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Determining antifungal, anti-biofilm and anti-cancer activities of "1,3-di(thiophen-2-yl) prop-2-en-1-one" chalcone derivative

"1,3-di(thiophen-2-yl) prop-2-en-1-one" kalkon türevinin antifungal, anti-biyofilm ve anti-kanser aktivitelerinin belirlenmesi

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SUMMARY

Objective: Our study aims to determine antifungal and anti-biofilm activity potential of five chalcone derivatives, which have been already reported in the literature, against *Candida albicans* and *Candida tropicalis*. In addition, it aimed to determine antioxidant property and anti-cancer activity of 1,3-di(thiophen-2-yl)prop-2-en-1-one chalcone derivative, whose antimicrobial activity was revealed within the study, in human breast cancer cell line (MCF-7) and human bone cancer cell line (MG63).

Method: Chalcones used in the study were diluted two times in dimethyl sulfoxide (DMSO) and prepared at concentration between 1000-15 µg/mL. Antifungal and anti-biofilm activity of chalcones determined by using microdilution method. Radical scavenging activity was tested by employing 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The cell viability was assessed using the cleavage of tetrazolium salts added to the culture medium.

Results: In our study, MIC values of "1,3-di(thiophen-2-yl) prop-2-en-1-one", which showed its antimicrobial activity on *C. albicans* and *C. tropicalis*, were found to be 0.06 mg/mL for both organisms, whereas its MBC values were found to be 0.12 mg/mL and 0.50 mg/mL respectively. MBIC activities were found to be 0.06 and 0.25 mg/mL respectively, whereas MBEC values were found to be 1 mg/mL. When MIC was applied to 48-hours biofilm formation, it was observed that the formation has decreased by 83% and 81%. IC50 value of "1,3-di(thiophen-2-yl) prop-2-en-1-one" was found to be 13,52 µg / mL. Regarding MCF-7 and MG63 cell line at 5 µg / mL concentration, 30% and 65% preserve its viability after 24 hours of incubation respectively.

Conclusions: According to these results, it can be said that chalcone compound that include thiophen ring has shown an inhibiting effect on the multiplication and both on the formation of the biofilm of *C. albicans* and *C. tropicalis* strains. The results of the study revealed that chalcone compound may be valuable as an antifungal, anti-biofilm and anti-cancer agent.

Keywords : Chalcone, antifungal, anti-biofilm, anti-cancer

ÖZET

Amaç: Çalışmamızın amacı, literatürde rapor edilen beş kalkon bileşiğinin *Candida albicans* ve *Candida tropicalis*'e karşı potansiyel antifungal ve antibiyofilm aktivitelerinin belirlenmesidir. Ayrıca antimikrobiyal aktivitesi belirlenen "1,3-di(thiophen-2-yl)prop-2-en-1-one" kalkon bileşiğinin antioksidan ve insan meme kanseri (MCF-7) ve kemik kanseri (MG63) hücre hatları üzerindeki etkilerinin belirlenmesi amaçlanmıştır.

Yöntem: Çalışmada kullanılan kalkonlar dimeti sülfoksit (DMSO) içinde iki kat seyreltilmiş ve 1000-15 µg/mL konsantrasyonda hazırlanmıştır. Kalkonların antifungal ve anti-biyofilm aktiviteleri mikrodilüsyon yöntemi kullanılarak

belirlenmiştir. Radikal süpürücü etkisi 2, 2-diphenyl-1-picrylhydrazyl (DPPH) ile test edilmiştir. Hücre canlılığı , kültür ortamına ilave edilen tetrazolium tuzları ile değerlendirilmiştir.
Bulgular: Çalışmamızda antimikrobiyal aktivite gösteren "1,3-di (tiyofen-2-il) prop-2-en-1-on" *C. albicans* ve *C. tropicalis* üzerinde MIC değerleri 0.06 mg/mL bulunmuştur. MBC değerleri ise sırasıyla 0.12 mg/mL ve 0.5 mg/mL olarak saptanmıştır. MBIC aktiviteleri ise sırasıyla 0.06 ve 0.25 mg/mL olarak bulunmuştur. MBEC değerleri her iki mikroorganizma için 1 mg/mL bulunmuştur. *C. albicans ve C. tropicalis'* in 48 saatlik biyofilm yapılarına MIC değeri uygulandığında yapının sırasıyla 83% ve 81% oranında azaldığı saptanmıştır. "1,3-di(thiophen-2-yl) prop-2-en-1-one" IC50 değeri 13,52 µg/L olarak bulundu. MCF-7 ve MG63 hücre hatlarında 5 µg/mL konsantrasyonda 24 saat inkübasyon sonrasında sırasıyla hücrelerin % 30'u ve % 65' i canlı kalmıştır.
Sonuç: Elde edilen sonuçlara göre tiyofen halkası içeren kalkon bileşiği *C. albicans* ve *C. tropicalis* suşları üzerinde hem üremelerini inhibe edici etki göstermiş hem de gerek biyofilm oluşumunda gerekse oluşan biofilm üzerinde etkili olduğu görülmüştür. Çalışmanın sonuçları tiyofen halkası içeren kalkon bileşiğinin potansiyel antimikrobiyal, antibiyofilm ve antikanser ajan olarak değerli olabileceğini ortaya koymuştur.
Anahtar sözcükler: Kalkon, antifungal, anti-biyofilm, anti-kanser

INTRODUCTION

Even though candida species live commensally with humans, recently they started to be seen as a frequent cause of local and systemic infections, which attracts more and more attention from a medical standpoint[1]. *Candida albicans* that causes candidiasis is regarded as the most common fungal infection agent in humans. The treatment gets quite difficult with the biofilm structure that they frequently form, and the mortality frequency may increase, especially among immunosuppressive patients[2].

Biofilm is defined as the colonies that microorganisms form by attaching to each other and to a surface. These colonies are covered by the extracellular polymeric substrate (EPS) that they have secreted to the environment, so they can protect themselves against many environmental factors and chemicals such as disinfectants and antimicrobial agents[3]. As many microorganisms, *C. albicans* and *C. tropicalis* can also form biofilms[4]. For the treatment of the sessile forms that formed biofilm, 1000 times more antibiotics should be used compared to planktonic forms[5].

Chalcones are compounds which can be obtained synthetically or can be isolated from plants. Owing to their pharmacological and biological activity, the number of studies about chalcones is increasing day by day. It is reported that the combination of many chalcone derivatives with antibiotics have revealed successful results. In addition to the studies about antimicrobial activities of chalcones, literature contains various works revealing their antioxidant, radical removing, antimalarial, cytotoxic, anticancer and anti-inflammatory properties[5-10].

Morever thiophene containing chalcones (Figure 1) were reported in various studies that it have bioactive potential such as antibacterial, apoptotic, anti-angiogenic and radical scavenging agent (11-15). On the other hand, there is no study on the antifungal and anti-biofilm activities of the 1,3-di(thiophen-2-yl)prop-2-en-1-one (4) according to our literature survey.

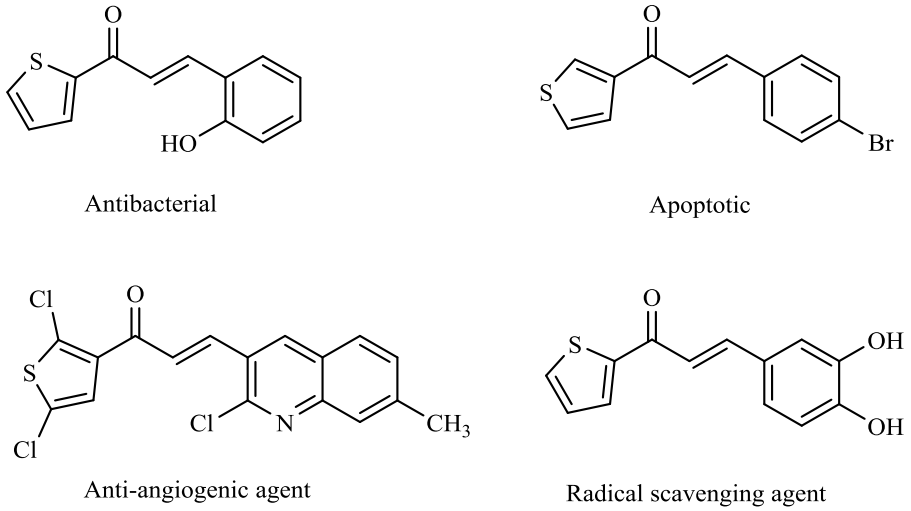


Figure 1. Some thiophene containing chalcones

Our study aims to research antifungal and anti-biofilm activities of four chalcone derivatives 1-(4-bromophenyl)-3-(4-methoxyphenyl) prop-2-en-1-one (**1**), 3-phenyl-1-(p-tolyl)prop-2-en-1-one (**2**), 1-phenyl-3-(m-tolyl)prop-2-en-1-one (**3**), 1,3-di(thiophen-2-yl)prop-2-en-1-one (**4**), which have been already reported in the literature, and a chalcone type compound 2-(4-bromobenzylidene)-2,3-dihydro-1H-inden-1-one (**5**) (Figure 2) against *C. albicans* and *C. tropicalis* and determine the potential of these compounds on this issue.

MATERIAL AND METHODS

Microorganisms: *C. albicans* (ATCC 10231) and *C. tropicalis* (ATCC 750) strains were used in the study.

Chalcones: Examined compounds were obtained from related aldehydes and ketones by Claisen-Schmidt condensation reaction that reported in the literature[16].

General Procedure for the Synthesis of Chalcone Derivatives: A solution of related aromatic ketone (1 mmol) in EtOH (20 mL) was added 5 M NaOH (2 mL) and stirred for a while. Afterwards, related aromatic aldehyde (1 mmol in 10 mL EtOH) was added to the mixture. The mixture was stirred for 3 hours at room temperature. At the end of the reaction the mixture was transferred to separation funnel, neutralized with dilute HCl (10 mL) and extracted with $CHCl_3$ (3x15 mL). The organic layer was dried (Na_2SO_4) and evaporated. The residue was crystallized in $CHCl_3$/hexane (3:1) or EtOH.

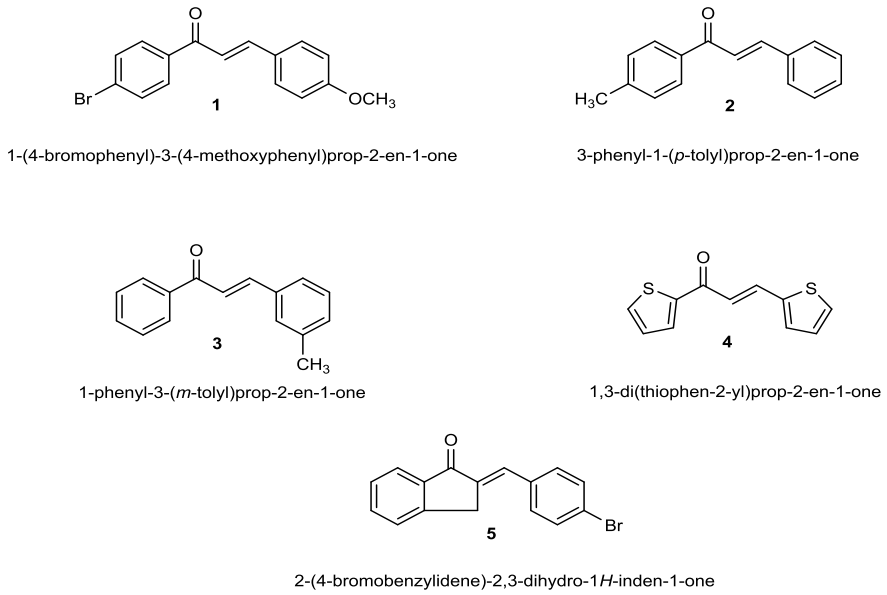


Figure 2. Compounds examined in terms of antifungal and anti-biofilm activities

Determination of Antifungal and Anti-Biofilm Activities: Minimal Inhibitory Concentration (MIC) was found according to the instructions of Clinical and Laboratory Standards Institute (CLSI), using microdilution method[17].

Fungus was prepared in Müller Hinton Broth (MHB), which is one of the 24-hours broth cultures, according to the 0.5 McFarland standard and diluted at a rate of 1/100. Chalcones used in the study were diluted two times in DMSO and prepared at concentration between 1000-15 µg/mL. 100 µL of microorganism and chalcone solutions were added to 96-well plates, incubated at 37 C°, for 24 hours. Fluconazole was used as positive control. The value of the well, where multiplication did not occur was set as the MIC. The samples taken from the wells exceeding MIC value were planted into Müller Hinton Agar medium, the concentration where the multiplication did not occur was set as Minimum Bactericidal Concentration (MBC).

Minimum biofilm inhibitory concentration (MBIC) is the lowest concentration where antimicrobial agents prevent biofilm formation. The MBIC test was carried out using the method of Adukwu et al[18]. Microorganisms were prepared in 1% glucose added MHB (one of the 24-hours culture) as described above and the steps of MIC test were repeated. Fluconazole was used as positive control, whereas MHB was used as negative control. Plates were incubated at 37 C°, for 48 hours and biofilm formation was monitored

by observing negative control. Following the formation of the biofilm, planktonic forms were removed from the plates by washing with PBS three times and left to drying for 1 hour. 0.1% crystal violet was applied to the wells, they were washed after 30 minutes and biofilm structure were colored. After adding 95% ethanol to the wells, MBIC value was determined by microplate reader at 550 nm.

Minimum Biofilm Eradication Concentration (MBEC) represents the lowest concentration that can break the structure of the biofilm formation. MBEC assay was carried out according to the method of Kuzma et al[19]. The test was prepared in a manner similar to MBIC application and chalcone derivatives were applied to 48-hours biofilm formation of the microorganisms. After 24 hours of incubation, washing and coloring operations were performed and MBEC was determined by reading at 550 nm. The percentage of biofilm eradication in the formation was calculated according to the following formulas.

Cell Culture: All cell lines were purchased from (ATCC). Three cell lines were used in this study: Human bone carcinoma cells (MG63), breast carcinoma cells (MCF-7), and mouse fibroblast (L929) cell lines. DMEM, which is the medium used in our study, consisted of 10% Fetal Bovine Serum (FBS), %1 L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin. Cell lines produced by DMEM medium were reproduced in the oven with 95% humidity 5% CO_2, at 37^0C[20].

Determination of DPPH: DPPH (2,2-difenil-1-pikrilhidrazil) radical removing activity of the chalcone compound was used by modifying the method developed by Ou et al[21]. Equal volume of (750 µL) DPPH and sample solutions were mixed and waited for 30 minutes in room temperature. Following the incubation, the absorbance of DPPH was read from the spectrophotometer at 517 nm wavelength. DPPH solution and the solvent in which the sample has been dissolved was used as negative control. In order to compare the results, ascorbic acid, which is a natural antioxidant, was used as positive control.

Statistical analysis: The results are presented as mean ± standard deviation (n=3) and assessed by one-way analysis of variance (ANOVA), while Student's ttest was applied for comparison between two groups. $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

As a result of the study, it was found that among the chalcone derivatives only "4" was effective on *C. albicans* and *C. tropicalis*. Accordingly, MIC values were found to be 0.06 mg/mL for both organisms. MBC values were found to be 0.12 mg/mL and 0.50 mg/mL respectively (Table 1). Conducted studies shows that compounds that include thiophen ring are effective on bacteria and fungus. Accordingly, it can be said that the effect of the mentioned compound ("**4**") on the mentioned microorganisms may be because of the thiophen ring that it possesses and this statement is also supported by the data in the literature[22,23]. Candida species are the fungus that may form strong biofilms[24]. MBIC activities of "3-di (thiophen-2-yl) prop-2-en-1-on" compound, which showed antimicrobial activity in our study, on the biofilm formation of *C. albicans* and *C. tropicalis* were found to be 0.06 and 0.25 mg/mL respectively. Accordingly, it was observed that the compound prevents biofilm formation of *C. albicans* at MIC level, whereas it prevents *C. tropicalis* just below MBC. MBEC were found to be 1 mg/mL for the two organisms. When MIC (0.6 mg/mL) was applied to 48-hours biofilm formation of *C. albicans* and *C. tropicalis*, it was observed that the formation has decreased by 83% and 81%. According to these results, chalcone compound that includes thiophen ring has shown an inhibiting effect on the multiplication of *C. albicans* and *C. tropicalis* standard strains, in addition it was effective both on the formation of the biofilm, and on the biofilm that has been already formed. These data showed that chalcones, which are seen as potential antimicrobial agents, may be valuable as anti-biofilm agents as well.

Table 1. Antimicrobial and antibiofilm activity of "1,3-di(thiophen-2-yl)prop-2-en-1-one" on *Candida albicans* and *Candida tropicalis*

Strain	ZI(mm)	MIC (mg/ml)	MBC (mg/ml)	MBIC (mg/ml)	MBEC (mg/ml)
Candida albicans	15.8±0.2	0.06	0.125	0.06	0.50
Candida tropicalis	14.3±0.5	0.06	0.50	0.25	1.00

Table 2. The percentage reduction in biofilm formation

Strain	MIC/2	MIC	2MIC	4 MIC	8 MIC
Candida albicans	70.3±2.0	88.3±0.5	89.0±2.6	91.3±2.5	94.3±2.0
Candida tropicalis	75.3±2.3	82.3±2.0	84.0±1.7	86.6±3.5	92.6±2.5

In order to evaluate antioxidant activity, radical scavenging effect was spectrophotometrically tested with DPPH, which is a commercially purchasable material. DPPH radical shows maximum absorbance values at 517 nm wavelength when interacted with antioxidant substance(s). The intensity of the purple color arising from DPPH decreases, resulting with the decrease of absorbance values.

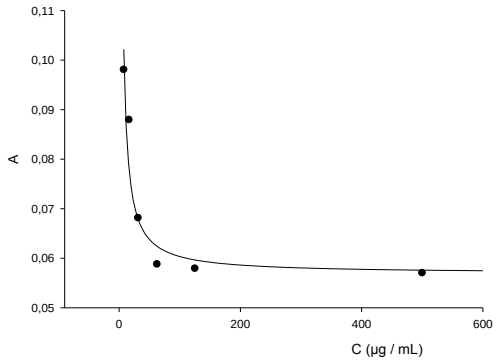


Figure 3a. DPPH graph of "1,3-di(thiophen-2-yl)prop-2-en-1-one"

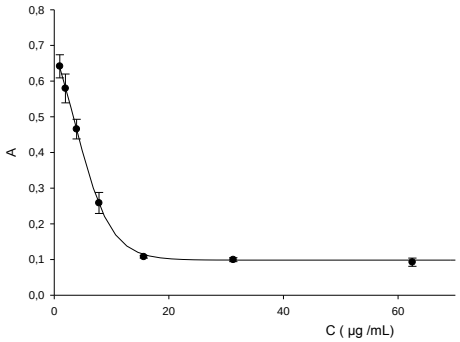


Figure 3b. DPPH graph of Ascorbic acid

DPPH result of "4" was calculated according to IC50 value. Ascorbic acid was used as positive control. IC50 value of "4" was found to be 13,52 µg / mL (Figure 3a), whereas IC50 value of Ascorbic acid, whose radical scavenging effect is very high, was found to be 5,02 µg / mL (Figure 3b). It was concluded that radical scavenging effect of "4" is high.

In this study, it has been worked on mouse healthy fibroblast cell line (L929) in order to examine the cytotoxicity of the compound. The compound was applied at four different concentration levels (80, 40, 20 and 5 µg/ml), for 24 and 48 hours of incubation times. In addition, it has been worked on MCF-7 and MG63 in order to examine its anti-cancer activity. XTT test was performed by applying this compound to MCF-7 and MG63 cell lines at four different concentration levels (80, 40, 20 and 5 µg/ml), for 24 and 48 hours of incubation times and the results were compared with the control group. The results were calculated as % viability. The viability of this compound at 80, 40,

20 and 5 µg/ml concentration levels were found as shown in the figure (Figure 4).

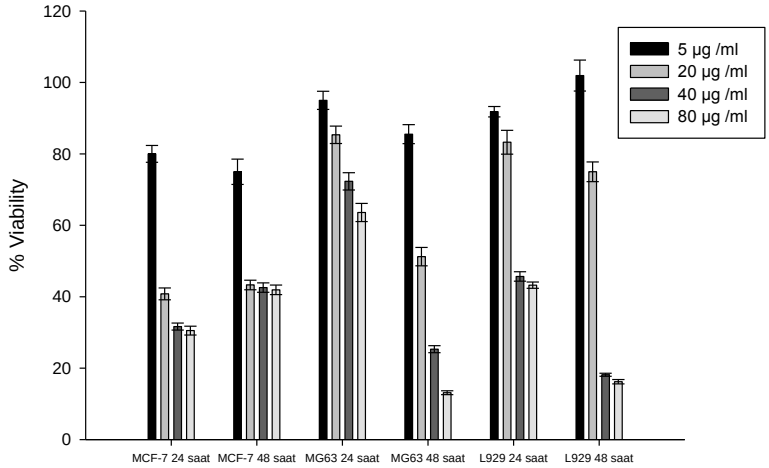


Figure 4. Cytotoxic and anti-cancer activities of "1,3-di(thiophen-2-yl) prop-2-en-1-one" chalcone derivative on MCF-7, MG63 cancer cell lines and L929 fibroblast cell line

At the end of 24 and 48-hours application, no toxic effect was observed for the concentrations below 20 µg/mL. Regarding MCF-7 cell line at 5 µg/mL concentration, 30% preserve its viability after 24 hours of incubation, whereas 45% preserve its viability after 48 hours of incubation. Regarding MG63 cell line at 5 µg/mL concentration, 65% preserve its viability after 24 hours of incubation, whereas 10% preserve its viability after 48 hours of incubation.

CONCLUSION

According to these results, It can be said that chalcone compound that include thiophen ring has shown an inhibiting effect on the multiplication of *C. albicans* and *C. tropicalis* strains, in addition it was observed that it has been effective both on the formation of the biofilm, and on the biofilm that was already formed. Additionally, the compounds were evaluated anticancer and radical scavenging activity in the MCF-7 and MG63 cell lines. The results of the study revealed that chalcone compound that include thiophen ring may be valuable as an antimicrobial, anti-biofilm and anti-cancer agent.

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