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Original scientific paper

HPTLC-direct bioautography-guided isolation of isogeranic acid as the main antibacterial constituent of *Artemisia santonicum* essential oil

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Abstract: This study was performed to determine the main antibacterial compounds of the essential oil (EO) of saltmarsh plant *Artemisia santonicum* (Asteraceae). The combination of HPTLC and direct bioautography was used for the activity guided isolation of isogeranic acid as the main antibacterial constituent with remarkable antimicrobial activity, although it was the minor component of the EO, present only in 0.2 %, as calculated from GC/FID. Its structure was determined by 1D- and 2D-NMR and GC–MS techniques. Antibacterial activity of isogeranic acid against all tested bacteria was significantly higher than EO and even than both controls streptomycin and ampicillin. In further investigation of antibiofilm and antiquorum sensing activity EO exhibited the best inhibition of the biofilm formation at 1/8 minimal inhibitory concentration (*MIC*) and isogeranic acid at 1/2 *MIC*. Both EO and isogeranic acid possessed pyocyanin inhibitory activity showing the reduction of pigment at 60.6 and 62.8 %, respectively, at 1/2 *MIC* concentrations.

Keywords: antimicrobial activity, direct bioautography, antibiofilm, antiquorum potential.

INTRODUCTION

Genus *Artemisia* belongs to family Asteraceae. Plants of this genus have long ethnopharmacological tradition and they were used in folk medicines of

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various countries. *A. absinthium* was used as antiparasitic plant and against gastric problems, especially prepared in alcoholic beverages (absinthe products); *A. abyssinica* as an anthelmintic, antirheumatic and antibacterial agent; *A. afra* against coughs, bronchitis, asthma, diabetes; *A. annua* as famous anti-malaric; *A. biennis* as antiseptic; *A. argui* as herbal medicine for liver, spleen and kidney; *A. campestris* as anti-inflammatory, anti-rheumatic and antibacterial agent.[1]

The most abundant secondary metabolites isolated from *Artemisia* species are coumarins, flavones, flavonols, phenolic acids and sesquiterpenes.[2] Famous sesquiterpene lactone, artemisinin is by far the best known compound from *Artemisia* species. Chinese scientist Tu Youyou isolated artemisinin from *A. annua*, found its great antimalarial activity and got Nobel prize in 2015. Nowadays it is considered promising as anticancer agent.[3]

There are several reports about antioxidant, antibacterial, antifungal, antimalarial, and antidiabetic activities of different *Artemisia* species. In the previous investigations of *A. santonicum* essential oil (EO) exhibited antibacterial effect against 24 of 25 bacterial strains, but constituents responsible for this activity have not been discovered yet.[4] High performance thin layer chromatography (HPTLC) in combination with bioautography is well known technique for the determination of active components, especially antimicrobial components of complex mixtures (extracts and EOs).[5]

Quorum sensing (QS) involves biofilm formation, bioluminescence, conjugation and virulence, antibiotic production, competence, conjugation, swarming, motility and sporulation. The biofilm, as well as inter-cellular microbial communication challenge many problems and is a growing threat to global public health throughout the world. The biofilm causes recalcitrant results in blood stream, urinary tract infection sand contamination leading to food spoilage.[6,7] One of the possible approaches to treat bacteria-caused diseases is investigation of intra-cellular microbial communication and mechanisms involved in microbial pathogenesis.[8,9] Many microorganisms are using QS mechanisms in regulation of virulence. The interruption of this bacterial communication gives a possibility of controlling bacterial infections by natural products. The most commonly studied objects are: *Pseudomonas aeruginosa* (PAO1), *Chromobacterium violaceum* (CV026), *Aliivibrio fischeri*, *Escherichia coli*, *Salmonella enterica*, *Acinetobacter* sp., *Aeromonas* sp., *Yersinia enterocollitica*, etc.[10] *Pseudomonas aeruginosa* is one of the resistant bacteria to antibiotic treatment. It may become multi-drug resistant using quorum sensing to coordinate the formation of biofilms, swarming motility, exopolysaccaride production, virulence, and cell aggregation.[11]

The aim of the present investigation was to determine antimicrobial, antibiofilm, and antiquorum sensing activity of the *A. santonicum* EO, find its active components, and isolate them.

EXPERIMENTAL

General methods

Anisaldehyde–sulphuric acid, used as spray reagent, was freshly made according to the literature procedure.[12] Aqueous 3 % solution of *p*-iodonitrotetrazolium violet (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; Sigma) was used as spray reagent for bioautography. Solvents used for the dry flash chromatography (toluene, ethyl acetate and *n*-hexane) were freshly distilled. HPTLC silica gel 60 F_{254} aluminum foil plates were obtained from Merck. HPTLC was conducted with a CAMAG HPTLC system (Muttenz, Switzerland) composed of Linomat 5, an ADC 2 automatic developing chamber, and a TLC visualiser. GC/ /FID and GC–MS analyses were carried out with an Agilent 7890A apparatus equipped with a 5975C MSD, FID, and a HP-5MSI fused-silica capillary column (30 m×0.25 mm, film thickness 0.25 μm). The oven temperature was programmed from 60 to 315 °C at 3 °C/min then held isothermal for 5 min. Injector: 250 °C; FID detector: 300 °C; carrier gas, He (1.0 mL/min at 210 °C); injection volume, 1 μl; split ratio, 3:1. EI-MS (70 eV), *m*/*z* range 40–550.

Compound identification. Identification of all compounds in analysis was performed by comparison of their linear retention indices (relative to C8–C36 *n*-alkanes on the HP-5MSI column) and MS spectra with those of authentic standards from NIST11 database. All NMR spectra (^{1}H, ^{13}C-DEPT, COSY, TOCSY, NOESY, HSQC, HMBC) were recorded on a Bruker Avance III 500 spectrometer at 500.26 MHz for ^{1}H and 125.80 MHz for ^{13}C, with chloroform-*d* as solvent and TMS as reference. Temperature was 298 K.

Analytical and spectral data are given in Supplementary material to this paper.

Plant material and isolation of EO

The plant material was collected in August 2017 from a saltmarsh in northern Serbia, near the city of Novi Bečej. The plant material was authenticated by Prof. Zora Dajić Stevanović and the voucher specimen BEOU17468 was deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Belgrade, Serbia. The EO was isolated by hydrodistillation of the aerial parts of dried *A. santonicum* using a Clevenger-type apparatus for 3h. Hydrodistillation of *A. santonicum* was performed six times (620 g of plant material in total) and EOs from all distillations were joined together. The quantity of 1331 mg of the essential oil was obtained in total, representing 0.20 mass %, based on dry plant material.

HPTLC-direct bioautography

HPTLC was performed on 10 cm×10 cm aluminum foil backed silica gel 60 F_{254} plates. EO was dissolved in methanol to give a 100 μL/mL solution. The position of the starting line was 1.50 cm from the left side and 0.80 cm from the bottom. Distance between tracks was 1.54 cm. On the plates 10 cm×10 cm, the oil sample aliquots of 2, 4 and 8 μL were applied. After the sample application, the plates were developed in a saturated chamber (ADC2 CAMAG) using toluene/ethyl acetate (92:8 volume ratio) solvent system. Saturation time was 10 min. Time of development was 20 min and separation distance 8 cm. After separation, the mobile phase was removed from the plates by drying under a cold air stream for 4 min. Four plates were used for bioautography, and one plate was sprayed with anisaldehyde reagent to mark the bands. The developed plate was dipped into ethanolic anisaldehyde reagent by CAMAG chromatogram immersion device III and then heated at 100 ºC for 5 min, until the bands became visible. Bioautography was performed according to Horvath *et al*.[13] The dried plates were sprayed with freshly prepared bacterial suspension (prepared at the day of experiment, 1.0×10^6 CFU/mL) in nutrient broth (Tryptic Soy Broth; Biolife Italiana S.r.l., Milano, Italia). The plates were incubated in a water-vapor chamber for 24 h at 37 °C and then sprayed

with aqueous sol. 3 % of *p*-iodonitrotetrazolium violet, stored for another 3 h and sprayed with 70 % EtOH to stop bacterial growth. Microbial growth inhibition appeared as clear zones against a pink background. The R_F values of the spots showing inhibition were determined.[13] The following Gram-negative bacteria were used: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and the following Gram-positive bacteria: *Micrococcus lutens* (ATCC 10240) and *Listeria monocytogenes* (NCTC 7973). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

Isolation of isogeranic acid

Dry-column flash chromatography fractionation of the EO was performed in a sintered glass column (300 mm×32 mm) packed with silica gel (100 g Merck, <0.08 mm) using water pump vacuum. The column was equilibrated with 750 mL of the mobile phase toluene/ethyl acetate (70:30 volume ratio) solvent system. The EO (m = 812 mg) was diluted with the mobile phase (1:1) before applying to the column. The elution was isocratic and 25 fractions of around 10 mL were collected. After the HPTLC-direct bioautography analysis of EO, the position of the active component (isogeranic acid), *i.e*., its R_F value was determined. Ordinary TLC chromatography of all of the fractions from dry flash chromatography revealed the fractions 7 and 8 which contained a spot of the same R_F value which exhibited antibacterial activity. These fractions, were joined, evaporated on the rotary vacuum evaporator (Ika-werke, RV10, Staufen, Germany) without heating, and further separated on the second dry-column flash chromatography packed with the silica gel (SiO_2, <0.08 mm, Merck), with the same dimensions as the first one. The elution was gradient, starting with the toluene–ethyl acetate (98:2 volume ratio) and ending with the toluene–ethyl acetate (95:5 volume ratio). Forty fractions were collected and the volume of each fraction was 10 mL. The last two fractions, which contained an "active spot", were grouped and evaporated on the rotary vacuum evaporator without heating, and the final separation was performed on the third dry-column flash chromatography in a sintered glass column (85 mm× 20 mm) packed with silica gel (SiO_2, <0.08 mm, Merck). The elution was isocratic with the mixture of solvents hexane/ethyl acetate (85:15 volume ratio). Volume of each fraction was 3 ml, and nineteen fractions where collected. The isogeranic acid (1.15 mg) was obtained from fractions 13–19 after evaporation on the rotary vacuum evaporator without heating.

Antibacterial activity

For the evaluation of antibacterial activity of the essential oil Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (human isolate), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 13311), and Gram-positive bacteria*: Listeria monocytogenes* (NCTC 7973), *Bacillus cereus* (clinical isolate), *Micrococcus lutens* (ATCC 10240) and *Staphylococcus aureus* (ATCC 11632) were used. The following bacteria: *E.coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *L. monocytogenes* (NCTC 7973) and *M. lutens* (ATCC 10240) were tested for the antibacterial activity investigation of isogeranic acid. The antibacterial assay was carried out by a microdilution method[14,15] using procedure described by Rasheda *et al.*[16] Ranges of concentrations of the essential oil and isogeranic acid were 1.99–24.88 mg/mL and 0.05–0.075 mg/mL, respectively. Streptomycin (Sigma–Aldrich S6501) and ampicillin (Sigma–Aldrich A9393) were used as positive controls (1 mg/mL in sterile physiological saline).

Biofilm formation

P. aeruginosa PA01 used in this study is from the collection of the Mycoteca, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. Bacteria were routinely grown in Luria-Bertani (LB) medium (1 % NaCl, 1 % tryptone, 0.5 % yeast extract) with shaking (220 rpm) and cultured at 37 °C. The effect of different subinhibitory concentrations of the essential oil (ranging from 1/8 to 1/2 of *MIC*; *MIC* was 4.0 mg/mL) and isogeranic acid (ranging from 1/8 to 1/2 of *MIC*; *MIC* was 0.075 mg/mL) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates as described by Mileski *et al.*[17] Briefly, 100 μL of overnight culture of *P. aeruginosa* (inoculum size was 10^8 CFU/mL) was added to each well of the plates in the presence of 100 μL sub-inhibitory concentrations (sub-*MIC*) of extracts (1/2, 1/4 and 1/8 *MIC*) or 100 μL medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 10 min with 0.1 % crystal violet in order to determine the biofilm mass. After drying, 200 μL of 95 % ethanol was added to solubilize the dye that had stained the biofilm cells. The excess stain was washed off with distilled H_2O. After 10 min, the content of the wells was homogenized and the absorbance at λ = 625 nm read on a Sunrise™-Tecan ELISA reader (Mannedorf, Switzerland). The experiment was done in triplicate and repeated two times and values were presented as a mean value ± *SE*.

Inhibition of twitching and flagella motility of P. aeruginosa

Inhibition of twitching and flagella motility of *P. aeruginosa* was determined by the procedure of Mileski *et al.*[17] The sub-*MIC* concentration of essential oil and isogeranic acid (2.00 and 0.0375 mg/mL) was mixed into 10 mL of molten MH (Mueller–Hinton) agar medium and poured immediately over the surface of a solidified LB agar plate as an overlay. The plate was point inoculated with an overnight culture of PAO1 once the overlaid agar had solidified and was incubated at 37 °C for 3 days. The extent of swimming was determined by measuring the area of the colony.[18] The experiment was done in triplicate and repeated two times. The colony diameters were measured three times in different direction and values were presented as a mean values ± *SE*.

Inhibition of synthesis of P. aeruginosa *PA01 pyocyanin*

Overnight culture of *P. aeruginosa* PA01 was diluted to OD 600 nm 0.2. Inhibition of synthesis of *P. aeruginosa* PA01 pyocyanin was determined by the procedure of Mileski *et al.*[17] The oil and isogeranic acid were added in sub-*MIC* concentrations (2.00 and 0.0375 mg/mL) to *P. aeruginosa* and incubated at 37 °C for 24 h. Absorbance of the extracted organic layer was measured at 520 nm using a Shimadzu UV1601 spectrophotometer (Kyoto, Japan).[18] The experiment was done in triplicate and repeated two times. The values for optical density were presented as a mean value ± *SE*.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The chemical composition of the isolated EO was established by GC–MS/FID. The relative amounts of the components are shown in Table S-I (Supplementary material). The number of 75 compounds were characterized, corresponding to 90.1 % of the total. Oxygenated monoterpenes were identified as the major class of compounds (67.5 %). Among the oxygenated monoterpenes the most abundant were 1,8-cineole (18.8 %), chrysanthenone (13.3 %), *cis*-thujone

(8.4 %), *trans*-sabinyl acetate (3.3 %), and camphor (3.3 %). Monoterpene hydrocarbons were present in amount of 8.2 %, sesquiterpene hydrocarbons 5.6 %, oxygenated sesquiterpenes 3.3 %, normonoterpenes 2.6 %, homomonoterpenes 1.0 %, and aromatics 1.9 %. Camphor and *cis*-thujone were previously reported as major constituents of *A. santonicum* EO.[4,19]

HPTLC-direct bioautography

HPTLC-direct bioautography was performed in order to find the antibacterial constituents of the EO. The mobile phase toluene–ethyl acetate (92:8 volume ratio) was chosen, and HPTLC separation on silica gel plates was performed. HPTLC-direct bioautography on Gram-negative *E. coli* and *P. aeruginosa*, and Gram-positive *L. monocytogenes* and *M. lutens* exhibited a white zone on violet background with the R_F value of 0.08 using aqueous sol. 3 % of *p*-iodonitrotetrazolium violet for visualization (Fig. 1). Another HPTLC plate was visualized using anisaldehyde reagent, where the active component appeared as a pink colored band. The R_F value and the color of the band was taken into account for further bioactivity-guided isolation.

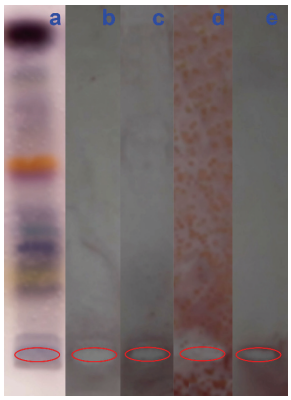

Fig. 1. HPTLC of the essential oil treated with: a – anisaldehyde and HPTLC-direct bioautography: b – *E. coli*, c – *P. aeruginosa*, d – *L. monocytogenes* and e – *M. lutens*.

Isolation and identification of isogeranic acid

The *A. santonicum* EO was fractionated by three consecutive dry-column silica gel flash chromatographies, using toluene/ethyl acetate and hexane/ethyl acetate solvent systems as mobile phases. Isogeranic acid as an active component (R_F 0.08), was isolated and its structure was elucidated using ^{1}H, ^{13}C-DEPT and 2D-NMR (Figs, S-2–S-7 and Table S-II, Supplementary material) and GC–MS. NOESY correlations revealed *Z* configuration (Fig. 2). GC/FID chromatograms of the EO and pure isogeranic acid are given in Fig. 3. The content of isogeranic acid in the EO was found to be 0.2 %, according to GC/FID. Isogeranic acid is a rarely investigated compound, only found in few EOs.[20,21]

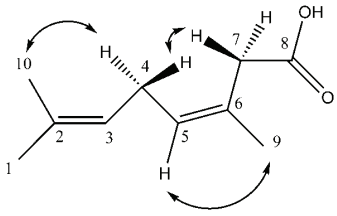

Fig. 2. The structure of isogeranic acid with the NOESY correlations.

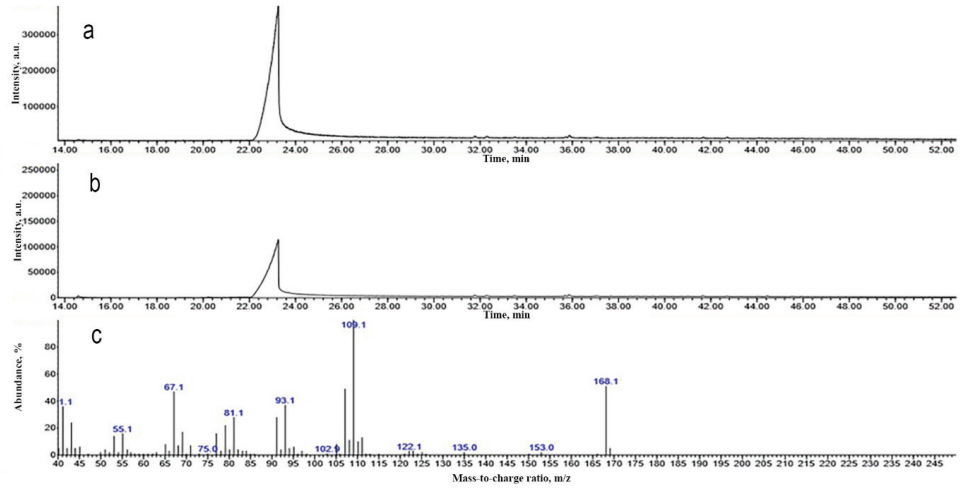

Fig. 3. a – GC/MS data; b – GC/FID; c – EI/MS spectrum of isogeranic acid.

Antibacterial activity of the essential oil and isogeranic acid

Antibacterial activity of the EO, isogeranic acid and known antibiotics used as controls are presented in Table S-III of the Supplementary material. EO showed moderate antibacterial activity in the range of 2–25 mg/mL for *MIC*, with the most pronounced effect on *B. cereus* and *P. aeruginosa*, while pure isogeranic acid exhibited strong antibacterial activity against all tested bacteria, in the range 0.05–0.075 mg/mL, higher than streptomycin and ampicillin (Table S-III).

Anti-QS activity of the essential oil and isogeranic acid

On swimming plates, the motile strain PAO1 was used as the 100 % standard (control) for motility, while the Petri dishes with the same strain plus oil and isogeranic acid were compared with the control.[22]

Inhibition of the biofilm formation. The *MIC* values of the EO and isogeranic acid against *P. aeruginosa* were determined by the microdilution method (4.00 and 0.075 mg/mL, respectively). The effect on biofilm formation of *P. aeruginosa* was tested at lower values than the *MIC*, *i.e*., 1/2 *MIC*, 1/4 *MIC* and 1/8 *MIC*. A concentration of 1/8 *MIC* of the EO allowed 42.54 % of inhibition of biofilm formation, while 1/4 *MIC* allowed activity of 1.52 % inhibition of biofilm

formation. Concentration of 1/2 *MIC* did not influence biofilm formation. A concentration of isogeranic acid of 1/2 *MIC* exhibited the best activity with 46.77 % of inhibition of biofilm formation. According to the obtained data a concluding remark could be highlighted: EO and isogeranic acid exhibited non-dose dependent activity on biofilm formation at subinhibitory concentrations, but certain activity undoubtedly exists. Both controls ampicillin and streptomyin exhibited the best activity for 1/8 *MIC* (Table S-IV, Supplementary material). Finally, isogeranic acid exhibited higher potential for inhibition of the biofilm formation than EO and lower than both positive controls.

Twitching and flagella motility. Tested oil and isogeranic acid completely reduced the twitching motility of *P. aeruginosa*. The normal colonies of *P. aeruginosa*, were flat with a rough appearance displaying irregular colony edges (Fig. 4e) and a hazy zone surrounding the colony. *P. aeruginosa* solution with oil and isogeranic acid was incapable of producing such a twitching zone and had almost round, smooth, regular colony edges, the flagella were completely reduced both in size and in numbers (Fig. 4a and b). Ampicillin and streptomycin reduced the flagellae completely (Fig. 4c and d). The EO, isogeranic acid and antibiotics also influence the white color of colony, in contrast with green control.

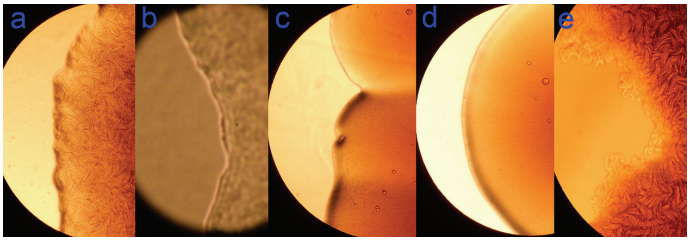

Fig. 4. Light microscopy of colony edges of *P. aeruginosa* in twitching motility plates, grown in the presence or absence of *A. santonicum* essential oil and isogeranic acid; magnification: 100×.

The EO and isogeranic acid provoked lower colony diameter (11.00 and 10.67 mm, respectively) than control of *P. aeruginosa* untreated (29.34 mm). Ampicillin and streptomycin reduced colony diameter in higher content (8.33 and 6.00 mm) (Table S-V, Supplementary material).

The bacterial colonies grown in the presence of 1/2 *MIC* of oil and isogeranic acid; *P. aeruginosa* colony in the presence of ampicillin (1/2 *MIC*) had a reduced protrusion; *P. aeruginosa* colony in the presence of streptomycin with a reduced protrusion; *P. aeruginosa* produced a flat, widely spread, irregularly shaped colony in the absence of EO and isogeranic acid.

Pyocyanin production. Tested EO and isogeranic acid showed pyocyanin inhibitory activity. The oil showed a reduction of pigment at 60.6 %, isogeranic acid 62.8 % while ampicillin and streptomycin decreased pyocyanin in higher

amount (32.5 and 23.8 %) in comparasion to untreated control (79.3 %, Fig. 5, Table S-VI, Supplementary material). These values for the reduction of pigment refer to 1/2 *MIC* concentrations (mg/mL).

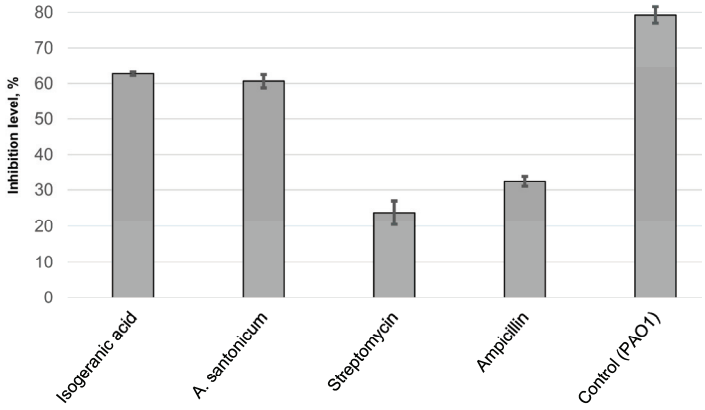

Fig. 5. Reduction of the pyocyanin production of *P. aeruginosa* PAO1 by *A. santonicum* essential oil, isogeranic acid, streptomycin and ampicillin tested at 1/2 *MIC* (mg/mL).

CONCLUSION

Activity guided isolation from the EO of saltmarsh plant *Artemisia santonicum* (Asteraceae) revealed isogeranic acid as a minor, but highly active ingredient with remarkable antimicrobial, antibiofilm, and antiquorum activities. Its antibacterial activity against all tested bacteria was significantly higher than EO and even than both controls streptomycin and ampicillin. Both EO and isogeranic acid possess pyocyanin inhibitory activity.

SUPPLEMENTARY MATERIAL

Additional data are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

HPTLC ДИРЕКТНО-БИОАУТОГРАФСКИ ВОЂЕНО ИЗОЛОВАЊЕ ИЗОГЕРАНИЛНЕ КИСЕЛИНЕ КАО ГЛАВНЕ АНТИБАКТЕРИЈСКЕ КОМПОНЕНТЕ ЕТАРСКОГ УЉА *Artemisia santonicum*

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Ово истраживање је спроведено у циљу одређивања главних антибактеријских компоненти етарског уља слатинске биљне врсте *Artemisia santonicum* (Asteraceae). Комбинација HPTLC методе и методе директне биоаутографије је коришћена за активношћу вођено изоловање изогеранилне киселине као главне компоненте са значајном антимикробном, антибиофилм и антикворум активношћу иако је била присутна у етарском уљу са само 0,2 %, израчунато из GC/FID. Њена структура је одређена 1D и 2D NMR и GC–MS техникама. Антибактеријска активност изогеранилне киселине на све тестиране бактерије је била значајно боља од етарског уља, и од обе контроле, стрептомицина и ампицилина. Етарско уље је показало најбољу инхибицију формирања биофилма у концентрацији 1/8 минималне инфибиторске концнетрације (*MIC* вредности), а изогеранилна киселина у концентрацији 1/2 *MIC* вредности. И етарско уље и изогеранилна киселина су имале пиоцијанин инхибиторну активност показавши смањење пигмента на 60,6 и 62,8 %, у концентрацији 1/2 *MIC* вредности, редом.

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