Botanica SERBICA 40 (1): (2016) 43-48

Original Scientific Paper

Screening of the antibacterial effect of *Juniperus sibirica* and *Juniperus sabina* essential oils in a microtitre plate-based MIC assay

Biljana Nikolić[1]*, Bojana Vasilijević[1], Dragana Mitić-Ćulafić[1], Marija Lesjak[2], Branka Vuković-Gačić[1], Neda Mimica Dukić[2] and Jelena Knežević-Vukčević[1]

1 Chair of Microbiology, Faculty of Biology, University of Belgrade, Studentski trg 16, Belgrade, Serbia

2 Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, Novi Sad, Serbia

ABSTRACT: The antibacterial effect of wild-growing *Juniperus sibirica* Burgsdorf and *Juniperus sabina* L. var. *sabina* essential oils was studied in a microtitre plate-based MIC assay. Bacterial growth was monitored by measuring turbidity of the sample (OD_{600}), as well as by following the colorimetric resazurin reaction. Essential oils were prepared from the needles of female plant samples and analysed by GC-MS. Hydrocarbon monoterpenes were determined as the dominant constituents; the compounds detected in the highest amounts were α-pinene (74.5%) and sabinene (54.3%) in *J. sibirica* and *J. sabina* oil, respectively. As indicator strains in the MIC assay, we used selected Gram-positive (*Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC6633 and *Listeria innocua* ATCC33090) and Gram-negative (*Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC14028, *Salmonella enteritidis* ATCC13076, *Aeromonas hydrophila* ATCC49140) bacteria. Bacterial inocula used in the MIC assay were adjusted to a 0.5 McFarland standard, corresponding to approximately 10^8 CFU/mL. The obtained results indicated that determination of turbidity decrease cannot be used to precisely quantify MIC values of the oils. The resazurin-incorporated MIC assay showed that the most susceptible strains were *A. hydrophila* and *B. subtilis*, with MIC values of 12.5 mg/mL and 6.25 mg/mL, respectively, for *J. sibirica*, and 6.25 mg/mL for both bacteria for *J. sabina*. The remaining bacteria were far less sensitive to *Juniperus* oils. In the range of tested concentrations, the effect of both oils was predominantly bactericidal, but *J. sibirica* oil showed a bacteriostatic effect against some Gram-negative bacteria.

Keywords: *Juniperus sibirica*, *Juniperus sabina*, essential oils, MIC assay

Received: 05 November 2015 Revision accepted: 23 December 2015

UDC: [582.477:665.52/.54]:615.281.9
DOI: 10.5281/zenodo.48858

INTRODUCTION

The use of antibiotics is considered to be among the most important achievements of the twentieth century, one that revolutionarily changed the medical treatment of microbial infections. Antibiotic therapy is widely practiced for the treatment of various microbiological infections. However, the acquisition of antimicrobial resistance by key microbial pathogens is increasing worldwide and reaching an alarming rate (Davies & Davies 2010). This makes it imperative to develop new antibacterial agents that can be substituents for the ineffective ones.

Different compounds of plant origin possess a significant antimicrobial potential and could be good sources for the development of new antimicrobial chemotherapeutics (Newman *et al.* 2000; Chin *et al.* 2006). In a preliminary search for new antibacterial agents of plant origin, we screened different extracts of less studied species from our region, ones belonging to the genera *Urtica*, *Parietaria*, *Allium* and *Juniperus,* against selected Gram-positive and

*correspondence: biljanan@bio.bg.ac.rs

Gram-negative bacteria. Preliminary results directed our study to essential oils (EOs) of *Juniperus* species.

The genus *Juniperus* belongs to the family Cupressaceae, which is widely distributed in the Northern Hemisphere (Adams 2014). EOs obtained from berry cones of *Juniperus* species, especially *J. communis*, are used for fragrance and flavouring in preparation of food and alcoholic beverages, as well as for medicinal, insecticidal and cosmetic purposes (Iida *et al.* 2007; Cabral *et al.* 2012). Study of their biological properties indicates that *Juniperus* species are endowed with numerous activities, including antimicrobial, antioxidant, antiseptic, diuretic, anticancer, antirheumatic, antihelminthic, anti-inflammatory, immunomodulatory, analgesic, antituberculotic and abortifacient activities (Swanston-Flatt *et al.* 1990; Glišić *et al.* 2007; Orphan *et al.* 2011).

The purpose of the present study was to examine the antibacterial effect of *Juniperus sibirica* Burgsdorf and *Juniperus sabina* L. var. *sabina* EOs obtained from needles against selected Gram-positive and Gram-negative bacteria. Among different *in vitro* antimicrobial assays developed so far (Choma & Grzelak 2011), we used a microtitre plate-based MIC assay to detect minimal inhibitory concentrations (MICs) of *Juniperus* EOs. Bacterial growth was monitored by measuring turbidity of the sample, as well as by following the colorimetric resazurin reaction (Sarker *et al.* 2007).

MATERIAL AND METHODS

Plant material and preparation of essential oils. Samples of wild-growing *J. sibirica* Burgsdorf and *J. sabina* L. var. *sabina* were collected from the Stara Planina Mountains in July 2008 and in Mavrovo, FYR of Macedonia, in July 2010. The voucher specimens (*J. sibirica* 2-1852 and *J. sabina* 2-1790) were prepared, identified and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Sciences, University of Novi Sad (Thiers 2013).

To prepare EOs, air-dried and smoothly ground needles of female *J. sibirica* and *J. sabina* plant samples were subjected to hydro-distillation using an apparatus of the Clevenger type. A weighed portion (300 g) of plant material was added to 1200 mL of dH_2O and distilled for 4 h, followed by removal of the recipient solvent (hexane) under reduced pressure. The yields of produced EO were 2.04 % and 2.54 % for *J. sibirica* and *J. sabina*, respectively. Produced EOs were stored at -20˚C prior to analysis and dissolved in hexane and dimethyl sulfoxide (DMSO) for GC-MS analysis and the MIC assay, respectively. EOs were analysed by GC-MS analysis as previously described by Lesjak *et al.* (2013).

Bacteria and growth conditions. The bacterial strains used in this study were *Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC14028, *Salmonella enteritidis* ATCC13076, *Aeromonas hydrophila* ATCC49140, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC6633 and *Listeria innocua* ATCC33090. They were cultivated at 37˚C in brain-heart infusion (BHI) and brain-heart agar (BHA) for *L. innocua* or in Mueller-Hinton broth (MHB) and Mueller–Hinton agar (MHA) for the other bacteria. Solid media (BHA and MHA) contained 1.5% (w/w) agar.

Table 1. Composition of *J. sabina* and *J. sibirica* EOs.

Compound (%)	*J. sibirica*	*J. sabina*
α-thujene	n.d.	2.0
α-pinene	74.5	2.7
sabinene	n.d.	54.3
β-pinene	4.8	n.d.
β-myrcene	2.8	4.3
α-terpinene	n.d.	2.8
Limonene	n.d.	3.1
β-phellandrene	3.5	n.d.
g-terpinene	n.d.	4.5
α-terpinolene	n.d.	1.7
4-terpineole	n.d.	6.6
Citronellal	n.d.	6.8
g-elemene	1.0	0.4
Germacrene D	4.3	n.d.
α-muurolen	1.0	0.8
d- cadinene	1.5	2.8
Germacrene B	4.0	1.0
α-cadinol	n.d.	1.2
Monoterpene hydrocarbons	86.7	75.4
Oxidised monoterpenes	0.0	13.4
Sesquiterepenes	11.3	3.3
Oxidised sesquiterpenes	2.0	7.9
Total	100.0	100.0

n.d.- not determined

MIC assay. Bacterial cultures were freshly prepared for every experiment by overnight cultivation at 37°C in the corresponding medium. Bacterial suspensions were centrifuged at 4000 rpm for 10 min and resuspended in

Table. 2. Antibacterial effect of *J. sibirica* and *J. sabina* EOs: MIC and MBC values.

Bacterial strains	*Juniperus sibirica*		*Juniperus sabina*		Streptomycin	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Aeromonas hydrophila	12.5	12.5	6.25	6.25	0.200	0.200
Escherichia coli	50	n.d.	25	50	0.050	0.100
Salmonella enteritidis	50	n.d.	50	50	0.025	0.025
Salmonella typhimurium	50	n.d.	50	50	0.200	0.400
Staphylococcus aureus	25	25	25	50	0.025	0.025
Listeria innocua	25	50	25	25	n.d.	n.d.
Enterococcus faecalis	25	50	50	n.d.	0.0125	0.100
Bacillus subtilis	6.25	6.25	6.25	6.25	0.025	0.025

n.d.-not determined in used concentration range

0.01M $MgSO_4$ to achieve turbidity of a 0.5 McFarland standard, corresponding to approximately 10^8 CFU/mL.

The MIC assay was performed in 96-well microtitre plates by making serial two-fold dilutions of test substances (concentration range of 50 – 0.39 mg/mL) in the corresponding media. As a positive control, we used the antibiotic streptomycin (CAS No. 3810-74-0) in a concentration range of 400 – 3.125 μg/mL. To each well was added 10 μL of bacterial suspension. The final volume of samples in the wells was 100 μL. The microtitre plates were wrapped with vapor film and incubated for 24 h at 37°C. After incubation, absorbance at 600 nm (OD_{600}) was measured to determine turbidity of the samples.

In determining MIC values by following the resazurin reaction, an aqueous solution of resazurin (CAS No. 62758-13-8; 0.675 mg/mL) was added to each well after OD_{600} measurement. The plates were incubated for 3 h, and MIC values were determined as the lowest concentrations that showed no visible colour change. Whether the effect was bacteriostatic or bactericidal was established by plating samples from wells without visible growth onto the corresponding agar medium and incubating for 24 h at 37°C. The lowest concentration which showed no visible growth after plating and incubation was determined as the minimal bactericidal concentration (MBC). For each bacterial strain, three individual experiments were performed in triplicate.

RESULTS AND DISCUSSION

The composition of EOs was determined by GC-MS analysis, which revealed monoterpenes, mainly hydrocarbon ones, as the dominant constituents (Table 1). The main constituents of *J. sibirica* and *J. sabina* EOs were the hydrocarbon monoterpenes α-pinene (74.5%) and sabinene (54.3%), respectively. Other components, present in moderate amounts, were: β-pinene (4.8%), germacrene D (4.3 %), germacrene B (4.0%) and β-phelandrene (3.5%) in *J. sibirica* EO; and citronellal (6.8%), 4-terpineole (6.6%), γ-terpinene (4.5%) and β-myrcene (4.3%) in *J. sabina* EO (Lesjak 2011).

In order to evaluate the antibacterial potential of *J. sibirica* and *J. sabina* EOs, we used *A. hydrophila*, *E. coli*, *S. enteritidis* and *S. thyphimurium* as Gram-negative indicator strains; and *S. aureus*, *L. innocua*, *E. faecalis* and *B. subtilis* as Gram-positive indicator strains. A microtitre plate-based antibacterial assay was performed, and bacterial growth was monitored by measuring absorbance of the samples at 600 nm (OD_{600}). Reduction of absorbance compared to the solvent control amounting to 90% (IC_{90}) can be approximated as MIC (Kronvall *et al.* 2006; Okeleye *et al.* 2013). The obtained OD_{600} values in relation to test-substance concentrations revealed that IC_{90} was reached only in a few cases (Fig. 1). This is due to the fact that dead cells, as well as the remains of lysed cells, contribute to the turbidity read-off by the spectrophotometer. For this reason, determination of turbidity decrease cannot be used to precisely quantify MIC values of EOs. We therefore determined MIC values by the above-described resazurin-incorporated MIC assay. The obtained results indicated that the antibacterial potential of both EOs was weak (Table 2). The most susceptible strains were *A. hydrophila* and *B. subtilis*, while the remaining bacteria were far less sensitive. In the range of tested concentrations, the effect of both EOs was predominantly bactericidal, which is in line with the proposed mechanism of antimicrobial action of terpenoids involving membrane disruption by lipophilic compounds (Cowan 1999). However, *J. sibirica* EO showed a bacteriostatic effect against some Gram-negative bacteria (Table 2).

To judge from chemical composition of the EOs in question, the obtained antibacterial effect could be at least partially attributed to α-pinene and sabinene for

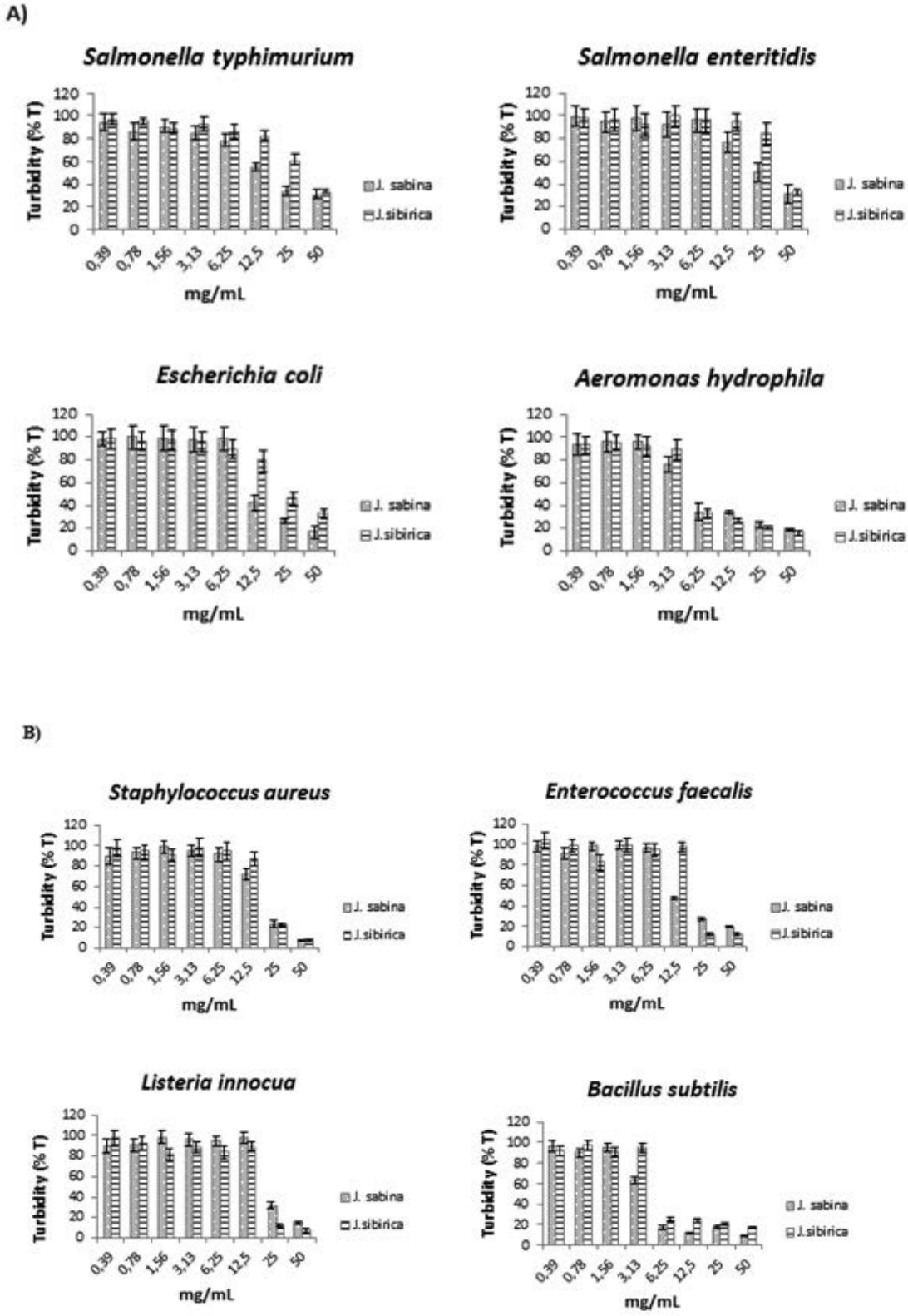

Figure 1. Antibacterial effect of *J. sibirica* and *J. sabina* EOs against Gram-negative (A) and Gram-positive (B) bacteria. The % of turbidity (%T) was calculated in relation to the solvent control.

J. sibirica and *J. sabina* EOs, respectively (Da Silva *et al.* 2012; Arunkumar *et al.* 2014), but contribution of other constituents is not excluded. The relatively low antibacterial potential could be attributed to the high proportion of hydrocarbon monoterpenes, which possess the lowest effect compared to other terpenoid compounds (Griffin *et al.* 1999). However, since the cell number in inocula affects MIC assessment, and in this study we used a high number of bacteria per well (approximately 10^6), it would be advantageous to further assess the antibacterial effect of *Juniperus* species using a lower and more precisely determined cell number, as recommended by Kolarević *et al.* (2016).

CONCLUSION

Using a resazurin-incorporated MIC assay, we showed that essential oils of *Juniperus sibirica* Burgsdorf and *Juniperus sabina* L. var. *sabina* exert a moderate antibacterial effect against *A. hydrophila* and *B. subtilis*, and possess a low antibacterial potential against *E. coli*, *S. typhimurium*, *S. enteritidis*, *E. faecalis*, *S. aureus* and *L. innocua*. In the range of tested concentrations, the effect was predominantly bactericidal.

Acknowledgement – This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. 172058.

REFERENCES

Adams RP. 2014. Junipers of the World: The genus *Juniperus*. 4th Edition. Trafford Publishing Co., Bloomington, IN, USA & Canada.

Arunkumar R, Nair SA, Rameshkumar KB & Subramoniam A. 2014.The essential oil constituents of *Zornia diphylla* (L.) Pers, and anti-inflammatory and antimicrobial activities of the oil. Rec. Nat. Prod. **8**: 385-393.

Cabral C, Francisco V, Cavaleiro C, Gonçalves MJ, Cruz MT, Sales F, Batista MT & Salgueiro L. 2012. Essential oil of *Juniperus communis* subsp. *alpina* (Suter) Čelak needles: Chemical composition, antifungal activity and cytotoxicity. Phytother. Res. **26**: 1352-1357.

Chin YW, Balunas MJ, Chai HB & Kinghorn AD. 2006. Drug discovery from natural sources. Aaps. J. **8**: 239-253.

Choma IM & Grzelak EM. 2011. Bioautography detection in thin-layer chromatography. J. Chromatogr. A. **1218**: 2684-2691.

Cowan MM. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. **12**: 564-582.

Davies J & Davies D. 2010.Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. R. **74**: 417-433.

Da Silva ACR, Lopes PM, Azevedo MMBD, Costa DCM, Alviano CS & Alviano DS. 2012. Biological activities of a-pinene and β-pinene enantiomers. Molecules **17**: 6305-6316.

Glišić S, Milojević S, Dimitrijević S, Orlović A & Skala D. 2007. Antimicrobial activity of the essential oil and different fractions of *Juniperus communis* L. and a comparison with some commercial antibiotics. J. Serb. Chem. Soc. **72**: 311–320.

Griffin SG, Wyllie SG, Markham JL & Leach DN. 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour. Frag. J. **14**: 322-332.

Iida N, Inatomi Y, Murata H, Inada A, Murata J, Lang FA, Matsuura N & Nakanishi T. 2007. A new flavone xyloside and two new flavan-3-ol glucosides from *Juniperus communis* var. *depressa*. Chem. Biodivers. **4**: 32–42.

Kolarević S, Milovanović D, Avdović M, Oalđe M, Kostić J, Sunjog K, Nikolić B, Knežević-Vukčević J, Vuković-Gačić B. 2016.Optimization of the microdilution method for detection of minimum inhibitory concentration values in selected bacteria. Bot. Serb. **40**: 29-36.

Kronvall G, Karlsson I, Walder M, Sörberg M & Nilsson LE. 2006. Epidemiological MIC cut-off values for tigecycline calculated from Etest MIC values using normalized resistance interpretation. J. Antimicrob. Chemoth. **57**: 498-505.

Lesjak MM, Beara IN, Orčić DZ, Ristić JD, Anačkov GT, Božin BN & Mimica-Dukić NM. 2013. Chemical characterisation and biological effects of *Juniperus foetidissima* Willd. 1806. LWT-Food Sci. Technol. **53**: 530-539.

Lesjak MM. 2011. Biopotential and chemical characterization of extracts and essential oils of species from *Juniperus* L. genus (Cupressaceae). PhD Thesis, Faculty of Sciences, University of Novi Sad, Serbia (in Serbian).

Newman DJ, Cragg GM & Snader KM. 2000.The influence of natural products upon drug discovery. Nat. Prod. Rep. **17**: 215-234.

Okeleye BI, Mkwetshana NT & Ndip RN. 2013. Evaluation of the antibacterial and antifungal potential of *Peltophorum africanum*: Toxicological effect on human Chang liver cell line. The ScientificWorld J. Article ID 878735. http://dx.doi.org/10.1155/2013/878735.

Orhan N, Orhan IE & Ergun F. 2011. Insights into cholinesterase inhibitory and antioxidant activities of five *Juniperus* species. Food Chem. Toxicol. **49**: 2305-2312.

Sarker SD, Nahar L & Kumarasamy Y. 2007.Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods **42**: 321-324.

Swanston-Flatt SK, Day C, Bailey CJ & Flatt PR. 1990. Traditional plant treatments for diabetes. Studies in normal and streptozotocin diabetic mice. Diabetologia **33**: 462-464.

Thiers B. 2013. Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium. http://sweetgum.nybg.org/ ih/

Botanica SERBICA

REZIME

Ispitivanje antibakterijskog efekta etarskih ulja *Juniperus sibirica* i *Juniperus sabina* primenom mikrodilucionog MIC testa

Biljana Nikolić, Bojana Vasilijević, Dragana Mitić-Ćulafić, Marija Lesjak, Branka Vuković-Gačić, Neda Mimica Dukić i Jelena Knežević-Vukčević

Antibakterijski efekat etarskih ulja samoniklih *Juniperus sibirica* Burgsdorf and *Juniperus sabina* L. var. *sabina* ispitivan je primenom mikrotitarskog MIC eseja. Rast bakterija je praćen merenjem stepena zamućenja uzoraka (OD_{600}) i preko kolorimetrijske reakcije resazurina. Etarska ulja su pripremljena od četina ženskih biljaka, a njihov hemijski sastav određen je primenom GC-MS analize. Monoterpenski ugljovodonici su bili dominantni sastojci oba ulja, a u najvećem procentu identifikovani su α-pinen (74.5% u ulju *J. sibirica*) i sabinen (54.3% u ulju *J. sabina*). Antibakterijski potencijal je određivan prema odabranim Gram-pozitivnim (*Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC6633 i *Listeria innocua* ATCC33090) i Gram-negativnim bakterijama (*Escherichia coli* ATCC25922, *Salmonella typhimurium* ATCC14028, *Salmonella enteritidis* ATCC13076, *Aeromonas hydrophila* ATCC49140). Gustina bakterijskih inokuluma je podešena prema McFarland standardu 0.5, što približno odgovara broju10^8 CFU/mL. Dobijeni rezultati su ukazali da stepen zamućenja uzoraka nije mogao biti iskorišćen za precizno određivanje MIC vrednosti. MIC esej sa resazurinom je pokazao da su najosetljiviji sojevi *A. hydrophila* i *B. subtilis*, za koje je MIC vrednost u slučaju *J. sibirica* iznosila 12.5 mg/mL i 6.25 mg/mL, a u slučaju *J. sabina* 6.25 mg/mL za oba bakterijska soja. Ostale bakterije su bile daleko manje osetljive. U opsegu testiranih koncentracija, oba etarska ulja pokazala su predominantno baktericidni efekat, mada je ulje *J. sibirica* pokazalo bakteriostatički efekat prema pojedinim Gram-negativnim bakterijama.

Ključne reči: *Juniperus sibirica*, *Juniperus sabina*, etarska ulja, MIC test