

## Determination of the antimicrobial and antibiofilm effects and 'Quorum Sensing' inhibition potentials of *Castanea sativa* Mill. extracts

Saliha EKŞİ<sup>1\*</sup>, Ülkü Zeynep ÜREYEN ESERTAŞ<sup>1</sup>, Ali Osman KILIÇ<sup>2</sup>,  
Nebahat EJDER<sup>1</sup>, Barış UZUNOK<sup>3</sup>

<sup>1</sup>Recep Tayyip Erdoğan University, The Faculty of Medicine, Department of Medical Microbiology, 53020 Rize, Turkey; [saliha.eksi@erdogan.edu.tr](mailto:saliha.eksi@erdogan.edu.tr) (\*corresponding author); [biyolog\\_ulku@hotmail.com](mailto:biyolog_ulku@hotmail.com); [nebahat.aydogan@erdogan.edu.tr](mailto:nebahat.aydogan@erdogan.edu.tr)  
<sup>2</sup>Karadeniz Technical University, Faculty of Medicine, Department of Medical Microbiology, 61080 Trabzon, Turkey; [akilic@ktu.edu.tr](mailto:akilic@ktu.edu.tr)  
<sup>3</sup>Uşak University, Faculty of Medicine, Department of Physiology, 64300 Uşak, Turkey; [Uzunok20@yahoo.com](mailto:Uzunok20@yahoo.com)

### Abstract

The rapid rise of resistance causes existing antibiotics to become dysfunctional. Therefore, search for new antimicrobial active ingredients has increased in recent years. In this study, flower extracts of *Castanea sativa* were examined for antimicrobial and anti-quorum sensing aspects. The antimicrobial properties of methanol, ethyl acetate, ethanol and hexane extracts of *C. sativa* against some gram-positive and gram-negative bacterial species, as well as yeasts (*Candida albicans* and *Candida parapsilosis*) were investigated by the agar well diffusion method. The minimum inhibition concentration (MIC) and minimum bactericidal concentrations (MBC) of *C. sativa* extracts were also determined. *Chromobacterium violaceum* ATCC 12472, *C. violaceum* 35352, *C. violaceum* VIR07 and *C. violaceum* CV026 indicator strains were used for determination of the quorum sensing inhibitions, and the *Pseudomonas aeruginosa* PAO1 strain was used for the swarming tests. Additionally, biofilm inhibition was detected by the spectrophotometric method using the *P. aeruginosa* PAO1 strain. Methanol, ethanol and ethyl acetate extracts of *C. sativa* was found to have high antibacterial and antifungal effects, while the methanol extract also had anti-quorum sensing, anti-swarming and biofilm inhibition effects, but no activity was found in the n-hexane extract. To the best of our knowledge this is the first report revealed that methanol extract obtained from *C. sativa* flowers induced anti-quorum sensing activities mainly inhibited the violacein production, swarming and biofilm formation. The present investigation provided evidence that the *C. sativa* flower extract maybe a potential source of antimicrobial agents. Therefore, much attention should be paid to *C. sativa* flower content, which could be used with high efficacy against microorganisms.

**Keywords:** antimicrobial activity; biofilms; *Castanea sativa*; quorum sensing

### Introduction

The effectiveness of current antibiotics on microorganisms has been decreasing rapidly in recent years. Moreover, it is obvious that antibiotic resistance will be one of the most important issues of the global agenda

in the future as it is today. Antibiotic resistance is increasingly diminishing the options available for treatment of diseases and requires a search for new sources of treatment. As a result of this, interest in herbal resources in modern medicine is increasingly growing (Paul *et al.*, 2010). In recent years, 22% of all newly approved drug molecules have been synthesized as natural product derivatives (semi-synthetic), 4% were produced as real natural products, and 13% were produced as pharmacophore of a natural product (Newman and Cragg, 2012). *C. sativa* is known as sweet chestnut and is a plant belonging to the *Fagaceae* family. From the past to the present, it has been used in folk medicine to treat a wide range of diseases such as the common cold, diarrhea, asthma, bronchitis, heart, back pain and rheumatism. It was also used as a bleeding inhibitor due to the high tannin content in its shell (Lim, 2013).

Body of study has been done using different part of *C. sativa*. Hao *et al.* (2012) examined the antimicrobial activities of the shell, membrane and leaves of Japanese (*Castanea crenata*), European (*Castanea sativa*) and Chinese (*Castanea mollissima*) chestnuts. The results showed that the chestnut membrane had more antimicrobial compounds namely flavanol, glycoside and terpenoid than its leaf and shell parts.

A study conducted in 2010, showed that the extracts obtained from leaf, pericarp and aciculate peel of *C. sativa* had high antimicrobial activity (Zitkovic *et al.*, 2010). Additionally, Spanish researchers characterized the compounds found in chestnut flowers, demonstrated their antioxidant potential and reported that it may be possible to extend the shelf life of products and reduce the consumption of chemical additives by coating foodstuffs with these molecules (Carocho *et al.*, 2014). Furthermore, many scientific papers have reported the antimicrobial activities of honey which is one of the oldest traditional medicines considered to be important in human health (Mcloone *et al.*, 2015). Even though a number of investigations suggested the antimicrobial properties of different honeys from around the world, very few studies have been done with chestnut honeys. Among those, Kolaylı *et al.* (2016) reported that chestnut honeys had potent antimicrobial activities against *S. aureus*, *E. coli*, *E. faecalis*, while no antifungal activity was recorded against *Candida albicans* and *Candida cerevisiae*. Moreover, another study carried out by Ronsisvalle *et al.* (2017) to evaluate antimicrobial activity of two different chestnut honeys. Interestingly, they also found strong antimicrobial activity against *S. aureus*, *E. coli* and *P. aeruginosa*.

*C. sativa* is the only native species of genus *Castanea* grows in Europe and Turkey. This species has a distribution from the Eastern Black Sea to Mediterranean Regions in Turkey (Ketenoglu *et al.*, 2010). The leaves of *C. sativa* are used in the treatment of pertussis in the Kalkandere district of the province of Rize, while it is used as tea by the public in dyspnea and cardiovascular diseases in the Hayrat area of the province of Trabzon (Yaldız *et al.*, 2010). According to our research in the literature, there is not a sufficient number of scientific studies about the flower of *C. sativa* which is widely used in the region and utilized by the local people for the health benefits. These benefits have certainly close association to some of the bioactive secondary compounds. However, it was determined by the researchers that polyphenolic compounds (Pacheco-Ordaz *et al.*, 2018) which have been reported to have antimicrobial effect in the literature are found in high percentage of the flower parts of *C. sativa* (Pereira *et al.*, 2013; Vasconcelos *et al.*, 2010). Therefore, this study investigated the effects of methanol, ethyl acetate, ethanol and hexane extracts obtained from the flowers of chestnut plant for the antimicrobial, anti-quorum sensing and biofilm inhibition properties.

## Materials and Methods

### *Collection of C. sativa plant and preparation of its extracts*

The flowers of *C. sativa* used in the study were collected from İkizdere (Rize, Turkey), cleaned and fresh flowers were stored at -20 °C until used. The extracts were obtained using methanol, ethyl acetate, ethanol and hexane by maceration method (Solanki and Nagori, 2012). Briefly, 10-20 g of the flower of chestnut stored at

-20 °C were weighed, ground into fine powder and mixed with 100 or 200 mL solvent. Extraction was conducted on a shaker at room temperature (RT) for 48 h. After that, the extracts were filtered through filter paper and the solvents were evaporated in an evaporator (LabTech. EV311) at 40 °C. Subsequently, the extracts were suspended at 50-200 mg/mL in Dimethyl sulfoxide (DMSO) and aliquots of these were stored at -20 °C until needed for further assays. Pre-experimental working concentrations were diluted to 10 mg/mL, and the concentrations to be used in the experiments were adjusted from these dilutions.

#### *Media and cultures conditions*

Bacteria and yeast strains used in the study are given in Table 1. The stocks cultures of the microorganisms were stored at -80 °C. Before the experiment, *Mycobacterium smegmatis* strain was prepared by 3-day incubation in Brain Heart Infusion Agar (BHIA), *Candida* was prepared by 2-day incubation in potato dextrose agar (PDA), and finally other microorganisms were prepared by 1-day incubation in Mueller Hinton Agar (MHA). Stocks of the positive controls (ampicillin, gentamicin, ciprofloxacin and amphotericin-B) were prepared at 200 µg/mL and aliquots stored at -20 °C for further antimicrobial testing. The necessary concentrations were added from these stocks during the experiments. As a negative control, the equivalent concentration to the extracts from DMSO was added to the corresponding wells.

**Table 1.** Microorganisms used for the antimicrobial activity

Type of microorganism
<i>Staphylococcus aureus</i> ATCC 25923
<i>Escherichia coli</i> ATCC 25922
<i>Pseudomonas aeruginosa</i> ATCC 27853
<i>Bacillus subtilis</i> ATCC 6633
<i>Enterococcus faecalis</i> ATCC 29212
<i>Enterobacter aerogenes</i> ATCC 13048
<i>Acinetobacter haemolyticus</i> ATCC 19002
<i>Klebsiella pneumoniae</i> ATCC 13883
<i>Salmonella typhimurium</i> ATCC 14028
<i>Candida albicans</i> ATCC 10231
<i>Candida parapsilosis</i> ATCC 22019
<i>Mycobacterium smegmatis</i> ATCC 607
<i>Chromobacterium violaceum</i> ATCC 12472
Bacterial strains used in Quorum sensing inhibition tests
<i>Chromobacterium violaceum</i> ATCC 12472
<i>Chromobacterium violaceum</i> ATCC 31352
<i>Chromobacterium violaceum</i> VIR07
<i>Chromobacterium violaceum</i> CVO26
<i>Pseudomonas aeruginosa</i> PAO1*

\*It was used in the tests of 'Quorum Sensing' inhibition / Biofilm inhibition

#### *Determination of antimicrobial activity by the agar well diffusion method*

Agar diffusion method determination was performed according to Denev *et al.* (2014). Briefly, all the microorganisms were seeded in media as mentioned above. Following that bacteria cultures inoculated at 37 °C. Meanwhile, *Candida* species were incubated at 35 °C. The density of *M. smegmatis* strain was adjusted to 0.5 McFarland in BHIB (Woods *et al.*, 2003), the density of the other microorganisms and *Candida* species were adjusted to 0.5 and 1.0 McFarland in a Phosphate buffered saline solution (PBS), respectively. Following that, *M. smegmatis* strain was spread onto BHIA plates, the other test microorganisms were inoculated onto

MHA plates and *Candida* species cultured onto MHA plates including 2% glucose and 0.5 µg / mL methylene blue dye (CLSI, 2009). *C. sativa* extracts of 10 mg/mL concentration were prepared in 3% DMSO. Wells with 6 mm diameter were punched on the agar media and each well filled with 50 µL of the extracts from different solvents. The positive or negative controls were also added to the corresponding wells.

The cultures were incubated at 37 °C for each microorganism at appropriate times, *Candida* species were incubated at 35 °C. the plates were examined visually for the formation of clear zone around the wells. The zone with a diameter greater than 6 mm were considered zones of inhibition.

#### *Determination of minimum inhibition concentration*

An extract having antimicrobial activity within the agar diffusion assay were further tested for the determination of the MIC (Minimum Inhibition Concentration) values. The microorganisms were incubated on the appropriate medium at appropriate times as indicated above. Subsequently, 0.5 McFarland suspensions for bacteria cultures and 1.0 McFarland from *Candida* cultures were prepared into appropriate medium (RPMI 1640 (0.2% glucose) for *Candida* species (CLSI, 2008), BHIB for *M. smegmatis* (Woods *et al.*, 2003), and MHB-II for other microorganisms were used (CLSI, 2006; Murray *et al.*, 2009).

Afterwards, 100 µL respective medium for each microorganism was added to each well of the microplates. Subsequently, 100 µL of *C. sativa* extracts from the 10 mg/mL stocks were added to the first wells of the plates. After positive controls (ampicillin, gentamicin, ciprofloxacin and Amphotericin-B) and negative control DMSO were applied into the corresponding wells, twofold dilutions were made. Next, 10<sup>6</sup> CFU / mL bacteria to be tested were added to the respective wells and the microplates were incubated at 37 °C for the relevant times for each microorganism. Each assay was performed in duplicate and repeated at least two times. The MIC was determined as the lowest concentration which showed no bacterial growth.

#### *Determination of minimum bactericidal concentration values*

To determine the minimum bactericidal concentration (MBC), 50 µL samples were taken out from the MIC well and the previous three wells and they were seeded onto the plates with relevant media and left to incubated at 37 °C for the relevant times for each microorganism. After evaluating the reproduction in Petri dishes, MBC was defined as the lowest concentration at which no viable cell growth on the plate (NCCLS, 1999).

#### *Determination of anti-quorum sensing*

To test anti-quorum sensing; all extracts were used for violacein inhibition assay, but only the methanol extract was used to determine the swarming and biofilm tests since it induced the high antimicrobial effect by the agar well diffusion method.

#### *Determination of violacein inhibition*

In order to determine pigment inhibition, after determining the Sub-MIC values of the strains of *C. violaceum* ATCC 12472, *C. violaceum* 35352, *C. violaceum* VIR07 and *C. violaceum* CV026 as above, *Chromobacterium* species were inoculated in 5 mL LB, and overnight cultures were prepared in a shaking incubator at 37 °C.

On the following day, the cultures were adjusted to 0.5 McFarland in PBS and the signaling molecules used for indicator strains were prepared in DMSO and ethyl acetate separately in the concentrations of C6-AHL 2 mg/mL, C7-AHL 2.1 mg/mL and C12-AHL 2.8 mg/mL (Torres *et al.*, 2013). Subsequently, 50 µL of each culture was inoculated into 5 mL of soft LB agar, and among the indicator strains, C6-AHL and C7-AHL were added for CV026, and C12-AHL 25 µL (1 mM) was added for VIR07. These cultures were poured onto LB agar plates and left to dry, then 50 µL of the determined Sub-Mic concentrations of each extract were added to the opened wells. As a result, the presence of anti-quorum sensing was determined by evaluating the zones

where the continuity of bacterial growth seen but the formation of purple pigment was suppressed (McLean *et al.*, 2004; Koh and Tham, 2011).

Additionally, the methanol extract which was effective on all *Chromobacterium* by the agar well diffusion method was also evaluated spectrophotometrically. The Sub-Mic and sub-concentrations of the extract were incubated with 0.5 McFarland of *C. violaceum* 12472 at 5 mL LB, and on the next day, violacein measurement was conducted at 585 nm (Hamidi, 2013).

#### *Determination of "swarming" suppression*

The swarming suppression property of the methanol extract was tested by modifying the methods reported by Rashid and Kornberg (2000) and by Uğurlu (2013) *P. aeruginosa* PAO1 strain was grown overnight on LB agar and 50 µL from the Sub-Mic or 25 µL from the sub-concentration (100 µg/mL, 50 µg/mL) of the *C. sativa* methanol extract was added in 5 mL of autoclaved but not solidified LB media. Then this mixture was poured onto LB agar plates, and the agar was allowed to solidify for a while. Subsequently, *P. aeruginosa* was inoculated in the middle of the plates with a sterile toothpick and incubated at 37 °C for 16-18 hours. At the end of incubation, sliding movement was determined by measuring the diameter of the spread from the point of inoculation to the environment.

#### *Testing inhibition of biofilm development*

*P. aeruginosa* PAO1 strain was grown for 8 h at 37 °C for shaking at 175 rpm in 5 mL of LB medium and 1% dilution of 0.5 McFarland, culture suspension was used for biofilm experiment. Briefly, microplate wells were filled with 125 µL of LB medium, in order to 40 µL of methanol extract and 35 µL of *P. aeruginosa* PAO1 cells. No extract was added to the control wells, while the same amounts of bacteria and medium were added. The prepared microplates were allowed to incubate at 37 °C for 24 h. Subsequently, the microplates were washed three times with distilled water, and stained with 0.3% crystal violet for 15 minutes. The microplates were then washed three times with distilled water and remaining crystal violet was removed with 95% ethanol for 15 minutes and measured on a spectrophotometer at 570 nm (Truchado *et al.*, 2009; Uğurlu, 2013). Biofilm inhibition was demonstrated by averaging three repeated.

## **Results**

#### *Antimicrobial effects of C. sativa extracts*

Methanolic extract of *C. sativa* flowers was tested against thirteen microbial strains for antibacterial activity by the agar well diffusion method, and its potential effect was determined by the presence or absence of the inhibition zones. According to results were presented in Table 2. among three gram-positive bacteria *S. aureus* was found to be the most sensitive strain with 23 mm inhibition zone. The extract exhibited a mild inhibition zone on *B. subtilis* (12 mm) but did not show any inhibition zone on *E. faecalis*. Similarly, the diameters of the zone of inhibition ranged from 7.3 mm to 23.0 for gram-negative bacteria. Among the 8 gram-negative bacteria *A. haemolyticus* and *P. aeruginosa* were found to be the most sensitive bacteria having the inhibition zones diameters, 23 and 12 mm, respectively. Those zones diameters are very close to the controls. Additionally, the extract also exhibited good inhibition zones against *M. smegmatis* (17 mm) and *C. violaceum* (20 mm). Moreover, the methanol extract of *C. sativa* flowers exhibited high antifungal activity with inhibition zones ranging between 24 mm to 27.3 mm (Table 1) and MIC of 31.2 µg/mL (Table 2). The highest activity was against to *C. albicans*.

Antimicrobial effect of the ethyl acetate extract from *C. sativa* flowers was investigated against bacteria as well as two *Candida* species including *C. albicans* and *C. parapsilosis*.

The extract demonstrated the antibacterial activity with inhibition zones of 17.3, 10.0, 15.0, 13.0, 14.0 mm against *S. aureus*, *B. subtilis*, *K. pneumoniae*, *C. violaceum*, *M. smegmatis*, respectively. These results revealed that among three gram-positive bacteria *S. aureus* is the most sensitive strain but *M. smegmatis* was found to be the most sensitive gram-negative bacteria. Moreover, the ethyl acetate extract of *C. sativa* flowers exhibited higher antifungal activity against *C. parapsilosis* than *C. albicans*. with inhibition zones of 20 mm and 13 mm, respectively (Table 2).

Ethanol extract of *C. sativa* flowers exhibited high antibacterial activity with inhibition zones of 17.3, 20.0, 17.0 mm against gram-positive *S. aureus*, gram-negative *A. baemolyticus* and *C. violaceum*, respectively. The extract exhibited moderate antimicrobial activity against *M. smegmatis* with an inhibition zone of 13.0 mm (Table 1) Moreover, ethanolic extract exhibited higher antifungal activity against *C. parapsilosis* than *C. albicans*. with inhibition zones of 19.0 mm and 13 mm, respectively (Table 2).

None of the extracts mentioned above and DMSO (negative control) demonstrated antimicrobial activity against *E. faecalis*, *E. aerogenes* and *S. typhimurium*. But methanol and ethanol extracts had very weak antimicrobial effect on growth of the gram-negative *E. coli*. In terms of antimicrobial aspect, the hexonic extract of *C. sativa* flowers did not show any antimicrobial activity against microorganisms used in the present study (Table 2).

**Table 2.** Agar well diffusion test results (mm)

Microorganisms	Extracts				
	Methanol	Ethyl acetate	Ethanol	Hexane	Controls
<i>S. aureus</i>	23.33±0.47	17.33±0.47	17.33±0.47	-	30.33±0.47
<i>B. subtilis</i>	12.0±0.81	10.0±0	-	-	22.33±0.47
<i>E. faecalis</i>	-	-	-	-	15.0±0.0
<i>E. coli</i>	7.33±0.47	-	8.0±1.63	-	25.0±0.0
<i>P. aeruginosa</i>	12.0±2.64	-	-	-	13.0±0.47
<i>A. baemolyticus</i>	23.0±0.81	-	20.0±0.0	-	23.0±0.0
<i>K. pneumoniae</i>	7.33±0.47	15.0±1.41	-	-	22.0±0.0
<i>E. aerogenes</i>	-	-	-	-	19.0±0.47
<i>S. typhimurium</i>	-	-	-	-	17.0±0.0
<i>C. violaceum</i> (ATCC12472)	20.33±0.47	13.0±0.0	17.0±1.41	-	30.33±0.47
<i>C. albicans</i>	27.33±0.47	13.0±0.0	13.0±0.81	-	28.0±0.0
<i>C. parapsilosis</i>	24.0±0.0	20.33±0.47	19.0±0.0	-	25.0±0.0
<i>M. smegmatis</i>	17.0±2.44	14.33±0.47	13.0±1.63	-	24.33±0.47

The experiments were repeated three times, the values of the zone diameters were averaged, and the results are presented in Table 2. DMSO (negative control), Ampicillin, Gentamicin, Amphotericin B and Ciprofloxacin (positive controls). 6-mm zone diameter correspond to the (-) in the table.

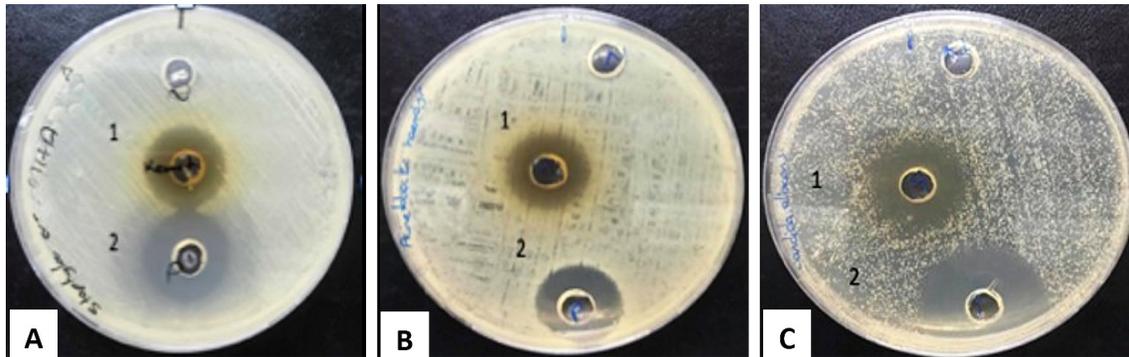
#### *The results of MIC and MBC*

The MIC and MBC were performed for only those methanol extracts which showed to have high antimicrobial activity by the agar well diffusion method.

#### *Violacein inhibition results*

The violacein inhibition results of the *C. sativa* extracts tested by the agar well diffusion method is presented in Table 4 and Figure 2. The results showed that methanol extract caused violacein inhibition in all *Chromobacterium* used. Moreover, ethyl acetate and ethanol extracts induced violacein inhibition only in *C.*

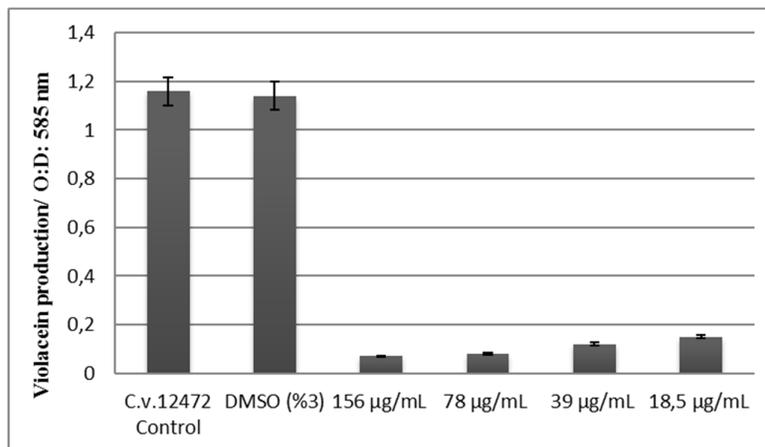
*violaceum* ATCC 12472 strain. In order to confirm the results above, violacein inhibition of the methanol extract was also measured spectrophotometrically. As shown in Figure 3, the extract inhibited the violacein production even at very low concentrations.



**Figure 1.** Agar well diffusion test results of methanol extract - A: *Staphylococcus aureus* ATCC 25923, A1: methanol extract, A2: Ampicillin; B: *Acinetobacter haemolyticus* ATCC 19002, B1: methanol extract, B2 Gentamicin; C: *Candida albicans* ATCC 10231, C1: methanol extract, C2: Amphotericin B



**Figure 2.** The effect of *C. sativa* methanol extract of *C. violaceum* ATCC 12472 violacein; (a-) violacein inhibition zone



**Figure 3.** The effect of *C. sativa* methanol extract on the production of *C. violaceum* ATCC 12472 violacein. (C.v. 12472; Only bacteria, DMSO (3%); Bacteria and 3% DMSO)

**Table 3.** MIC and MBC results of methanol extract ( $\mu\text{g/mL}$ )

Microorganisms	MIC* ( $\mu\text{g/mL}$ )	MBC** ( $\mu\text{g/mL}$ )
<i>S. aureus</i> ATCC 25923	62.5	125
<i>A. haemolyticus</i> 19002ATCC	62.5	125
<i>M. smegmatis</i> ATCC607	62.5	125
<i>C. albicans</i> ATCC 10231	31.2	62.5
<i>C. parapsilosis</i> ATCC22019	31.2	62.5

\*MIC: Minimum inhibitory concentration

\*\*MBC: Minimum bactericidal concentration

Summarizes the MIC and MBC values for the methanol extract of *C. sativa* flowers. The extract was very effective against all tested bacterial strains and displayed the MIC and MBC values of 65.5  $\mu\text{g/mL}$  and 125  $\mu\text{g/mL}$ , respectively. Moreover, its best activity was against the yeast with the MIC and MBC values of 31.2  $\mu\text{g/mL}$  and 62.5  $\mu\text{g/mL}$ , respectively

**Table 4.** Violacein inhibition results at a concentration of *C. sativa* of 156  $\mu\text{g/mL}$ 

Microorganisms	Extracts			
	Methanol	Ethyl acetate	Ethanol	Hexane
<i>C. violaceum</i> ATCC 12472	+	+	+	-
<i>C. violaceum</i> 31352	+	-	-	-
<i>C. violaceum</i> VIR07	+	-	-	-
<i>C. violaceum</i> CV026	+	-	-	-

#### “Swarming” suppression test results

The swarming inhibition of *C. sativa* methanol extract in the *P. aeruginosa* PAO1 strain was tested, and the results are given in Table 5 and Figure 4. The results from the experiment indicated that *C. sativa* methanol extract inhibited the “swarming” activity of *P. aeruginosa* PAO1 at 100  $\mu\text{g/mL}$  and 50  $\mu\text{g/mL}$  concentrations (Table 5, Figure 4).

**Table 5.** “Swarming” suppression results with the *C. sativa* methanol extract (100/50  $\mu\text{g/mL}$ ) in the *P. aeruginosa* PAO1 strain

Test plate	Diameter of zone (mm)
Bacteria control	29
Bacteria + 100 $\mu\text{g/mL}$ methanol extract	9
Bacteria + 50 $\mu\text{g/mL}$ methanol extract	11
Bacteria + 3% DMSO	27

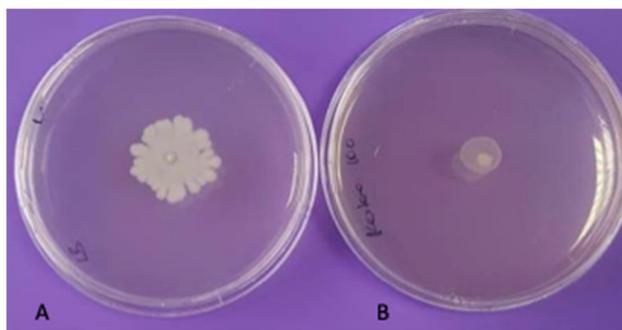
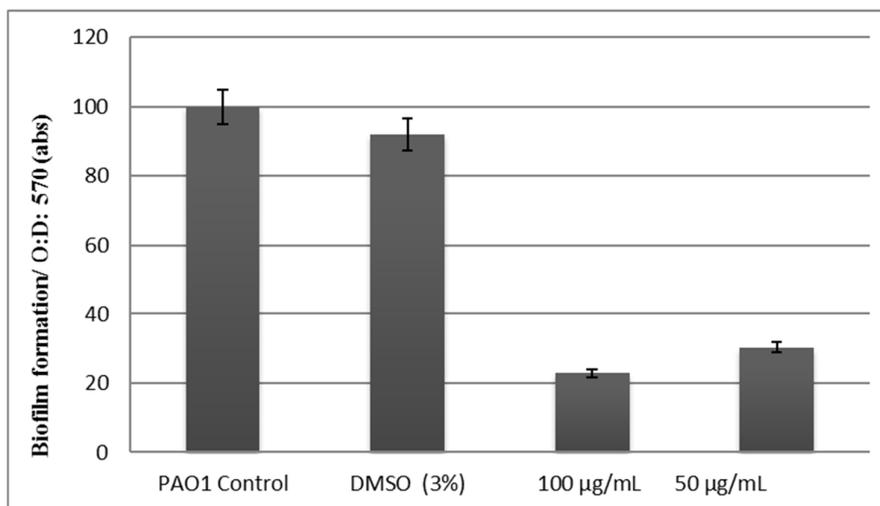


Fig. 4. “Swarming” suppression results of the *C. sativa* methanol extract 100  $\mu\text{g/mL}$  - A-) Positive (*P. aeruginosa* PAO1) Control; B-) Bacteria+ 100  $\mu\text{g/mL}$  methanol extract

*Biofilm inhibition results*

Anti-biofilm activity of the *C. sativa* methanol extract against *P. aeruginosa* PAO1 strain was tested and the results are presented in Figure 5. Compared to control, the *C. sativa* methanol extract was found to have very high biofilm suppressing activity at 100 µg/mL and 50 µg/mL concentrations.



**Figure 5.** Biofilm results of *C. sativa* methanol extract. (PAO1 Control; Only Bacteria, DMSO (3%); Bacteria and 3% DMSO)

**Discussion**

Antibiotic resistance has become an important problem in almost every country, particularly in Turkey. Hence, there is an urgent need to find alternative agents to treat resistant microorganisms. As a natural consequence of this, scientists have turned towards medicinal plants especially in recent times. Plants have great richness in their secondary phytochemicals. More importantly, they reported that polyphenols, tannins, terpenes and alkaloids have antimicrobial activities (Cowan-Murphy, 1999).

The current study assayed the antimicrobial potential of the extracts from *C. sativa* flowers. The results obtained in this study demonstrated the methanol and ethanol extracts from *C. sativa* flowers exhibited high antibacterial effects on gram-positive *S. aureus*, and gram-negative *A. baumannii*. On the other hand, the ethyl acetate extract was effective on gram-positive *S. aureus* and gram-negative *K. pneumoniae*. However, no antimicrobial activities of any extract were determined on *E. faecalis*, *E. coli*, *E. aerogenes* or *S. typhimurium* used in the study (Table 2). Avşar *et al.* (2016) reported that the methanol extracts obtained from the pollen grains of chestnut were completely ineffective on *K. pneumoniae*, similar to our study. On the other hand, our results indicated that ethyl acetate extract causes growth inhibition in *K. pneumoniae* which is an encapsulated bacteria and it is also one of the most important opportunist bacteria in clinic infections. More importantly, it has been shown that *K. pneumoniae* gains resistance worldwide (Niu *et al.*, 2019; Kohler *et al.*, 2017). Because of this aspect we think that our finding may be important to provide evidence that the ethyl acetate extract from *C. sativa* flowers might have some compound/compounds that may induce specific antimicrobial effect against *K. pneumoniae*.

In the literature, it has been reported that polar secondary metabolites are mostly concentrated in ethanol and methanol extracts. However, semi-polar secondary metabolites are mostly concentrated in ethyl acetate extracts (Houghton and Raman, 1998). Due to this fact that the ethyl acetate extract may have some

different secondary metabolites in its chemical content. Therefore, those secondary metabolites may cause growth inhibition in *K. pneumoniae*. Further work is needed to identify the bioactive components that may induce antimicrobial effect against *K. pneumoniae*.

Even though, all three extracts induced varying degree of antimicrobial activity against *M. smegmatis*, the methanol extract of *C. sativa* flowers exhibited marked growth inhibition against this bacterium with MIC value 62.5 µg/mL (Table 3). Determination of MIC value of a plant extract has been reported as an indicator of the strong activity of that extract (Ristic *et al.*, 2000). Although *M. smegmatis* is known as a non-pathogenic bacterium, but occasionally associated to human infections (Best and Best, 2009). Additionally, the fact that *M. smegmatis* is in the same genus as *M. tuberculosis*, which is known to cause a fatal disease, increases the importance of these results. The major contribution of the study to the literature may be achieved by determining the phyto-components in the extract content and their possible antimicrobial effects on other species of the genus *Mycobacterium*. Fokou *et al.* (2016) investigated antimicrobial effects of 65 extracts from 27 plant species against *M. ulcerans* and *M. smegmatis*. Their results showed that only 3 extracts had antimicrobial effects on *M. smegmatis*. These data may indicate that *M. smegmatis* is not very sensitive to secondary metabolites obtained from different plants. More importantly, it may suggest that very specific bioactive compound can induce antimicrobial activity against *M. smegmatis*.

Therefore, we think it may be important to investigate the methanol extract of chestnut flowers in terms of bioactive secondary compounds that may be significant to develop antimicrobial agent against *M. smegmatis* or against other members of *Mycobacterium* genus.

In our investigation, the extracts obtained from chestnut flowers have exhibited antifungal activity against *C. albicans* and *C. parapsilosis* species, and in particular, the methanol extract was found to have a strong antifungal effect with MIC values 31.2 µg/mL (Table 3). In the literature, the ability to determine the MIC value of a plant extract has been reported as an indicator of the strength of the activity of that extract (Ristic *et al.*, 2000).

Avşar *et al.* (2016) found that methanol extract obtained from chestnut pollen grains had a mild antifungal effect on *C. albicans* and *C. parapsilosis*. The major difference between two studies was that they used only the pollen grains of the flowers, however, in our study, we extracted whole flowers using the methanol as a solvent. Literature studies well documented that different organs of the plants produce so many different secondary metabolites with different biological functions (Li *et al.*, 2015). Therefore, differences in the extracts contents may determine the strong or the mild antifungal activity.

Although there is not much data showing the antimicrobial efficacy of chestnut flower with MIC values, Carochó *et al.* (2014) found that the extract obtained by boiling from chestnut flowers was effective on both gram-positive and gram-negative bacteria. On the other hand, the extract obtained by brewing induced higher antimicrobial effects on *Aspergillus* and *Penicillium* fungi. Those our results together, it is seen that the flower extracts of chestnut may have high antimicrobial potential and it may be affective on different groups of microorganisms.

Bacteria are social organisms and they communicate one to each other with 'quorum sensing' (QS), which is an intercellular signaling and gene regulatory mechanism used by bacteria (Quave *et al.*, 2011). Some of those mechanisms are swarming, violacein and biofilm production. The mechanisms of QS are treated as major agents in the pathogenicity and virulence of microorganisms. In recent years, researchers have predicted that infection development of antibiotic-resistant pathogens may be controlled by inhibiting the mechanism of QS (Quave *et al.*, 2011). Secondary metabolite production, pathogenicity, swarming, and biofilm formation are common behaviors among microorganisms in communication with signal molecules against resistance to stress. Last two decades anti-Quorum sensing activities of natural products got much attention and they reported that small molecules found in the structure of plants have a good quorum sensing suppression potential. More importantly, antimicrobial agents to be isolated from the plants can also work as quorum

sensing inhibitor in pathogenic microorganisms (Koh *et al.*, 2013; Bacha *et al.*, 2016) In this respect, in the present study we investigated the effect of the *C. sativa* flowers extracts on the swarming, violacein and biofilm mechanisms of the QS.

To date, there is a limited amount of reports on anti-QS activity of the extracts obtained from *C. sativa*. Among those, Quave *et al.* (2011) reported ethanol extract from *C. sativa* inflorescence had anti-QS activity against *Staphylococcus aureus* (MRSA) by inhibiting  $\delta$ -hemolysin which is one of the products of agr (*S. aureus* accessory gene regulator) locus in methicillin-resistant *S. aureus*. In another study, Quave *et al.* (2015) screened the methanol extracts of *C. sativa* leaf for QS inhibition. Based on the results, they demonstrated that oleanene and ursene derivatives rich methanol extract showed high quorum-sensing inhibition against *S. aureus* by affecting the agr alleles. Moreover, Truchado *et al.* (2009) investigated the anti-QS activity of methanol and aqueous extracts obtained from chestnut honey. As a result, they found that honey, and its extracts induced anti-QS activity against gram- bacteria by inhibiting the N-Acyl-L-homoserine lactones (AHLs) which are known main bacterial signaling molecules in gram-negative bacteria.

In the present study, inhibition of violacein production was demonstrated in all *Chromobacterium* species. Moreover, biofilm and swarming inhibition were observed in *P. aeruginosa* PAO1 strain. All these results revealed that the *C. sativa* methanol extract from flowers not only show antimicrobial effect, but also induce strong anti-quorum sensing activities. It is most meaning full that this is the first work in which *C. sativa* methanol extracts were screened for QS inhibition against *Chromobacterium* spp. and *P. aeruginosa* PAO1 strain.

## Conclusions

Altogether, the present study revealed that *C. sativa*'s flower extracts may contain various bioactive secondary substances that may induce good extent of antimicrobial properties. It would be useful to design new lead compounds against *K. pneumoniae*, *M. smegmatis* or against other members of *Mycobacterium* genus. Moreover, anti-QS activity of *C. sativa*'s flower extracts could be used in development of novel anti-pathogenic agents since it has been reported that anti-quorum sensing compounds isolated from plants have a great potential in the fight against multidrug resistant microorganisms because of their drug-like function. To underline this aspect of the extracts as well as their active components further studies are needed.

## Acknowledgements

This study was supported by the Project No: TDK-2018-959, by the BAP unit of Recep Tayyip Erdoğan University.

## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article

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