Dear Dr Dejan Opsenica:

Thank you for review of the manuscript entitled: "HPTLC Bioautography-Guided Isolation of Isogeranic Acid as the Main Antibacterial Constituent of Artemisia

santonicum Essential Oil"

We have done the corrections as suggested by Reviewers.

Here are the responds to specific questions:

Reviewer A:

Authors should change the title to “Bioautography-Guided Isolation of

Isogeranic Acid as the Main Antibacterial Constituent of Artemisia

santonicum Essential Oil.

**-The title has been changed as it was suggested by reviewer 2.**

Add RF instead of Rf

**-„Rf“ has been replaced with „RF“.**

Line 43. HPTLC is well known technique for the determination of active

components,… better write bioautography is well known technique for the

identification of bioactive components.

* **The combination of HPTLC with bioautography is well known technique for the**

**identification of bioactive components. Appropriate correction has been intorduced.**

In the introduction, the authors did not mention specialized metabolites

already identified from Artemisia species responsible for biological

activity based on literature as well as ethnopharmacological use of

Artemisia species.

**-Two paragraphs have been added in the introduction.**

Line 43. Authors should introduce an abbreviation of High-performance

thin-layer chromatography (HPTLC).

**-An abbreviation has been introduced.**

Line 46. The aim of the study is not convincingly written, authors should

correct it.

**-The aim of the study has been rewritten.**

RF value it too low, Authors did not optimizes chromatography system well,

especially for bioautography. Also, response is hardly visible.

**-RF value for isogeranic acid is too low, but the conditions for higher RF value would cause missing of some other potentialy active components.**

Line 83. Authors should mention the saturation time.

**-Saturation time was 10 minutes and it was added in the text.**

Line 170. Change v/v to V/V

**-Appropriate change has been made.**

Line 173. p-Jodonitrotetrazolium , p- Jodonitrotetrazolium, p- should be

Italic

**-Appropriate correction has been made.**

Fig.1. Based on Fig.1 HPTLC system is not appropriate for isogeranic acid,

and RF value is of target compound is 0.08, close to start band. Why authors

did not optimized bioautography?

-**RF value for isogeranic acid is too low, but the conditions for higher RF value would cause missing of some other potentialy active components.**

biological response of bioactive band is not clear. Based on Fig. 1, living cells are in down part of the plate…there is not clear different between a background of plate and bioactive bands..just in case of d-L. monocytogenes. Assay in this form was not reliable.

- **There is a difference between a background of plate and bioactive band in case of d-L. monocytogenes, but the contrast is realy lower than those in other 3 experiments.**

Why authors did not use positive control (ampicillin and streptomyin) for

bioautography?

**-There is no need to take any antibiotics for this tipe of test, because Bioautographic assay is usually used to screen for antimicrobial activity by separating components onto the surface of chromatographic plates and overlaying the TLC plate with molten bacterial/fungal agar. In this test we wanted to find compounds with potential antimicrobial activity.**

Reviewer B:

I suggest to alter the beginning of the title to HPTLC-Direct

Bioautography. The hyphen between HPTLC and direct bioautography should be

used throughout the text.

**-The title has been changed. Appropriate corrections have been made throughout the text.**

Please introduce the abbreviation QS.

**-Appropriate corrections have been made.**

Also I recommend the deeper description of the QS including the listing of the bacterial population behaviors regulated by QS.

**-Deeper description of QS has been given.**

It is important to clear how can the anti-QS activity be measured without the analysis of QS signaling molecule production and QS gene expression. So antibiofilm activity is also an indicator of anti QS at the used bacterial strain, so please revise the abstract in this view (lines 21-22).

**-The abstract has been corrected.**

In the last sentence in the abstract the used concentration corresponding those inhibition should be given.

**-Appropriate corrections have been made.**

- line 70: please add the collected plant organ – flowered herba? Was the

hydrodistillation performed with the use of dried plant material?

**-Aerial parts of the plant were used. Appropriate correction has been made**.

- line 78: HPTLC-direct bioautography

- line 84: please replace “ADS2” by “ADC2”

- line 90: please expand “freshly prepared bacterial” suspension?

- line 91: please correct “for in”

- line 95: Reference 5 should be moved to the appropriate place – maybe

its method was used as a base? If so, then please give this info.

**-All corrections have been made.** “**Freshly prepared bacterial suspension is the one prepared at the day of experiment“.**

- Lines 102 and 107: please give the instruments (flash chromatograph,

rotary vacuum evaporator) and their sources

**-There were no instrument for the dry column flash chromatography. We have just added in the text that „water pump vacuum“ was used. For rotary vacuum evaporator the producer has been added.**

- How were the flash fractions checked to determine the active ones (that

contained isogeranic acid)?

**-The following sentences have been added in the text: “After the HPTLC-direct bioautography analysis of EO it was determined the postion of the active component(isogeranic acid) ie. its RF value. 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 RF value which exhibited antibacterial activity.“ Additional corrections have been added at former lines 107 and 112 of the original article.**

- lines 121-126: please shorten the name of the bacterial strains if they

have been presented in the text previously

**-Appropriate corrections have been made.**

- Please briefly describe the methods of biofilm formation and twitching and

flagella motility inhibition.

**-The method of biofilm formation has been described.**

- Please check the method of the pyocyanin synthesis test. Was the optical

density (cell concentration) measured? Not the absorbance of the pyocyanin?

What was the used wavelength?

**-Additional corrections have been made.**

- Table S1: the retention time of isogeranic acid is 21.99 min according to

the table S1. However, in figs. S1 and 3 the peaks are at 22.2-23 min. How

can it be?

**-Two reason can led to the shifted peak: The first is polarity mismatch of the stationary phase and solute. The second is that there is an influence of matrix in the EO and peak is distored and shifted.**

- line 167: is it possible that there are more chemotypes of A. santonicum?

**-There are not more phenotypes of A. santonicum. Different localities and different collection time can cause the difference of the essential oil or extract composition.**

- line 173: please correct the dye solution to “3% aqueous solution of

p-iodonitrotetrazolium”

**-Appropriate correction has been made.**

- At the Rf of the active components pink color was observed after

derivatization with anisaldehyde. However, this reagent is not so sensitive

and the isogeranic acid content of the EO is only 0.2%. Furthermore at so

low Rf the co-elution is common. I suggest to check whether the standard

isogeranic acid really give pink color after the use of the reagent. Also it

would be nice to see a chromatogram with parallel development of the EO and

isogeranic acid.

**-** **pink color was also observed after derivatization of isolated isogeranic acid with anisaldehyde. We used** **this reagent during whole isolation procedure where various polarities of mobile phase used. Target compound was always appired as pink color spot. We agree co-elution is common, but in our case, no co-elution occurred.**

- Fig. 1: please revise the legend; TLC should be replaced by HPTLC; ‘a’

is not bioautography

**-The caption of Fig.1 has been revised.**

- The active component was identified as isogeranic acid. This compound has

two geometric isomers Z and E. Z and E are generally separated by HPTLC as

well as by GC. Moreover, they are usually not stable can be transformed to

each other even in solution. Both isomers were detected? One of them had

higher concentration? Did NMR observe both? Why the peak of isogeranic acid

had such shape in GC?

**-According to the NOESY correlations Z configuration of isogeranic acid was determined. We have isolated only Z-isogeranic acid. These findings have been introduced in the text and Fig.2 has been changed. The peak of isogeranic acid in GC has such shape because of the polarity mismatch of the column and solute, here coulumn HP-5 and isogeranic acid. Carboxylic acids are well known for producing tailing on most columns. At the end,these are reasons of wide GC peak of isogeranic acid and not two isomers.**

- line 188: Fig. 1 should be replaced by fig. 3 or S1?

**-„Fig.1“ has been replaced with „Fig.3“.**

- Fig. 3: please mark the chromatograms and spectrum by a to c. ‘a’ is a

chromatogram, please correct it.

**-Chromatograms and spectrum at Fig.3 have been marked.**

- line 199: please correct ‘isogeranic acids’ to ‘isogeranic acid’

**-Correction has been made.**

- lines 205-208: please give more info how anti-QS activity was determined

- lines 213-217: it is strange that lower concentration had higher

anti-biofilm activity in the cases of EO and positive controls. I propose an

interpretation of this observation.

**-The following sentence has been added: ”According to the obtained data a concluding remark could be highlighted: EO and isogeranic acid exhibited non-dose dependant activity on biofilm formation at subinhibitory concentrations, but certain activity undoubtedly exists.”**

- line 219: I suggest to replace “both controls” by “both positive

controls”

**-The word “positive“ has been added.**

- line 222: P. aeruginosa should be in italic

**-Correction has been made.**

- Fig. 4: please use small a to e in the legend as the pictures were marked

**-Appropriate correction has been made.**

- in pyocyanin production test what was the 100%? What was the used

concentrations? In the experimental part only the concentration of EO and

isogeranic acid was given.

**-The missing data have been added.**

In the legend of Fig. 5. this value is also

missing “at subMICs (mg/mL)”. Please add this info.

**-Info about concentration has been added.**