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# The Evaluation of Antimicrobial and Antibiofilm Activity of Bioactive Compounds Obtained from *Aspergillus Sclerotiorum*

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**ABSTRACT:** This study was concerned with the screening of antimicrobial products from fungi collected from soil and evalution of their antibiofilm activity. The isolate having antimicrobial and antibiofilm compounds was characterized by the molecular methods and identified as *Aspergillus sclerotiorum*. *A. sclerotiorum* was grown in yeast peptone glucose (YPG) medium and extracellular medium was extraction by 1:1 ethyl acetate. Crude extraction characterized through thin layer chromatography (TLC) on silica gel 60 HF254 and was detected five bands. Agar diffusion and TLC overlay assays were done against Gram-positive (*Staphylococcus aureus* ATCC 25923, meticilin resistance *S. aureus* (MRSA) and *Enterococcus faecalis* ATCC 29212) and Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853). The most dense band (Rf:0.42) showed the best inhibition zone on TLC overlay. The results showed that the most dense band can potential source for antimicrobial compound. After the most dense band in silica gel was scraped and dissolved ethyl acetate for minimum inhibitory concentration (MIC) determination and crystal violet assay against *S. aureus* and MRSA. These results indicate that fungi, *A. sclerotiorum*, isolated from soil was potential source for antimicrobial and antibiofilm compounds.

**Keywords:** Antimicrobial, Antibiofilm, *Aspergillus sclerotiorum*, Fungi, Thin layer chromatography.

## *Aspergillus sclerotiorum'dan* Elde Edilen Biyoaktif Bileşiklerin Antimikrobiyal ve Antibiyofilm Aktivitelerinin Değerlendirilmesi

**ÖZET:** Bu çalışma, topraktan izole edilen funguslardan antimikrobiyal bileşiklerin taranmasını ve bu bileşiklerin antibiyofilm aktivitelerinin değerlendirilmesini kapsamaktadır. Antimikrobiyal ve antibiyofilm özellikteki bileşiklere sahip izolat, moleküler olarak karakterize edilmiş ve *Aspergillus sclerotiorum* olarak tanılanmıştır. *A. sclerotiorum* maya pepton glikoz (YPG) besiyerinde geliştirilmiş ve ekstrasellüler besiyeri 1:1 oranında etil asetat ile ekstrakte edilmiştir. Ekstraksiyon ürünü ince tabaka kromotografisi (TLC, silica gel 60 HF254) ile karakterize edilmiş ve 5 bant elde edilmiştir. Gram pozitif (*Staphylococcus aureus* ATCC 25923, metisiline dirençli *S. aureus* (MRSA), *Enterococcus faecalis* ATCC 29212) ve Gram negatif (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) bakterilere karşı agar difüzyon ve TLC kapama deneyleri yapılmıştır. Rf değeri 0.42 olan bantın antimikrobiyal aktivite gösterdiği bulunmuştur. Bu bant TLC plaktan geri kazanılarak, minimum inhibitör konsantrasyonu (MIC) belirleme ve kristal viyole testi ile *S. aureus* ve MRSA' ya karşı antibiyofilm aktivitesi değerlendirilmiştir. Bu sonuçlar, topraktan izole edilen fungus, *A. sclerotiorum*'un antimikrobiyal ve antibiyofilm bileşikleri için potansiyel bir kaynak olduğunu göstermektedir.

**Anahtar Kelimeler:** Antimikrobiyal, Antibiyofilm, *Aspergillus sclerotiorum*, Fungus, İnce tabaka kromotografisi.

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## INTRODUCTION

Since ancient times, fungi have been used as food and medical purposes. Nowadays, natural resources, especially fungi, are valuable in that they contain promising compounds in the treatment of diseases. It is estimated that there are approximately 5 million fungus species in the world. An average of 100 000 of them were identified. A few of these fungi were examined for their pharmacological properties. Studies on bioactive molecule for the medical use of microscopic fungi have great potential (Blackwell, 2011).

Recently, existing health systems are fighting against resistant bacteria and their infections. Especially, infections caused by biofilm lead major health problems (Gootz, 2010). Biofilms are a collection of microorganisms that coexist in their extracellular matrix by attaching to an inanimate or living surface (Song et al., 2018). Extracellular matrix contains polysaccharides, proteins, and extracellular DNA. These structures serve as a shield for microbial cells and at the same time help to infect other areas. An organism that forms a biofilm is always more resistant to environmental conditions than planktonic cells (Simoes et al., 2010). The biofilm layer can be formed in many environments where moisture and air are present, and even the simplest biofilm layer has a complex dynamic (Dongari-Bagtzoglou, 2008).

Antibiofilm studies have focused on the treatment of various bacterial and fungal infections and, after the first report on Zobell's (1943) biofilms, concerns about food, biomedical and environmental issues are still maintains its importance (Parsek and Singh, 2003; Marques et al., 2007) The biofilm medium can provide a suitable environment for increasing the antibiotic resistance. Thus, it is urgently needed to obtain new drugs and biocides that inhibit biofilm formation and have microbicidal activity on living cells (Bueno, 2014).

Discovery of new antibiofilm and the search for resources to combat biofilms remain important. Bioactive compounds from plant, fungus, for use in antibiofilm strategies are an effective alternative in this regard. Therefore, natural sources have been preferred for the search for new antibiofilm production in this study. For the search for new and effective substances, soil is an enormous medium in terms of metabolites produced by microorganisms. In this context, natural products obtained from filamentous fungi are known to have strong antimicrobial activity (Svahn et al., 2012). This makes them attractive for the development of new antibiofilm strategies. Kang et al. (2005) reported that *A. sclerotiorum* showed very effective antifungal activity against phytopathogenic fungi, *Phytophthora* spp. Such studies have encouraged the evaluation of the antibiofilm properties of this fungus. In the literature, there is no information on the activity of antibiofilm about this fungus.

The aim of this study was to investigate the potential antibiofilm activity of the extracellular compounds produced by *A.sclerotiorum*, a strain isolated from soil. The results showed that natural production from *A.sclerotiorum* may be a potential antibiofilm agent.

## MATERIAL AND METHODS

### Bacterial Strains and Culture Conditions

The microorganisms used in this study were *Staphylococcus aureus* ATCC 25923, meticilin resistance *S. aureus* (MRSA), *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. All strains were maintained in Mueller-Hinton agar (MHA) at 37 °C.

### Isolation of Filamentous Fungi

Soil samples were collected at 2016 to 2017 in Erzurum, Turkey, according to Pepper et

al., 2011. Briefly, 1 gram of soil sample was diluted in 10 mL distile water containing %0.02 Tween 20. After five-fold serial dilution, 100 µL were spread on potato dextrose agar (PDA) plate at 25°C. After incubation, colonies were taken in new PDA plates.

**Identification of Fungi**

The fungi with antimicrobial and antibiofilm acitivity were identified based on their morphological and molecular characteristics. After 7 days of incubation in the PDA flask, DNA isolation from solid culture was performed by the EcoPure Genomic DNA Isolation Kit (EcoTech, Turkey). The universal ITS1 primer (5 'TCC GTT GGT GAA CCA GCG G 3') and ITS 4 primer (5 'TCC TCC GCT TAT TGA TAT GC 3 ') for the amplification of the internal transcribed spacer (ITS) region, which contains a partion of the 5.8S, 18S and 25S rDNA, was used. The PCR conditions include; initial denaturation (95 °C for 2 min); 35 cycles (95 °C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min) and a final extension at 72 °C for 10 min. The amplicon was verified with agarose gel electroporesis and sequenced using ITS1 and ITS4 primers in two different sequencing reactions. Using Bioedit sequence assembly program, sequence was deposited with the GenBank database (Zhang et al., 2000).

**Preparation of the Extracts**

Isolate was grown in PDA Petri for 7 days at 25 °C. After 7 days, 100 ml of 6 erlenmeyer flasks was inoculated with yeast peptone glucose medium (YPG) containing 2 g $L^{-1}$ D-glucose, 1 g $L^{-1}$ yeast extract and 2 g $L^{-1}$ peptone with minimum salt solution ($K_2HPO_4$; 0.4 g, $KH_2PO_4$; 0.14 g, $(NH_4)_2SO_4$; 0.6 g, $MgSO_4$; 75 mg, NaCl; 75 mg, $CuCl_2.H_2O$; 1,5 mg, $ZnSO_4.7H_2O$; 7.5 µg, $FeSO_4.7H_2O$; 7.5 µg, $MnSO_4.H_2O$; 7.5 µg $L^{-1}$) broth, which was brought to pH 6.2. Six discs were taken from the solid culture with 6 mm cork borer and inoculated into 100 ml of YPG medium. Then the liquid culture was grown in shaker at 150 rpm at 25 °C for 7 days.

At the end of the incubation, ethyl acetate extraction is performed from growing liquid cultures. The liquid cultures were passed through a 4-layer filter paper before extraction. After liquid cultures were filtrated by the PES membrane 0.22 µm syringe filter to obtain cell free supernatant (CFS). CFS were shaken with 1:1 ethyl acetate for 30 minutes. After that, the ethyl acetate phase was taken up and the ethyl acetate was completely evaporated at 150 rpm at 45 °C. (Santos et al., 2015). After, the residue remaining in the tube was dissolved with 2 ml of DMSO to obtain a substance extract.

**Antimicrobial Activity Determination**

Agar-well diffusion bioassay was performed to evaluate the antimicrobial activity of the obtained extract. Wells were opened in agar Petri which inoculated with 1-day liquid bacteria culture ($OD_{625}$, optical density = 0.1-0.2) with 6 mm sterile cork borer. 200 µl of extract and CFS were added to wells as a comparison. Petri plates were allowed to incubate overnight at 37 °C, 16 hours (Balouiri et al., 2016).

**Thin-Layer Chromatography(TLC) and TLC Overlay Assay**

The components of the extracts were separated on aluminum supported thin layer chromatography (TLC) plates (Merck, silica gel 60 F254). 7:3 ethyl acetate: dichloromethane was used as the solvent system. This method has been modified and applied according to the Hamburger and others working in 1987 (Hamburger et al., 1987).

45 µl of the extract was loaded onto the previously prepared TLC plate for overlay assay. The extract was left in the same solvent medium and then dried. After the image was taken in UV, TLC plate was inverted into soft MHB medium containing one day of liquid bacteria culture to

see zone formation and allowed to incubate overnight at 37 °C.

**MIC Determination**

Antimicrobial composition purified from TLC was added to 96-well plates at a concentration of 0.5 McFarland in 75 µl of bacterial cells and at specific concentrations (0, 25, 0.5, 1, 2, 4, 8 mg $ml^{-1}$). Total volume was 150 µL. Mueller-Hinton Broth (MHB) was added as a negative control, and bacteria without compound was added as a positive control. This Petri was evaluated as a concentration-adjusted MIC value without colony development after 24 hours of incubation at 37 °C.

**Crystal viole (CV) Assay**

The CV assay was performed against moderate biofilm producer strains, *S. aureus* ATCC 25923 and MRSA. Briefly, $1\times10^6$ CFU/mL bacterial cell in MHB and purfied compound with increasing concentration (0.25, 1, 2, 4 µg $ml^{-1}$) were seed on 96 well plate. The plate incubated for 48 h at 37 °C in static incubator. Then, it was washed three times with phosphate buffer saline (PBS) to remove unbound cells and 0.5 % CV dye was added for 20 min. Then, CV was removed with three times washing steps. The plates rinsed with 30% acetic acid. OD was read at 590 nm with spectrometer (Feoktistova et al., 2016).

**RESULTS AND DISCUSSION**

**Selection and Identification of Antimicrobial Compounds Producer Isolate**

Molecular identification has been done for fungal isolate having antimicrobial activity. rDNA sequence data were interpreted using BLASTN 2.8.1+ and bioedit program, then filamentous fungus was identified as *A.sclerotiorum* with accession number MH345718.

**Antimicrobial Activity Determination**

The ethyl acetate extract of *A. sclerotiorum* has been shown to be effective against human pathogens such as *S. aureus, E. coli, E. feacalis, P. aeruginosa* bacteria and was evaluated in terms of antimicrobial potential. This evaluation was carried out with the most common agar-well diffusion bioassay used in antimicrobial tests. (Table 1. and Figure 1.) In the well diffusion test, the antibacterial activity of *A. sclerotiorum* was 35 mm, which is the highest zone diameter against *S. aureus*. Furthermore, 30 mm zone for *E. coli*, 26 mm zone for *P. aeruginosa* and 27 mm zone for *E. feacalis* were observed. In addition to the ethyl acetate extract of the fungus, the CFS was also used for comparison. The results showed that the ethyl acetate extract produced a much more effective inhibition diameter than the CFS. Indicated that the antimicrobial content produced by the fungus is efficiently released by ethyl acetate extraction.

Using the same method, Murali et al. (2017) have reported that evaluated the antibacterial potential of *Aspergillus sulphureus* isolated from Sida Acuta. Similar data were obtained for *S. aureus* in this study. Santos et al. (2015) reported that among the 18 fungi isolates, *Nigrospora sphaerica* (URM-6060) and *Pestalotiopsis maculans* (URM6061) gave the best results against test pathogens similarly.

**Table 1.** Inhibition zone diameters of the CFS of the fungus and the substance resulting from ethyl acetate extraction

| Bacterial Strains | Zone value (Extract) | Zone value (CFS) |
|---|---|---|
| ***P. aeruginosa* ATCC 27853** | 26 mm | 12 mm |
| ***S. aureus* ATCC 25923** | 35 mm | 24 mm |
| ***E. coli* ATCC 25922** | 30 mm | 16 mm |
| ***E. faecalis* ATCC 29212** | 27 mm | 19 mm |

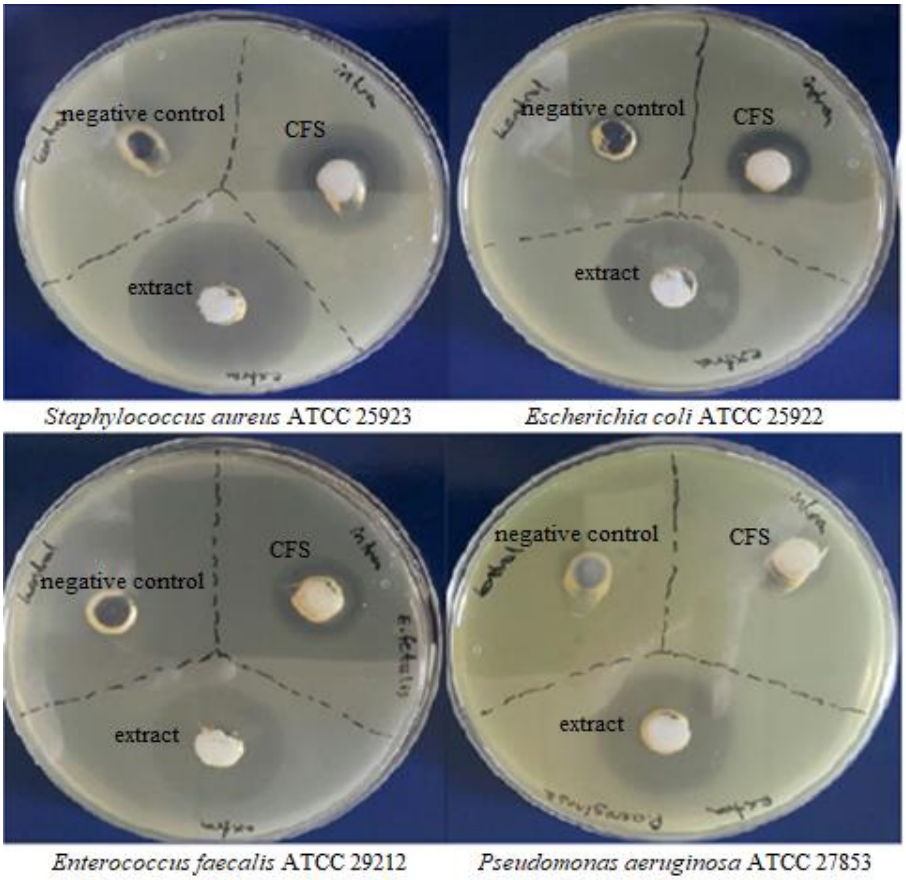



**Figure 1.** (A) Inhibition zones of fungus extract against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853

## Thin-layer Chromatography(TLC) and Overlay

Murali et al. (2017) reported that TLC bioautography is the ideal method for separation of bioactive compounds. Extraction of culture filtrate with ethyl acetate is a good solvent for distinguishing bioactive compounds from other substances that may be present in filtrate. In order to distinguish the bioactive metabolites of ethyl acetate extract from *A. sclerotiorum*, a TLC based biotechnology was performed and the antimicrobial potentials after decomposition were examined. 7:3 ratio of dichloromethane and ethyl acetate as the solvent system. However, 5:5, 6:4, 8:2 ratios were also tried to optimize the system used and 7:3 ratio phase solvent system was chosen because similar band profiles appeared. A total of 5 bands with Rf values of 0.25, 0, 27, 0.33, 0.42, 0.52, were obtained (Figure 2). The bands at TLC 0.42 showed effective and greater antibacterial potential against *S. aureus* shown in TLC overlay assay.

Our aim is to identify the bioactive components of the *A. sclerotiorum* extract by TLC bioautography and determine the antimicrobial activity by the resulting band profiles. As a result of the TLC overlay, the band with the highest antimicrobial profile gave way to work as a strong antimicrobial and antibiofilm substance. Interestingly, although the ethyl acetate content had an antimicrobial effect against *P. aeruginosa, S. aureus, E. coli* and *E. faecalis*, gave only a zone of inhibition against *S. aureus* in the TLC overlay test. The band of Rf value: 0.42 was selected for our study by natural product antibiofilm obtained by TLC scraping method.

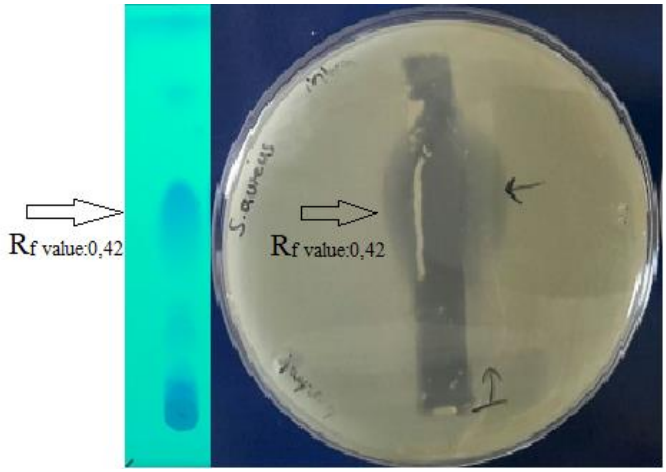
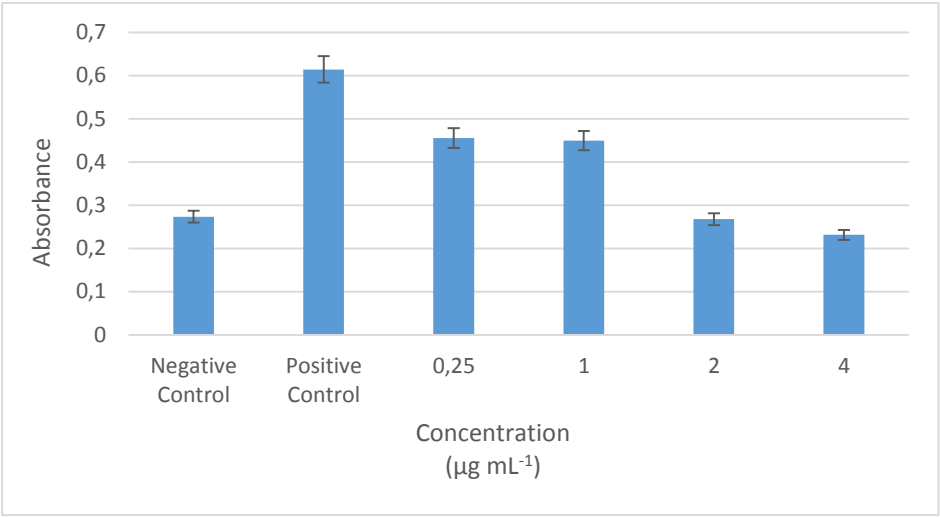



**Figure 2.** TLC-bioautography of ethyl acetate extract of *A. sclerotiorum* (MH345718) and TLC overlay result

A




B

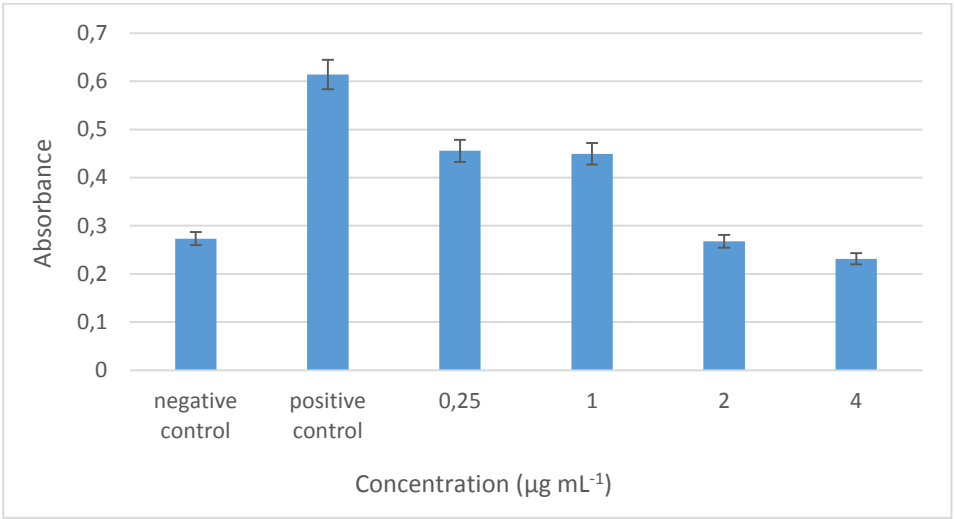



**Figure 3.** Antibiofilm results of the substance obtained from *A. sclerotiorum* against A. *S. aureus* B. MRSA

**Antimicrobial Activity**

MIC determination results against *S. aureus* and MRSA were 4 μg $ml^{-1}$, 4 μg $ml^{-1}$ respectively. Murali et al. (2017) reported that MIC results of the ethyl acetate extract of *A. sulphureus* revealed of 15.6, 62.5, 15.6 and 62.5 μg $ml^{-1}$ against *S. aureus, B. subtilis, S. typhi* and *E. coli*, respectively. Similarly, Lihan et al. (2014) reported MIC determination studies were performed in the range of 0.0625-1 mg $ml^{-1}$.

**Antibiofilm Activity**

Scraped and purified bioactive compound with an RF value of 0.42 was tested against *S. aureus* and MRSA which are moderate biofilm producers as seen in the Figure 3 A and B, respectively. The bioactive compound produced by *A. sclerotiorum* prevented growth of biofilm cells. We studied the concentration range of 0.25-4 μg $ml^{-1}$.

We used the TLC excavation as the bioactive compound that we suspect is strongly antibiofilm activity, that is, the method of obtaining the most intense banding compound. We have seen effective antibiofilm activity from the material obtained from this process as seen in the Figure 3.

Biofilm inhibition percentages of the bioactive compound was 62%, 79% against *S. aureus* and MRSA respectively. But there must be different methods for purification at the higher concentrations of the densest band to achieve more effective results and to keep the work going. Similarly, Wang et al. (2017) reported that 18 components obtained from marine-derived fungi showed *S. aureus* growth at a concentration of 100 μg $ml^{-1}$ and an antibiofilm activity of 50% on biofilm formation.

## CONCLUSIONS

Antibiofilm activity of natural compounds from *A. sclerotiorum* was determinated against *S. aureus* and MRSA for the first time. It was found that the natural production obtained from *A. sclerotiorum* inhibited biofilm formation. Other methods of substance purification can be performed to increase the concentration of the substance obtained by TLC. Antimicrobial content could not be obtained effectively by this method. This problem is overcome if the substance is obtained at a higher concentration. In addition, Q-TOF / MS characterization methods should be used for further studies in order to identify the scraped band from TLC. With this study, we were able to hope for the discovery of new antibiofilm drugs.

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