Antimicrobial and anti-quorum sensing activities of giant fennel (Ferula elaeochytris Korovin) from the Hatay region

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Received: 28.03.2020, Accepted: 13.08.2020 *etufekci@kastamonu.edu.tr

Hatay bölgesinde çıkarılan (Ferula elaeochytris Korovin) antimikrobiryal ve anti-quorum sensing aktiviteleri

Eurasian J Vet Sci, 2020, 36, 3, 214-220 DOI: 10.15312/EurasianJ VetSci.2020.281

Abstract

Aim: Today, increasing antibiotic resistance has focused the science world on the discovery of new antimicrobial molecules or the development of alternative methods of struggle. One of the alternative methods is thought to be inhibition of bacterial quorum sensing (QS). Because the QS system performs a crucial part in the synthesis of virulence factors in bacteria. Numerous medicinal plants are known to have promising antimicrobial and anti-QS activities. One of these plants may be giant fennel (Ferula elaeochytris Korovin), which has been extensively used in Anatolia for years. Therefore, it was aimed to investigate the antimicrobial and anti-QS activities of the root extract of F. elaeochytris Korovin in this study.

Materials and Methods: The antimicrobial activity of the extract was screened by disc diffusion assay against various microorganisms. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract against sensitive strain(s) were determined using broth microdilution assay. Anti-QS activity investigated on inhibition of violacein and pyocyanin production in Chromobacterium violaceum and Pseudomonas aeruginosa bioreporter strains, respectively.

Results: The extract exhibited the antimicrobial activity against only Staphylococcus aureus (zone of inhibition (ZOI) = 9.3±0.6 mm). The MIC and the MBC values were determined as 4.4±1.9 mg/mL and >105 mg/mL, respectively. No inhibition of violacein production was detected. Pyocyanin production was reduced by 60% and 92% at sub-MIC concentrations (at 1.64 mg/mL and 3.28 mg/mL, respectively) as against the control (p<0.05) without suppressing bacterial growth.

Conclusion: This study shows that the root extract of F. elaeochytris Korovin may be a good candidate to develop antibiotic and anti-QS agents.

Keywords: Antimicrobial, extract, Ferula elaeochytris Korovin, pyocyanin, quorum sensing

RESEARCH ARTICLE

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Öz

Amaç: Gümüşde giderek artan antibiyotik direnci, bilim dünyasını yeni antimikrobial moleküllerin keşfi veya alternatif mücadele yöntemlerinin geliştirilmesi üzerine odaklanmıştır. Bu alternatif mücadele yöntemlerinden bir tanesi olan bakterilerde skorum sensing (QS) (coğurluq algılama) inhibisyonu olacağını düşündürmektedir. Günlük QS sistemi bakterilerle virülsans faktörlerinin sentezinde önemli bir rol oynar. Medikal öneme sahip birçok bitkinin umut verici antimikrobial ve anti-QS aktiviteleri sahipliği beklenmektedir. Bu bitkilerden birininin de Anatolideki yıldızlık olarak kullanılan çığ namo (Ferula elaeochytris Korovin) üzerinde araştırılmıştır. Bu nedenle, bu çalışmada F. elaeochytris Korovin’i kök özünün antimikrobial ve anti-QS aktivitelerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Öztünün antimikrobial aktivitesi, çeşitli mikroorganizmalarla karşı disk difüzyon yöntemi ile taramıştır. Öztünün etkili suş veya suşla karşı karşıya minimum inhibitory konsantrasyonu (MIC) ve minimum bakterisidal konsantrasyonu (MKB) değerleri svi mikrodüya yöntemi ile belirlenmiştir. Anti-QS aktivite, Pseudomonas aeruginosa ve Chromobacterium violaceum biyoyapıtoru suşlarında sızıntısı piyoksayin ve piyolazin pigmentleri üretimiinin inhibisyonu üzerine çalışılmıştır.

Bulgular: Öztünün sadexe Staphylococcus aureus suşuna karşı antimikrobial aktivitesi (ızon alanı = 9.3±0.6 mm) tespit edilmişdir. MIC ve MKB değerleri sırasıyla 4.4±1.9 mg/mL ve >105 mg/mL olarak saptanmıştır. Piyolazin üretim inhibisyonu belirlenmemiştir. Öztünün MIC altı konsantrasyon değerleri (1.64 µg/mL ve 3.28 µg/mL) bakteri üremesini baskılamadan piyoksayinin üretimini sırasıyla %60 ve %82 oranında inhibe etmiştir.

Öneri: Bu çalışmamızın sonuçları F. elaeochytris Korovin’in kök özünün antistatifik özellik ve anti-QS ajanların geliştirilmesi için iyi bir aday olarak olduğu göstermektedir.

Anahtar kelimeler: Antimikrobial, ekstrakt, Ferula elaeochytris Korovin, piyoksayin, skorum sensing
Introduction

The genus *Ferula* is found in the Apicae family and has about 170 species on earth. They grow naturally in the Mediterranean region east to central Asia. *Ferula* species have a long story of therapeutic use, and their biological properties demonstrated in various studies (Kose et al 2010, Ozek et al 2008, Khoury et al 2017). *Ferula* species are known to contain compounds such as sesquiterpenes, sesquiterpene coumarins, sesquiterpene lactones and sulfur-containing compounds (Akaberi et al 2015). *Ferula elaeochytris* Korovin (giant fennel) is widely used among the *Ferula* species and has commercial preparations. In Anatolia, the leaves and roots of this plant have been consumed as a tea for aphrodisiac purposes (Altundag and Ozturk 2011, Guzel et al 2015, Sargin 2015).

Nowadays, increasing antibiotic resistance has led scientists to discover new antibacterial molecules and to find alternative treatment strategies to combat bacteria. Besides, the discovery of new antimicrobial molecules has declined in recent years. Quorum sensing (QS) is a system by which bacteria communicate with each other to regulate most of their pathogenic behaviors and synthesis of virulence factors via small signaling molecules. Anti-QS agents may be considered alternatives to antibiotics because of their capacity to interfere with the synthesis of virulence factors in bacteria (Jiang et al 2019).

Various signal molecules related to QS have been described in bacteria. Autoinducing peptides (AIP) used by Gram-positive bacteria, N-acyl homoserine lactone (AHL) used by Gram-negative bacteria, and autoinducer-2 (AI-2) signaling molecules used by both Gram-negative and Gram-positive bacteria have the most common of these. The QS system can also interfere in many ways. These are prevention of signal molecule production; destruction of signal molecules upon release; and prevention of signal molecule uptake into the cell (Lade et al 2014).

We need to come up with solutions to combat antibiotic resistance, which has become a major problem today. To the best of our knowledge, no investigations on antimicrobial and anti-QS activities, have been reported on the root extract of *F. elaeochytris* Korovin. Thus, we aimed to investigate the antimicrobial and anti-QS activities of the root extract of *F. elaeochytris* Korovin in the current study.

Material and Methods

Plant material and preparation of the extract

The roots of *F. elaeochytris* Korovin were collected in Hatay, Turkey, in September 2019. The root extract of *F. elaeochytris* Korovin was prepared described previously (Lin et al 1999).

The collected root samples were properly cleaned and dried at room temperature conditions for 30 days. The dried samples were ground using a laboratory mill and passed through 1 mm mesh sieves to dark glass jars. Then, 100 g of the ground roots was extracted using 1 L from methanol (40% w/v) at 250 rev/min for 24 hours at room temperature. Then, the extract was percolated into a sterile container using Whatman No. 1 filter paper. The solvent was evaporated using a vacuum rotary evaporator at 60°C. The obtained extract was dissolved in sterile distilled water as 210 mg/mL final concentration and used to investigate antimicrobial and anti-QS activities.

Microorganism strains and growth conditions

The antibacterial activity of the extract was tested against two Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), two Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923), and one fungus (*Candida albicans* ATCC 10231) as representative pathogens.

The anti-QS activity of the extract was tested against *Chromobacterium violaceum* ATCC 12472 and *P. aeruginosa* PA01. *Chromobacterium violaceum* ATCC 12472 generates a bluish-purple pigment called violacein, using long-chain AHL molecules (C10-C16) as QS signal molecules and is widely used as a reference strain for anti-QS agents (Morohoshi et al 2008). *Pseudomonas aeruginosa* PA01 produces a bluish pigment called pyocyanin, using short-chain AHL molecules (C4-AHL) and is used as a bioreporter strain like *C. violaceum* (Kella et al 2015).

Pseudomonas aeruginosa PA01 is an opportunistic pathogen and widely used as a reference strain in laboratories. It was acquired from the Department of Medical Microbiology, Faculty of Medicine, Kocaeli University. The other strains were ATCC (American Type Culture Collection) reference strains and were get from the culture collection of the Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University.

Unless and otherwise stated, *P. aeruginosa* PA01 and *C. violaceum* and strains were cultured in peptone water and Luria Bertani (LB; NZYTech, Lisbon, Portugal) media. The others were cultured on Mueller hinton agar (MHA; Merck, Darmstadt, Germany) and potato dextrose agar (PDA; Pronadisa, Spain). All culture processes were conducted aerobically at 37°C.

Disc diffusion assay

The antimicrobial activity of the extract was screened according to EUCAST disc diffusion methodology (EUCAST 2020). Shortly, overnight cultures of each strain grown on agar me-
dia were adjusted to 0.5 McFarland turbidity standard (1.0 – 2.0 × 10^8 CFU/mL for bacteria and 1.0 – 2.0 × 10^6 CFU/mL for Candida albicans) with colony suspension method. Then, a sterile cotton swab was submerged to the suspension and seeded by swabbing in three directions on MHA for bacteria and PDA for C. albicans. Blank discs (6 mm diameter) were set on the surface of the seeded agar media and impregnated with 10 μL extract (210 mg/mL). Imipenem (10 μg/disc; Bioanalyse®, Turkey) and erythromycin (15 μg/disc, only for S. aureus; Bioanalyse®, Turkey) were used as positive controls according to EUCAST. No antifungal agent was used as a positive control. Distilled water was used as negative control.

The cultures were incubated at 18 hours for bacteria and 48 hours for C. albicans. After the incubation, zone of inhibition (ZOI) surrounding the discs were measured using a ruler (mm).

**Broth microdilution assay**

The minimum inhibitory concentration (MIC) was determined using the broth microdilution assay as described by Wiegand et al. (2008) with some modifications. Briefly, the assay was performed in 96-well microtiter plate containing 100 μL/well of Mueller hinton broth (MHB; Merck, Darmstadt, Germany). One hundred microliters from the stock concentration of the extract (210 mg/mL) was transferred to the first well and two-fold diluted to obtain concentrations ranging from 105 mg/mL to 0.20 mg/mL. Erythromycin (Molekula, UK) solution as a control antibiotic was used, and its tested concentration ranged from 64 μg/mL to 0.125 μg/mL. The cells suspension adjusted to 0.5 McFarland density as described above was added to each well in a volume of 5 μL except sterility control wells. The plate was sealed with a sterile lid and incubated for 18 hours. The lowest concentration of the extract which there was no visible growth of the microorganisms was determined as the MIC value.

The minimum bactericidal concentration (MBC) value was found after the MIC determination. For this, viable cell counts were performed on MHA with subculturing method from concentrations of MIC and above. The first concentration counted as below 100 colonies on the agar medium was interpreted as the MBC value.

**Violacein inhibition assay**

Violacein inhibitory activity of the extract was investigated against C. violaceum ATCC 12472 using the soft agar method described as McClean et al. (1997) with some modifications. Briefly, five milliliters of molten soft LB agar (0.5% w/v) was transferred with 50 μL of C. violaceum overnight culture in LB broth. The soft agar-culture mixture was gently vortexed and directly poured over the surface of prewarmed LB agar plates. When the soft agar had solidified, wells of 6 mm in diameter were punched in the agar with a sterile cork borer and 50 μL of the extract (210 mg/mL) was pipetted into the agar wells. Vanillin (500 μg/mL; Merck, Darmstadt, Germany) and distilled water were used as positive and negative controls, respectively. The culture was incubated for 18 hours, then analysed for violacein production. Quorum sensing inhibition was determined by a white, opaque, but viable halo surrounding the wells.

**Pyocyanin inhibition assay**

Pyocyanin inhibitory activity of the extract was investigated against P. aeruginosa PA01 using pyocyanin extraction method described as Essar et al. (1990) with slight modifications. Prior to this experiment, the MIC value of the extract against P. aeruginosa PA01 was determined. The sub-MIC concentrations of the extract were used as final concentrations in this assay to avoid any antibacterial effect.

Overnight culture of P. aeruginosa PA01 was adjusted to OD600 _nm_ 0.1 and transferred to sterile two culture tubes, in volumes of 4922 μL and 4961 μL. The extract was added to one of the tubes in a volume of 78 μL (for the final concentration of 3.28 mg/mL) and to the other in the volume of 39 μL (for the final concentration of 1.64 mg/mL). Thus, the final volume was completed to 5 mL. Besides, one tube was also used as a negative control (sterile distilled water in the same volume). After the tubes were wrapped in aluminum foil, they were incubated for 24 hours at 150 rev/min shaker. Then, 1.5 mL culture from each tube was centrifuged at 10 000 rev/min for 5 min to obtain cell-free supernatant. Chloroform (0.9 mL; Merck, Darmstadt, Germany) was added to the supernatant and mixed vigorously. The chloroform layer was then taken to a new sterile microcentrifuge tube and re-extracted with 0.3 mL of 0.2 N hydrochloric acid (HCl). After centrifuge process, 0.2 mL volume of the top layer (HCl) was transferred to 96-well microplate. The absorbance was determined at 520 nm against 0.2 N HCl using a UV-visible spectrophotometer (BioTek Epoch, Vermont, USA).

Although the extract was studied at sub-MIC concentrations, whether the cell growth was suppressed was confirmed by viable cell count from cultures after 24 hours. The cultures were serially diluted to factors of 10^{-5}–10^{-7} in serum physiological solution, and 50 μL from 10^{-7} dilutions were spread on MHA plates. The plates were incubated for 24 hours, and colony counts were compared with the control.

**Statistical analysis**

All the experiments were carried out at least three times and results were presented as mean ± standard deviation (SD) values. The data were analysed for normality using the model Wilks-Shapiro test (Shapiro and Wilks, 1965). Paramet-
ric data was analysed with independent samples t-test using IBM-SPSS statistics version 23.0 (IBM Inc., Armonk, NY, USA), and p<0.05 was recognized as statistically significant.

Results

The antimicrobial activity of the root extract of *F. elaeochytris* Korovin was tested against various microorganisms by the disc diffusion assay and results are shown in Table 1. The extract possessed the antimicrobial activity against *S. aureus* (ZOI = 9.3±0.6 mm; Figure 1). The extract had no antimicrobial activity against other tested microorganisms. After the antimicrobial activity was determined by the disc diffusion method, the MIC value was detected by the broth microdilution assay. The MIC and MBC values of the extract against *S. aureus* were found to be 4.4±1.9 mg/mL and >105 mg/mL, respectively (Table 1).

Anti-QS activity of the extract was investigated against *P. aeruginosa* PAO1 and *C. violaceum* ATCC 12472 bioreporter strains, which produced pyocyanin and violacein pigments interacting with the QS mechanism, respectively. Pyocyanin production was reduced by 60% and 82% at concentrations of 1.64 mg/mL and 3.28 mg/mL compared with the control (p<0.05) without interfering with bacterial growth (Figure 2). No inhibition of violacein pigmentation was observed.

Discussion

The emergence of antibiotic-resistant pathogens makes the cure and control of infectious diseases difficult (Nellums et al 2018). The commercial antibiotics may also be insufficient to combat these resistant pathogens (Towse et al 2017). For this reason, it is very important to find compounds that will support antibiotics or strengthen their effects. We believe that traditional medicinal plants have very significant potential in this regard. In our study, the antimicrobial activity of the root extract of *F. elaeochytris* Korovin was screened against several reference strains representing medically important pathogens by the disc diffusion method.

The antimicrobial activity of the extract was observed only

| Table 1. Antimicrobial activity of the root extract of *F. elaeochytris.* |

| Microorganism                  | ZOI (mm)  | MIC (mg/mL) | MBC (mg/mL) |
|-------------------------------|-----------|-------------|-------------|
| *S. aureus* ATCC 25923        | 9.3±0.6   | 4.4±1.9     | >105        |
| *E. faecalis* ATCC 29212      | 0         | -           | -           |
| *E. coli* ATCC 25922          | 0         | -           | -           |
| *P. aeruginosa* ATCC 27853    | 0         | -           | -           |
| *C. albicans* ATCC 10231      | 0         | -           | -           |

* Data are presented as the mean ± SD. -, not tested.
against *S. aureus*. However, the antimicrobial activity of the extract against *S. aureus* was rather weak compared to erythromycin (ZOI = 28.3 ± 0.6 mm) used as a positive control (Figure 1). The MIC value of the extract against *S. aureus* was found to be 4.4 ± 1.9 mg/mL while the MBC value was >105 mg/mL. This figure suggests that the root extract of *F. elaeochytris* has a bacteriostatic effect. Meanwhile, the MIC value of erythromycin against *S. aureus* was 0.25 μg/mL.

To the best of our knowledge, there is no study in the literature on the antimicrobial activity of the root extract of *F. elaeochytris* Korovin. Therefore, our study will be the first to evaluate the antimicrobial activity of the root extract of *F. elaeochytris*. In only one study conducted by Khoury et al. (2017), the antimicrobial activity of the essential oils of *F. elaeochytris* fruits was tested against *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *C. albicans* ATCC 10231, Cryptococcus neoformans SNB-CN1, Candida parapsilosis ATCC 22019, *Aspergillus fumigatus* SNB-AFI, Trichophyton tonsurans SNB-1T1, Trichophyton soudanense SNB-TS1, Trichophyton mentagrophytes SNB-TM1, Trichophyton rubrum SNB-TR1, and Trichophyton violaceum SNB-TV1. They detected antimicrobial activity against all tested microorganisms except *E. coli* ATCC 25922, *C. parapsilosis* ATCC 22019, and *A. fumigatus* SNB-AFI.

Ghasemi et al. (2005) found that the essential oil from the fruits of *Ferula gummosa* possessed high antibacterial and antifungal activities against Gram-positive (*Staphylococcus epidermidis* PTCC 1114, *S. aureus* PTCC 1112, and *Bacillus subtilis* PTCC 1023) and Gram-negative (*E. coli* PTCC 1338, *Salmonella Typhi* PTCC 1609, and *Pseudomonas aeruginosa* PTCC 1074) bacteria and fungi. *C. albicans* ATCC 14053 (respectively, in qualitative screening on violacein inhibition) that the extract could not inhibit the production of violacein. Pyocyanin production was inhibited by 60% and 82% at two sub-MIC concentrations compared with the control (p<0.05) without interfering with bacterial growth. Testing the extract at sub-MIC concentrations and the absence of a decrease in viable cells indicated that the suppression of pyocyanin production in *P. aeruginosa* was due to the QS inhibitory activity of the extract.

In particular, *Ferula asafoetida* extracts including oleo-gum-resin and essential oil were researched for anti-quorum sensing activity in before reports. These extracts of *F. asafoetida* were effective in different levels on tested bioreporter strains. Sepahi et al. (2015) and Kambhala et al. (2016) reported that the essential oil of *F. asafoetida* inhibited violacein and pyocyanin production in *C. violaceum* and *P. aeruginosa*, respectively. Also, Sepahi et al. (2015) stated that elastase, pyoverdine, and biofilm formation was decreased on *P. aeruginosa* by the essential oil of *F. asafoetida*. Jomehpour et al. (2016) investigated the effect of *F. asafoetida’s* oleo-gum resin on the expression of pathogenesis-related *tst* and *hld* genes regulated by QS in MRSA and MSSA (mecillin-sensitive *S. aureus*) strains. They determined that the decrease of the *hld* gene expression on MRSA.

Violacein production in *C. violaceum* ATCC 12472 is regulated by long-chain (C10-C16) AHL molecules while pyocyanin production in *P. aeruginosa* PAO1 is regulated by short-chain AHL (C4-AHL) molecules. In our study, the fact that the extract was not able to inhibit the production of violacein pigment and inhibited the production of pyocyanin pigment suggested that compounds of the extract affected short-chain AHL molecules. Apart from pyocyanin, *P. aeruginosa* strains also regulate the expression of genes responsible for elastase, siderophore, and rhamnolipid synthesis with C4-AHL signal molecules. Therefore, if it is assumed that these virulence factors will also be inhibited, the root extract of *F. elaeochytris* Korovin may have significant potential in controlling...
the pathogenesis of \textit{P. aeruginosa}. However, uncontrolled and overuse of the consumption of this plant may cause liver toxicity. For this reason, it should be kept in mind that this plant may cause liver toxicity when excessively used in various forms such as tea.

Limitations of the study: It is necessary to identify the compounds responsible for the antibacterial activity by performing a phytochemical analysis of the extract. We also recommend combining bioactive compounds with commercial antibiotics and testing against strains that exhibit a specific resistance phenotype such as MRSA (methicillin-resistant \textit{Staphylococcus aureus}) in future work.

**Conclusion**

To the best of our knowledge, this research is the first study to investigate the antimicrobial and anti-QS activities of the root extract of \textit{F. elaeochytris} Korovin. The results showed that the root extract of \textit{F. elaeochytris} Korovin has the potential to develop an antistaphylococcal agent. Furthermore, the root extract of \textit{F. elaeochytris} Korovin showed that it is a good candidate for the development of anti-QS agent. All the data obtained in this study are preliminary results. The determination of the chemical contents of the extract and the effectiveness of bioactive molecules in combination with reference antibiotics should be evaluated.

**Acknowledgement**

The authors would like to thank Prof. Dr. Ali Osman Kılıç and Prof. Dr. Fetiye Kolaylı for test microorganisms and Kastamonu University Central Research Laboratory for the opportunities and support they provided.

**Conflict of Interest**

The authors did not report any conflict of interest or financial support.

**Funding**

During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and/or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study, or no moral support.

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Ethical Approval

The data and information presented in this article were obtained within the framework of academic and ethical rules. Ethical declaration that the evaluation results were in accordance with scientific and ethic rules, was received from the authors.

CITE THIS ARTICLE: Tufekci EF, Alkateeb A, Akar S, Corum O, et al., 2020. Antimicrobial and anti-quorum sensing activities of giant fennel (Ferula elaeochytris Korovin) from the Hatay Region. Eurasian J Vet Sci, 36, 3, 214-220

CITE THIS ARTICLE: Tüfekci EF, Alkateeb A, Akar S, Çorum O, et al., 2020. Anti-microbial and anti-quorum sensing activities of giant fennel (Ferula elaeochytris Korovin) from the Hatay Region. Eurasian J Vet Sci, 36, 3, 214-220