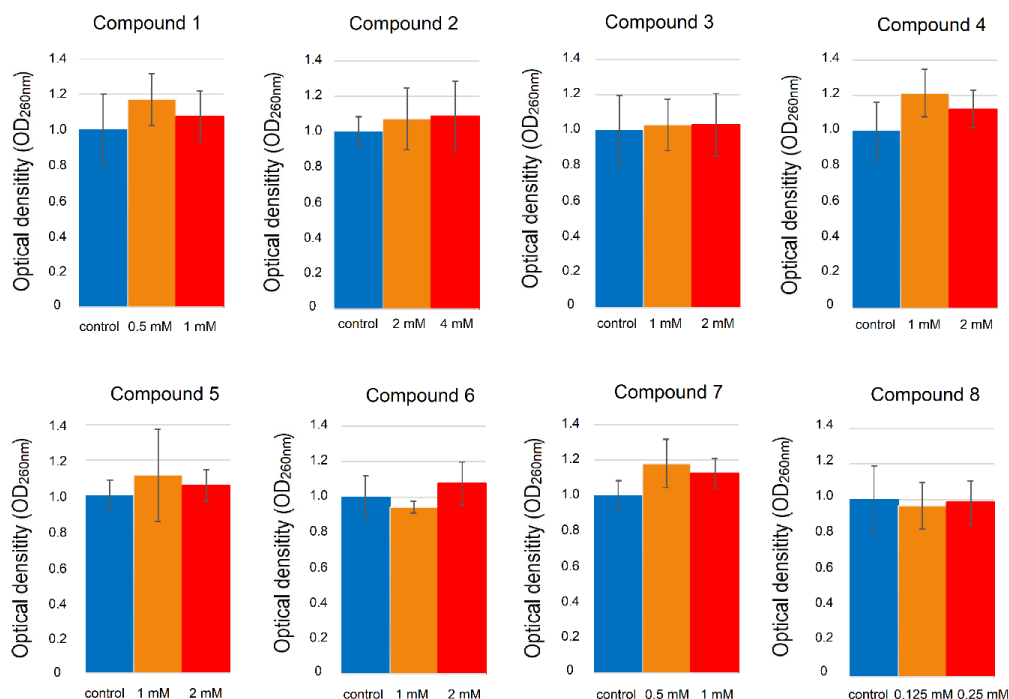


Figure 4.8: Effects of chalcones on the leakage of 260nm absorbing cellular material out of *S. cerevisiae* cells.

4.3. FTIR Spectral Changes in Yeast Cells

After the treatment of yeast cells with chalcone compounds, the changes in the biochemical structure were investigated (Figures 4.9 and 4.10). If cells that weren't treated with chalcones are compared to cells treated with chalcone compounds, it can be seen that they preserved the characteristic bands at 3268 cm^{-1} (hydroxyl vibrations of carbohydrates and NH vibrations of proteins), 2926 cm^{-1} (CH_2 asymmetric stretching of fatty acids), 1628 cm^{-1} ($\text{C}=\text{O}$ stretching of amide groups of proteins), 1546 cm^{-1} (CONH bending of amide groups of proteins), $1455\text{-}1390\text{ cm}^{-1}$ (C-N stretching and NH bending vibrations of amide groups of proteins), $1130\text{-}1040\text{ cm}^{-1}$ (C-O stretching of polysaccharides). Due to the results obtained from the experiment performed, current data available is not conclusive enough to reveal any correlation between these differences and the activity of the corresponding chalcone derivative, despite the differences in the intensities of bands. The most influential effect of chalcones were on the peaks in the region $1240\text{-}1275\text{ cm}^{-1}$ that can be attributed to the asymmetric stretching of PO_4^{2-} groups of nucleic acids. Thus, chalcones lead to some alterations in the biochemical composition of yeast cells that can be identified by FTIR measurements.

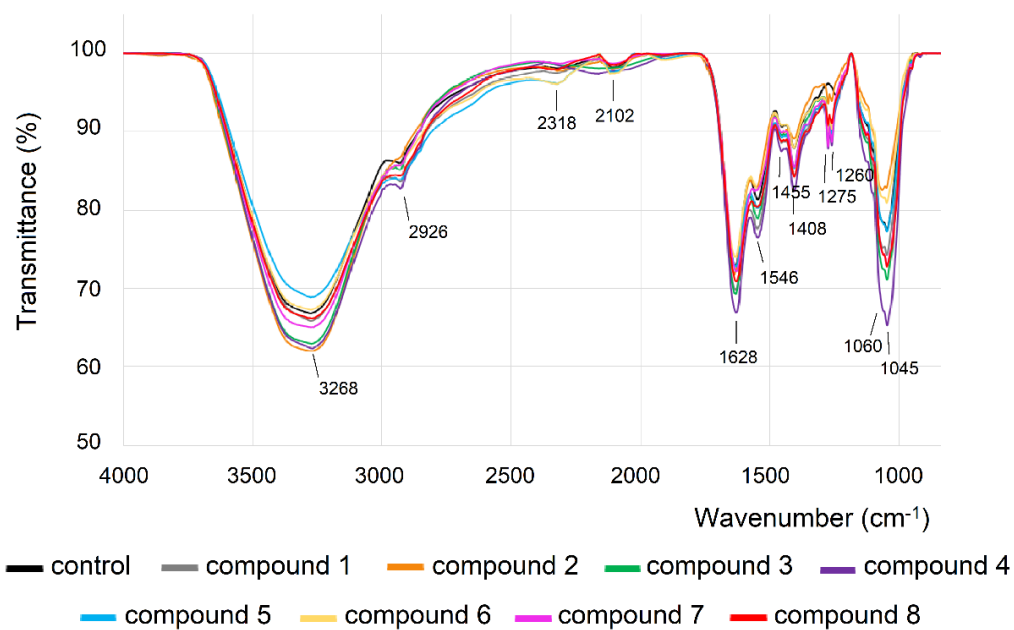


Figure 4.9: FTIR spectra of *S. cerevisiae* cells treated with compounds **1-8**.

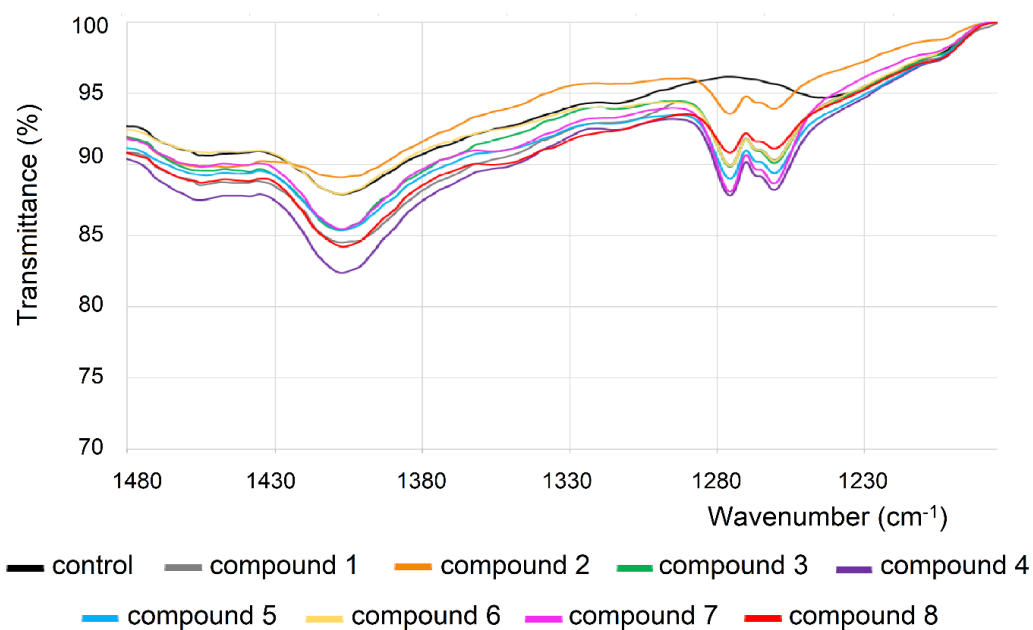


Figure 4.10: FTIR spectra of *S. cerevisiae* cells treated with compounds **1-8**, 1480-1200 cm⁻¹ region.

4.4. Chalcones Interact with the Calf Thymus DNA

To have a close look to the interaction between chalcones and DNA, UV-vis spectroscopy measurements were carried out on compounds **1** and **8** treated with increasing amounts of ctDNA (Figure 4.11). The working concentrations of chalcones were kept at 10 μM , while concentration of ctDNA varied from 0 to 20 μM . The gradual addition of ctDNA did not shift the absorption maxima, but rather caused a decrease in the intensity of absorption bands, indicating that the chalcones interact with ctDNA [43,44].

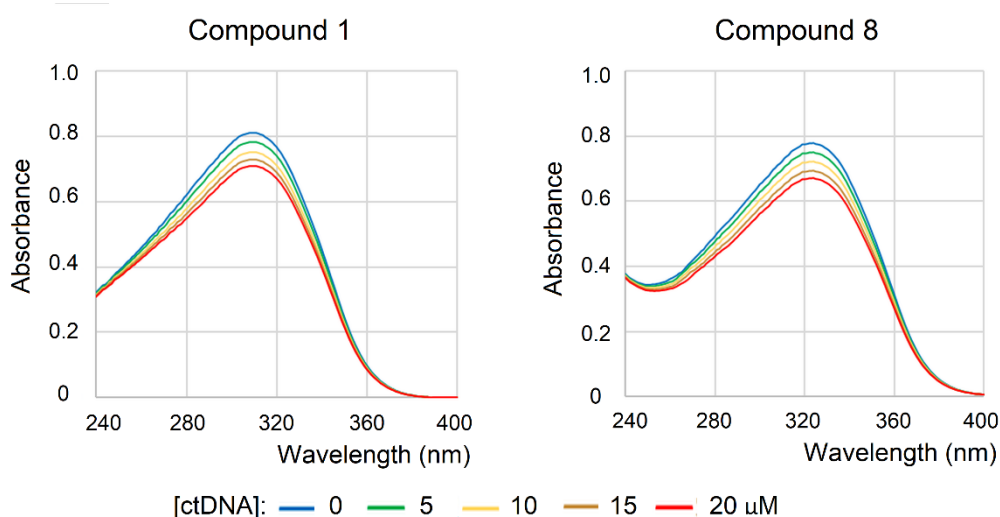


Figure 4.11: UV-vis spectra of compounds **1** and **8** (10 μM), treated with 0 to 20 μM ctDNA.

5. CONCLUSIONS

Chalcones are bioactive compounds exist in many natural resources such as fruits and vegetables, they are also promising compounds showing different levels of antibacterial activity depending on the position of different chemical groups in the chalcone structure. In this study, we focused on the investigation of antifungal activity of various chalcone compounds against the yeast *S. cerevisiae*. According to our MIC and MFC experiments, and the data obtained, it has been demonstrated that -NO₂, -OCH₃ or -OH groups substitution at the para position of the B ring of chalcones structure, decreased the antifungal activity, as in compounds **2**, **3**, and **4** (Table 1). Likewise, -NO₂ or -OCH₃ substitution at the para position of the A ring of chalcones decreased the antifungal activity as in compounds **5**, and **6**, while the presence of -OH substitute of the ring A did not affect the antifungal activity as in compound **7**. Among tested chalcone compounds, compound **8** that has dihydroxy substitutes in ortho and para position of the ring A, exhibited the highest antifungal activity.

The cell membrane of yeast cells was also among the vital structures we focused on in our study. As an example, compound **1** or the parent chalcone did not have effect on the cell membrane of yeast cells. In addition, compounds **5**, and **6** with -NO₂ or -OCH₃ substitution at the para position of the A ring of chalcones caused ion leakage, while hydroxy group substitution -OH of the ring A as in compound **7** did not cause defect in the cell membrane. So, we concluded that the effects of these substituents on the antifungal activity was not in compliance with the cell membrane damage capacity. Contrary to the previous observation, dihydroxy substitution in the para and ortho positions of the ring A of the chalcone as in compound **8**, caused the highest level of antifungal activity and the highest level of ion leakage. Ergosterol effect assay experiment results proved that the defect in yeast cell membrane was independent on ergosterol.

FTIR spectroscopy is a powerful method used in molecular biology and chemistry to identify the functional groups of chemical molecules within living cells. This technique was used in our study in order to investigate the effect of chalcone compounds of yeast cells and the macromolecular composition of them. FTIR spectral region we used in our study has been identified in previous studies, and it was divided

into five distinct regions: fatty acids 3000-2800 cm^{-1} , amides 1800-1500 cm^{-1} , protein-fatty acid mixed region 1500-1200 cm^{-1} , polysaccharides 1200-900 cm^{-1} and fingerprint region 900-700 cm^{-1} [45]. In our study, we focused on the spectra between 900-4000 cm^{-1} since the spectra portion 900-700 cm^{-1} usually not considered in analyzes, as its response does not correlate with the effect of the antifungal activity [45]. FTIR spectroscopy analysis results have showed that main macromolecular contents of yeast cells remain as is. The spectra were mainly different than the control at the mixed region at 1275-1260 cm^{-1} . The FTIR spectrum of the untreated cells, displays a weak band at 1240 cm^{-1} , that may be attributed to the hypophosphate vibrations largely found in the structure of nucleic acids [45]. On the contrary, the chalcone treated spectra contain the peaks at 1275-1260 cm^{-1} . This difference may be due to an alteration of the nucleic acids [46-48].

Next, we tested the interaction of chalcones and DNA. Thus, UV-vis spectroscopy was performed on compounds **1** and **8** titrated with high amounts of ctDNA. Obtained results have proved that chalcones bind to the ctDNA [43,44] and this may explain the difference in antifungal activity of these compounds.

As a conclusion, we studied the effect of eight chalcone compounds on the yeast *S. cerevisiae*, along with studying the effect of these compounds on other cellular vital structure such as cell membrane, and cell wall. Although we investigated the powerful ability of many of the tested chalcones in causing membrane defect and hence ion leakage out of the cell, we did not observe any link between the cell membrane damage and the antifungal efficiency. For this reason, we believe that the cell membrane defect or damage is not the main mode of action of these eight compounds. Instead, we can suggest according to our FTIR and UV-vis spectroscopy analysis, that these compounds may target the nucleic acids, as the results indicated a possible damage in the nucleic acid content of the yeast cells.

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PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Alregeb S., Ergüden B., (2023), “Structure Activity Relationship for Antifungal Activity of Chalcone Derivatives”, BIO Türkiye, İstanbul, 28-30 September.

Alregeb S., Ergüden B., (2024), “Chalcone Derivatives Disrupt Cell Membrane Integrity of *Saccharomyces cerevisiae* Cells and Alter Their Biochemical Compositions”, Archives of Microbiology, 206, 34.

