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Bioactivity of *Juniperus communis* essential oil and post-distillation waste: assessment of selective toxicity against food contaminants

Biljana Nikolić[1,*], Bojana Vasilijević[1], Ana Ćirić[2], Dragana Mitić-Ćulafić[1], Stefana Cvetković[1], Ana Džamić[1] and Jelena Knežević-Vukčević[1]

[1] *Faculty of Biology, University of Belgrade*, Studentski trg 16, 11000 Belgrade, Serbia

[2] *Institute for Biological Research "Siniša Stanković", University of Belgrade*, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia

***Corresponding author**: biljanan@bio.bg.ac.rs

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Abstract: Previously chemically characterized *Juniperus communis* essential oil (EO) and post-distillation waste (PDW) were tested for cytotoxicity and antimicrobial activity against food contaminants. Microdilution assay showed that PDW induced moderate antifungal (minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values, ranging between 0.118-0.900 mg mL^{-1}), and an antibacterial effect against *Listeria monocytogenes* (MIC and minimum bactericidal concentration (MBC) were 0.39 and 0.74 mg mL^{-1}, respectively). Combinations of EO/PDW with selected antibiotics induced synergistic antilisterial activity in the checkerboard assay. The MTT assay determined that cytotoxicity against colon cancer cells was high for the EO but negligible for PDW (IC_{50} values were 0.087-0.106 and 1.450-6.840 mg mL^{-1}, respectively). The selectivity indices indicated high selectivity of PDW against tested fungi and *L. monocytogenes*. In the adhesion-inhibition assay, PDW reduced *in vitro* adhesion of *L. monocytogenes* to colon cells (29-62% of inhibition). In conclusion, PDW exhibited an antimicrobial effect against important food spoilage and poisoning fungi and *L. monocytogenes*, and also reduced *in vitro* adhesion of *L. monocytogenes* to colon cells. The results indicate that *J. communis* PDW could be considered as natural preservative against food spoilage and poisonous fungi, and as an adjuvant to conventional therapy of listeriosis.

Keywords: *Juniperus communis*; essential oil; post-distillation waste; selective antimicrobial effect; adhesion-inhibition properties

INTRODUCTION

The search for new antimicrobial agents has become a necessity because growing resistance to existing antibiotics is posing a serious problem to global public health [1]. Similarly, the increased use of antifungal agents has resulted in the rapid development of fungal resistance and made it a major clinical problem, due to the limited arsenal of available systemic antifungal agents [2].

Plants are considered as an extremely important source of antimicrobials and many species used in traditional medicine are currently under investigation [3]. Junipers (*Juniperus* spp.), which contain numerous active compounds, are among the most widespread species in the Northern Hemisphere. The use of these plants, especially their seed cones ('berries'), in folk medicine and manufacturing is extensive [4]. In traditional medicine they are used as diuretics, appetizers, carminatives, stomachics, anticonvulsants and antihypertensive agents, as well as in the treatment of headaches, fever, bronchitis, asthma and some gynecological disorders [5,6]. Literature data also indicate remarkable antioxidant, antimicrobial and hypoglycemic activities of these berries [7-10]. Furthermore, juniper extracts have found wide applications in pharmaceutical industry, perfumery and aromatherapy [11].

Juniperus communis L. is well known as the only food spice derived from conifers [12]. Juniper berry

extracts are used as a flavoring agent in food and alcoholic beverage industries. The most famous alcoholic beverage containing juniper is gin but locally manufactured juniper brandies are also very popular [13,14]. In addition, juniper berries are used in European and particularly in Scandinavian cuisine. They are used in meat preparation, especially wild birds and game, and to flavor dishes prepared with pork, cabbage and sauerkraut [15,16].

In recent years, interest in plant-derived food additives is in great expansion, since they possess different health-promoting properties [17]. Furthermore, particular adverse effects such as immunologic hypersensitivity have been reported for some synthetic food additives [18], and this has additionally stimulated the search for natural replacements. Among natural compounds that could be used as alternatives to antimicrobial synthetic food additives, essential oils (EOs) are considered as effective candidates [19]. In addition, various plant waste materials, including post-distillation waste (PDW), are receiving increased attention since they contain numerous bioactive compounds available for further extraction [20].

The aim of this work was to study the antimicrobial effect of *J. communis* EO and PDW against selected food-borne pathogenic and spoilage bacteria, as well as food-poisoning and pathogenic fungi. The antibacterial effect was determined for EO and PDW alone or in combination with common antibiotics. To estimate the selective toxicity to microbial strains, the cytotoxic potential against colon cancer cells was also determined and selectivity indices were calculated. Finally, the inhibitory potential of PDW against bacterial adhesion to colon cells was examined *in vitro* using the most sensitive bacterial strain.

MATERIALS AND METHODS

Plant material, EO and PDW preparation

Plant material (seed cones of *Juniperus communis* L. var. saxatilis Pall.) was collected on Mt. Stara Planina, Serbia. The voucher specimen (No. 16693) was prepared, identified by Nemanja Rajčević (PhD in botany), and deposited at the Herbarium of the University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac" (BEOU Herbarium). Air-dried and finely ground seed cones were submitted to hydrodistillation in a Clevenger-type apparatus, as previously described [21]. The EO was dissolved in dimethyl sulfoxide (DMSO) for all performed bioassays. After distillation of the EO, the residual aqueous solution was evaporated in vacuum at 45°C, resulting in a dry PDW extract. Distilled water (dH_2O) was used as a solvent for the PDW extract.

Bacterial and fungal strains and human cell cultures

The antimicrobial effect was determined against: (i) Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *S. aureus* MRSA (ATCC43300), *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes* (ATCC 19111), (ii) Gram-negative bacteria *Escherichia coli* (ATCC 8739), *Shigella flexneri* (ATCC 9199), *Salmonella enteritidis* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 15442) and (iii) fungi *Aspergillus fumigatus* (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), and *Penicillium verrucosum* var. cyclopium (food isolate). The human cell lines used in cytotoxicity and adhesion-inhibition assays were colorectal carcinoma cells HT-29 (ATCC HTB-38) and HCT116 (ATCC CCL-247).

Chemicals, media and growth conditions

Ampicillin sodium salt (Amp, Cas No. 69-52-3, Sigma-Aldrich, St. Louis, USA), streptomycin sulfate salt (Str, Cas No. 3810-74-0, Sigma-Aldrich) and azithromycin dihydrate (Azm, Cas No. 117772-70-0, Sigma-Aldrich, St. Louis, USA) were used in antibacterial microdilution and checkerboard assays. Stock solutions of antibiotics were 1 mg mL^{-1}, prepared in sterile dH_2O (Amp and Str) or 5% DMSO (Azm). Fungicides bifonazole (Bfz, Cas No.60628-96-8, Sigma Aldrich, St. Louis, USA) and ketoconazole (Kcz, Cas No.65277-42-1, Sigma Aldrich, St. Louis, USA) were used in antifungal microdilution assays. Stock solutions of fungicides were 50 mg mL^{-1} in 0.9% saline solution and 25mg mL^{-1} in 0.9% saline solution, for Bfz and Kcz, respectively. Resazurin sodium salt (Cas No. 62758-13-8; Sigma Aldrich, St.

Louis, USA, stock solution 0,675 mg mL^{-1} in sterile dH_2O) was used as a growth indicator in antibacterial microdilution and checkerboard assays.

Bacteria were cultivated at 37°C in brain hart infusion broth (BHI, LAB M, Lancashire, UK) and brain heart agar (BHA) for *L. monocytogenes* and *E. faecalis*, or in Müller-Hinton broth (MHB, Himedia, Mumbai, India) and Müller-Hinton Agar (MHA) for *S. aureus,* MRSA*, E. coli, S. flexneri*, *S. enteritidis* and *P. aeruginosa*. Fungal strains were cultivated at 28°C in malt broth (MB, Institute of Immunology and Virology, Torlak, Belgrade, Serbia) and malt agar (MA). All solid media (BHA, MHA and MA) contained 1.5% (w/w) agar (LAB M, Lancashire, UK).

The human cells (HT-29 and HCT116) were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% glucose and 2 mM L-glutamine, supplemented with 10% fetal bovine serum (FBS) and a penicillin/streptomycin cocktail. Cells were maintained in an incubator at 37°C with 5.0% CO_2 in a humidified atmosphere. The cells growing attached to the surface were subcultured at 90% confluence twice a week. Single cell suspensions for subculturing and for experiments were obtained using 0.1% trypsin (from porcine pancreas). Cell viability in suspensions was inspected by the trypan blue dye exclusion method. In the cytotoxicity assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cas. No 298-93-1), 5-fluorouracil (5-FU, Cas No. 51-21-8) and phosphate buffered saline (PBS) were used as indicators of cell viability, positive control and to wash cells, respectively. All media and reagents used to grow and manipulate human cells were purchased from Sigma-Aldrich, St. Louis, USA.

Microdilution assay

The antimicrobial properties of the EO and PDW were determined in microdilution assay performed in 96-well microtiter plates. The serial two-fold dilutions of test substances were made in corresponding medium (BHI for *L. monocytogenes* and *E. faecalis*, MHB for other bacteria, and MB for fungi). Suspensions of indicator strains were adjusted to 10^6 CFU mL^{-1} by preparing an exponential bacterial culture or by washing fungal spores with sterile 0.85% saline containing 0.1% Tween 80 (v/v) from the surface of MA plates. EO was tested in concentration range 0.39-50 mg mL^{-1} for both bacteria and fungi, while PDW was tested in the ranges 0.195-25 mg mL^{-1} and 7-900 μg mL^{-1} for bacteria and fungi, respectively. Assays were performed in triplicate in three individual experiments.

A slightly modified resazurin-incorporated microdilution assay, performed as previously described [22], was used to evaluate the antibacterial properties. Briefly, test substances were serially two-fold diluted along the columns. Bacterial inoculum (10^5 CFU mL^{-1}) was added to each well except the sterility control. The growth indicator resazurin (final concentration 0.067 mg mL^{-1}) was added to the wells and after 24 h of incubation at 37°C, MICs were determined as the lowest concentrations that did not induce color change. After plating by inoculation loop from each well without visible growth on solid media (BHA/MHA) and incubation (24 h at 37°C), MBCs were determined. As positive controls, conventional antibiotics Str, Amp and Azm were applied in a concentration range of 0.78-100 μg mL^{-1}. Sterilized solvent (5% DMSO and dH_2O for EO and PDW, respectively) was used as a negative control.

The microdilution assay, performed as previously described [23], was used to evaluate the antifungal potential. Briefly, the fungal spore suspensions (1.0×10^5 CFU mL^{-1}) were added to each well containing graded concentrations of test substances. After 72 h of incubation at 28°C, MICs were determined with a binocular microscope as the lowest concentrations without visible growth in seeded wells. The MFCs were determined by the same procedure after serial subcultivation from each well without visible growth into microtiter plates. Standard fungicides Bfz and Kcz, both applied in the concentration range 4-512 μg mL^{-1}, served as positive controls, while a solvent (sterile 0.85% saline containing 0.1% Tween 80 and dH_2O for EO and PDW, respectively) was used as a negative control.

Checkerboard assay

A slightly modified checkerboard assay was performed as previously described [24]. It was used to determine the mode of interactions between the test substances (EO/PDW and antibiotics). One test substance was

serially two-fold diluted along the vertical, while the second one was serially two-fold diluted along the horizontal line of 96-well microtiter plates. In combinations prepared with PDW, the concentrations ranged between (1/32)×MIC-4×MIC, while in combinations prepared with EO the concentrations ranged between (1/64)×MIC-2×MIC. The MIC values of combinations were determined by adding resazurin (final concentration 0.067 mg mL^{-1}) and inspecting for color changes. Combinations that did not induce color change of resazurin were used for the calculation of the fractional inhibitory concentration index (FICI) for two antimicrobials in combination. The FICI was calculated according to equation (1), where substance A was EO or PDW, and substance B was the antibiotic (Str, Amp, Azm).

$$FICI = \frac{MIC_{A\ in\ comb.}}{MIC_{A\ alone}} + \frac{MIC_{B\ in\ comb.}}{MIC_{B\ alone}} \quad (1)$$

FICI was used to distinguish between the mode of interactions as follows: FICI≤0.5 – synergistic; 0.5<FICI≤1 – additive; 1<FICI≤4 – indifferent; FICI<4 – antagonistic effect [24]. The checkerboard assay was performed in triplicate in two individual experiments.

Cytotoxicity assay

Cytotoxicity was determined by the MTT reduction assay, performed as previously described [23]. Briefly, the assay was performed on HT-29 and HCT116 cells inoculated in 96-well plates at a density of $5x10^4$ cells/well and incubated until they formed a monolayer; EO and PDW were serially two-fold diluted in tested concentration ranges (0.016-0.500 and 0.313-20 mg mL^{-1}, respectively). After 24 h of incubation, the medium was removed and replaced with the MTT solution (final concentration 0.5 mg mL^{-1} in DMEM); the plates were additionally incubated for 3 h to allow for mitochondrial reduction of MTT into formazan, performed in viable cells. After this step, the medium was carefully removed and the formazan crystals were dissolved in DMSO. Cell viability was determined by measuring the absorbance at 570 nm, using a microplate reading spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Shanghai, China). The cytotoxic activity was evaluated by comparing the absorbance of the wells containing the test substances to that containing the vehicle (DMSO or dH_2O for EO and PDW, respectively). 5-fluorouracil (5-FU) was used as a positive control. For each test substance, two independent experiments with six wells per treatment point were performed.

Selectivity index

In order to estimate the selective toxicity of test substances, a relationship between cytotoxic and antimicrobial effects was determined through the selectivity index (SI). The SI was calculated as previously described [25] using the following equation (2):

$$SI = \log IC50/MIC \quad (2)$$

Positive values of SI indicate higher toxicity to bacteria or fungi, while negative values indicate higher toxicity to colon cells.

***In vitro* adhesion-inhibition assay**

The potential to influence bacterial adhesion to colon cells was determined for *L. monocytogenes*. Both HT-29 and HCT116 cells were used as a model of colon epithelium. The adhesion-inhibition assay was performed using a slightly modified protocol previously described [23]. Colon cells were inoculated into 12-well plates at a density $8x10^4$ cells/well and incubated at 37°C in 5% CO_2 to form a monolayer. After this step, the medium was removed and the cell monolayers were washed twice with PBS. Two wells were used to enumerate the cell number by trypan blue dye exclusion. *L. monocytogenes* suspension prepared in DMEM with PDW (concentration equal to ½ MIC) or without it, was added to each well. The final ratio of human and bacterial cell numbers was about 1/10 (the number of bacteria was 10-fold higher than the number of HT-29/HCT116 cells). To allow adhesion, human cells were co-incubated with bacteria for 1 h at 37°C in 5% CO_2. The medium was removed and the cell monolayer was washed twice to remove the non-adhering bacterial cells. To enumerate the adhered bacteria, the monolayer was treated with 0.1% trypsin and appropriate dilutions of obtained cell suspensions were plated in triplicate onto BHA and incubated at 37°C for 24 h. The numbers of bacterial cells in suspensions added initially to the cell monolayer were determined in order to calculate the proportion of adhering bacteria. The effect on adhesion was estimated by comparing the

percentage of adhering bacteria in the medium with and without the test substance. For each cell line, two independent experiments in triplicate were performed.

Statistical analysis

Experimental data were analyzed by Student's *t*-Test. The level of statistical significance was defined as $p<0.05$.

RESULTS

Antimicrobial properties

The antimicrobial potential of *J. communis* EO and PDW was determined against selected bacterial and fungal strains. While the antifungal effect of EO was negligible, PDW demonstrated remarkable activity, with MICs and MFCs ranging between 119-900 μg mL^{-1} and 250-900 μg mL^{-1}, respectively (Table 1). The highest activity was recorded against *A. versicolor*. On the other hand, the antibacterial effect of both EO and PDW was weak, with MICs mainly above 3 mg mL^{-1} (Table 2). The most sensitive bacterium was *L. monocytogenes* with MIC and MBC values of 3.30 mg mL^{-1} and 6.25 mg mL^{-1} for EO, respectively, and 0.39 mg mL^{-1} and 0.74 mg mL^{-1} for PDW, respectively.

The effect of EO and PDW against *L. monocytogenes* was further monitored in the checkerboard assay, where they were combined with conventional antibiotics Str, Amp and Azm. The results showed that certain combinations of EO with Str and Amp, as well as of PDW with all three tested antibiotics, induced a synergistic antimicrobial effect (Table 3).

Table 1. Antifungal activity of *Juniperus communis* EO, PDW and conventional fungicides.

Strains	EO (mg mL^{-1})		PDW (μg mL^{-1})		Bfz (μg mL^{-1})		Kcz (μg mL^{-1})	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus fumigatus	11.11±2.76	nd	425.00±75.00	900.00±0.00	156.44±56.44	213.33±64.00	8.89±2.67	17.78±5.33
Aspergillus versicolor	11.81±2.08	nd	119.13±47.05	250.00±75.00	106.67±32.00	199.11±67.46	4.00±0.00	7.11±1.76
Aspergillus ochraceus	12.50±0.00	nd	400.00±99.22	850.00±150.00	142.22±42.67	184.89±67.46	9.78±3.53	19.56±7.06
Aspergillus niger	13.89±4.17	nd	nd	nd	156.44±56.44	199.11±67.46	8.00±0.00	19.56±7.06
Trichoderma viride	11.81±2.08	nd	225.00±0.00	550.00±198.43	142.22±42.67	213.33±64.00	19.56±7,06	39.11±14.11
Penicillium ochrochloron	11.81±2.08	nd	250.00±75.00	450.00±0.00	199.11±67.46	241.78±42.67	4.44±1.33	9.78±3.53
Penicillium funiculosum	12.50±0.00	nd	425.00±75.00	900.00±0.00	213.33±64.00	241.78±42.67	4.44±1.33	15.11±2.67
Penicillium verrucosum var. *cyclopium*	11.11±2.76	nd	900.00±0.00	nd	184.89±67.46	284.47±85.33	5.78±2.11	17.78±5.33

nd – not determined in the applied concentration range

Table 2. Antibacterial activity of *Juniperus communis* EO, PDW and conventional antibiotics.

Strains	EO (mg mL^{-1})		PDW (mg mL^{-1})		Str (μg mL^{-1})		Amp (μg mL^{-1})		Azm (μg mL^{-1})	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus	5.90±1.04	13.89±4.17	3.47±1.04	11.81±2.08	6.94±3.41	13.19±4.88	12.50±5.41	26.39±9.77	2.26±0.83	23.61±4.17
MRSA	6.25±2.71	11.81±2.08	3.82±1.38	11.81±2.08	13.19±4.88	13.19±4.88	nd	nd	nd	nd
Enterococcus faecalis	11.11±2.71	13.89±4.17	12.50±0.00	23.61±4.17	nd	nd	nd	nd	29.17±12.50	31.94±14.13
Listeria monocytogenes	3.30±1.22	6.25±0.00	0.39±0.17	0.74±0.13	13.19±4.88	13.19±4.88	52.78±19.54	61.11±22.05	3.82±1.38	7.64±2.76
Escherichia coli	nd	nd	6.25±0.00	nd	6.94±2.08	7.64±2.76	nd	nd	52.78±19.54	94.44±16.67
Shigella flexneri	11.81±2.08	23.61±4.17	2.96±0.50	nd	1.91±0.69	2.08±0.78	55.56±16.66	88.89±22.05	5.90±2.90	12.50±5.41
Salmonella enteritidis	nd	nd	13.89±4.17	nd	3.82±1.38	13.89±4.17	nd	nd	47.22±8.33	nd
Pseudomonas aeruginosa	nd	nd	3.47±1.04	nd	13.89±4.17	55.55±16.66	nd	nd	88.89±22.05	nd

nd – not determined in the applied concentration range

Table 3. Antilisterial effects of combinations of *J. communis* EO/PDW with different antibiotics in the checkerboard assay.

EO (MIC)	Str (MIC)	FICI	Interpretation
2	1/32	2.031	Indifferent
1	1/8	1.125	Indifferent
1/2	1/4	0.750	Additive
1/4	**1/4**	**0.500**	**Synergistic**
1/8	**1/4**	**0.375**	**Synergistic**
1/16	**1/4**	**0.312**	**Synergistic**
1/32	2	2.031	Indifferent
1/64	2	2.015	Indifferent
EO (MIC)	**Amp (MIC)**	**FICI**	**Interpretation**
2	1/64	2.015	Indifferent
1	1/16	1.062	Indifferent
1/2	1/8	0.625	Additive
1/4	**1/8**	**0.375**	**Synergistic**
1/8	**1/8**	**0.25**	**Synergistic**
1/16	**1/8**	**0.187**	**Synergistic**
1/32	2	2.031	Indifferent
1/64	2	2.015	Indifferent
EO (MIC)	**Azm (MIC)**	**FICI**	**Interpretation**
2	1/8	2.125	Indifferent
1	1/2	1.5	Indifferent
1/2	1/2	1	Additive
1/4	1/2	0.75	Additive
1/8	1	1.125	Indifferent
1/16	1	1.062	Indifferent
1/32	2	2.031	Indifferent
1/64	2	2.015	Indifferent
PDW (MIC)	**Str (MIC)**	**FICI**	**Interpretation**
4	1/32	4.031	Antagonistic
2	1/32	2.031	Indifferent
1	1/32	1.031	Indifferent
1/2	1/32	0.531	Additive
1/4	**1/32**	**0.281**	**Synergistic**
1/8	**1/32**	**0.156**	**Synergistic**
1/16	1	1.062	Indifferent
1/32	1	1.031	Indifferent
PDW (MIC)	**Amp (MIC)**	**FICI**	**Interpretation**
4	1/32	4.031	Antagonistic
2	1/32	2.031	Indifferent
1	1/32	1.031	Indifferent
1/2	1/32	0.531	Additive
1/4	**1/32**	**0.281**	**Synergistic**
1/8	**1/4**	**0.375**	**Synergistic**
1/16	1/2	0.562	Additive
1/32	1/2	0.531	Additive
PDW (MIC)	**Azm (MIC)**	**FICI**	**Interpretation**
4	1/32	4.031	Antagonistic
2	1/32	2.031	Indifferent
1	1/32	1.031	Indifferent
1/2	1/32	0.531	Additive
1/4	**1/32**	**0.281**	**Synergistic**
1/8	**1/4**	**0.375**	**Synergistic**
1/16	**1/4**	**0.312**	**Synergistic**
1/32	**1/4**	**0.281**	**Synergistic**

Type of interaction expressed by FICI values is considered to be synergistic if FICI≤0.5, additive if 0.5<FICI≤1, indifferent if 1<FICI≤4, and antagonistic if FICI>4.0.

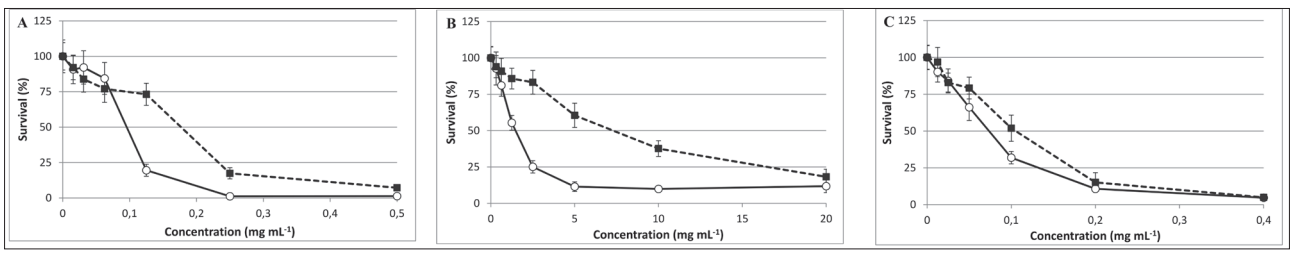

Fig. 1. Cytotoxicity of EO (**A**), PDW (**B**) and positive control 5-FU (**C**) against colon carcinoma cell lines HT-29 (o) and HCT116 (▪). Concentrations of test-substances are expressed as mg mL^{-1}. Student's *t*-Test used to analyze the experimental data, revealed that a statistically significant difference ($p<0.05$) corresponding to the control was observed for the following concentrations: **A** – for EO at 0.125 and 0.0625 mg mL^{-1} and all lower ones for HT-29 and HCT116 cells, respectively; **B** – for PDW at 0.625 and 1.25 mg mL^{-1} and all lower ones for HT-29 and HCT116 cells, respectively; (**C**) for 5-FU at 0.05 and 0.025 mg mL^{-1} and all lower ones for HT-29 and HCT116 cells, respectively.

Cytotoxic potential

Evaluation of cytotoxicity, performed on colon carcinoma HT-29 and HCT116 cells, revealed that both EO and PDW possess a cytotoxic potential (Fig. 1). The estimated IC_{50} values (concentrations that decreased cell viability to 50%) pointed at a significantly higher cytotoxicity of EO, comparable to the common cytostatic 5-FU (Table 4). PDW induced much lower cytotoxicity, with IC_{50} values approximately 20- and 60-fold higher than those of 5-FU in HT-29 and HCT116 cells, respectively. The results also showed higher sensitivity of HT-29 cells to all test substances, with the most pronounced discrepancy between cell lines determined for the effect of PDW.

Table 4. IC50 concentrations (mg mL^{-1}) of *J. communis* EO and PDW and the positive control, 5-FU against colon carcinoma cell lines HT-29 and HCT116.

	HT-29	HCT 116
EO	0.090	0.160
PDW	1.450	6.840
5-FU	0.075	0.110

Table 5. Selectivity Index (SI)* values of *J. communis* EO and PDW, corresponding to HT-29 and HCT116 cells.

Microbial strains	EO		PDW	
	HT-29	HCT116	HT-29	HCT116
Staphylococcus aureus	-1.9	-1.6	-0.3	0.3
MRSA	-1.9	-1.6	-0.3	0.3
Enterococcus faecalis	-2.2	-1.9	-0.9	-0.3
Listeria monocytogenes	-1.6	-1.3	0.6	1.2
Escherichia coli	nd	nd	-0.6	0.04
Shigella flexneri	-2.2	-1.9	-0.3	0.3
Salmonella enteritidis	nd	nd	-0.9	-0.3
Pseudomonas aeruginosa	nd	nd	-0.3	0.3
Aspergillus fumigatus	-2.2	-1.9	0.5	1.2
Aspergillus versicolor	-2.2	-1.9	1.1	1.8
Aspergillus ochraceus	-2.2	-1.9	0.5	1.2
Aspergillus niger	-2.2	-1.9	nd	nd
Trichoderma viride	-2.2	-1.9	0.8	1.5
Penicillium ochrochloron	-2.2	-1.9	0.8	1.5
Penicillium funiculosum	-2.2	-1.9	0.5	1.2
Penicillium verrucosum var. cyclopium	-2.2	-1.9	0.2	0.9

Nd – not determined; SI=log (IC50/MIC); the positive values indicate higher toxicity to bacteria or fungi, while negative values indicate higher toxicity to colon cells.

Selective toxicity

Calculation of the SI indicated the absence of selective toxicity of EO to all tested microorganisms, while SI values obtained for PDW were variable. They were almost all positive in respect to HCT116 cells (indicating selectivity against microorganisms), but in respect to HT-29, positive values were determined for *L. monocytogenes* and almost all fungal strains (Table 5).

Anti-adhesive properties

Taking into account that SI values for PDW indicated higher toxicity to *L. monocytogenes* than to both human colon cells, we performed the *in vitro* adhesion-inhibition assay and monitored the potential of PDW to reduce the ability of *L. monocytogenes* to adhere to the colon cells. The obtained results indicated that PDW significantly decreased adhesion of *L. monocytogenes* to both HT-29 and HCT116 cells (Table 6). The inhibitory potential was more prominent in the case of HCT116 cells: 62% inhibition vs 29% inhibition in HT-29 cells.

DISCUSSION

Previous studies indicated that plants from *Juniperus* genus possess antibacterial and antifungal potential [10,26,27]. Although the antimicrobial potential of *J. communis* extracts has been previously reported, this work specifically monitored their effect against food-borne pathogenic, spoilage and poisoning microorganisms and additionally estimated their selective toxicity. The EO and PDW used in this work have been chemically characterized previously [21]. The main constituents determined in EO were α-pinene (23.61%), δ-cadinene (10.71%), sabinene (9.53%), germacrene D (7.25%), α-murolene (6.58%), γ-cadinene (5.87%), germacrene B (4.56%) and β-elemene (4.37%). Interestingly, all EO constituents were monoterpenoid (40.7%) and sesquiterpenoid (59.3%) hydrocarbons, with no oxygenated terpenes. On the other hand, only 3.2% of the total PDW content was identified, and rutin (12.2 mg/g), quinic acid (11.1 mg/g), catechin (5.53 mg/g) and epicatechin (1.74 mg/g) were the most abundant.

Concerning the antifungal properties tested in this work, a general observation was that EO induced

Table 6. Anti-adhesive properties of PDW against *L. monocytogenes* on HT-29 and HCT 116 cells.

	HT-29			HCT116		
	Bacteria added	Adhered without PDW	Adhered with PDW	Bacteria added	Adhered without PDW	Adhered with PDW
	262±18*	77±14*	54±5*	130±19*	18.5±2*	7±1*
% of adhesion		29.3%	20.7%		13.7%	5.2
Inhibition of adhesion			**29%**			**62%**

* x 10^6/mL; concentration of PDW in treatment was ½MIC=0.192 mg mL^{-1}

only a weak, almost negligible effect, while the effect of PDW was remarkably higher. Literature data also indicates a weak antifungal effect of EO prepared from *J. communis* berries [28] and a slightly higher effect of oil prepared from needles [29]. To the best of our knowledge, the antifungal effect of PDW has not been previously tested, but literature data show the antifungal effect of the hydroalcoholic extract of *J. communis* [30]. According to Holetz et al. [31] who classified the antimicrobial agents according to MIC values into groups with high (MIC<0.1 mg mL^{-1}), moderate (0.1<MIC<0.5 mg mL^{-1}) and weak (0.5<MIC<1 mg mL^{-1}) antimicrobial activities, the antifungal effect of PDW could be considered as moderate. Bearing in mind that toxigenic fungi associated with food spoilage and poisoning belong mainly to three genera, *Aspergillus*, *Fusarium* and *Penicillium*, and that *Trichoderma* species could also be important food contaminants [32, 33], the antifungal activity of PDW obtained in this work could be of special interest for food preservation. According to SI values, the cytotoxicity of PDW against human colon cells was markedly lower than its antifungal effect, especially in the case of the more resistant HCT116 cells. The different sensitivities of the two cell lines could be attributed to their intrinsic differences [34], and they show the importance of using more than one cell line for SI assessment. The generally high SI values determined for almost all tested fungi are particularly important because biocontrol of toxigenic fungi present in foods has to make it safe for human use.

The antibacterial effect of *J. communis* EO and PDW was generally weak. Similar to our results, Glišić et al. [28] revealed low antibacterial activity of *J. communis* EO, but higher activity of different preparations of EO fractions. However, good antibacterial properties of some *J. communis* EOs were also previously reported [10,35], indicating that the origin and distilling procedure significantly influences the chemical composition and consequently the biological activities of the oils. The low antimicrobial activity of our EO could be due to the unusually high content of hydrocarbon terpenes, which commonly possess a lower antimicrobial potential than oxygenated terpenoids [36]. On the other hand, the higher antimicrobial activity of PDW, especially against micromycetes and *L. monocytogenes*, could be attributed to the antimicrobial properties of polyphenolics which are well established [37].

L. monocytogenes is an invasive food-borne pathogen that commonly enters the host by consumption of contaminated food. In our experiments, *L. monocytogenes* was the most sensitive bacterium to *J. communis* EO and especially PDW. Even more important is the finding that EO and PDW could act synergistically with the tested antibiotics against *L. monocytogenes*, lowering their MICs. While EO decreased the MICs of Str and Amp 4- and 8-fold, respectively, PDW decreased the MICs of all three antibiotics 32-fold. Considering the growing problem of antibiotic resistance, the use of natural compounds as adjuvants capable of increasing the efficacy of conventional antibiotics could be an important strategy to combat infections [38]. Amplified antibiotic activity could consequently decrease their therapeutic doses, which is additionally important in terms of their registered side effects [39].

J. communis PDW showed selective toxicity against *L. monocytogenes* and reduction of adhesion of this bacterium to intestinal cells *in vitro*. This is another important finding since *L. monocytogenes* and many other food-borne pathogens need to cross the epithelial barrier of the intestine to cause a systemic disease. The first step in this process is attachment to host intestinal cells, thus the search for natural products with anti-adhesive properties is encouraged [23,40]. The ability of *J. communis* products to inhibit adhesion of *Campylobacter jejuni* to polystyrene has been recently reported [41]. Moreover, in the same study, anti-adhesive properties were even increased in co-cultured *C. jejuni* and *L. monocytogenes*. Yet, to the best of our knowledge this is the first report indicating the potential of *J. communis* PDW to reduce adhesion of *L. monocytogenes* to intestinal cell lines.

In conclusion, the moderate antimicrobial potential of PDW of *Juniperus communis* against *Aspergillus*, *Penicillium* and *Trichoderma* species, as well as against *Listeria monocytogenes*, were demonstrated. Both EO and PDW synergistically potentiated the effect of conventional antibiotics against *L. monocytogenes*. Regarding the colon cell lines used, high selective toxicity of PDW to tested micromycetes and *L. monocytogenes* was detected. PDW also significantly reduced *in vitro* adhesion of *L. monocytogenes* to HT-29 and HCT116 colon cells. The obtained results indicate that material remaining after the distillation of *J. communis* EO contains bioactive compounds and endorse it

for further study as a potential natural antimicrobial preservative, as well as an adjuvant in conventional therapy of listeriosis.

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REFERENCES

1. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015;13(1):42-51.
2. Wiederhold NP. Antifungal resistance: current trends and future strategies to combat. Infect Drug Resist. 2017;10:249-59.
3. Savoia D. Plant-derived antimicrobial compounds: alternatives to antibiotics. Future microbial. 2012;7(8):979-90.
4. Adams RP. Junipers of the world: the genus *Juniperus*. 4th ed. Waco, USA: Trafford Publishing; 2014. 422 p.
5. Khan M, Khan AU, Gilani AH. Pharmacological explanation for the medicinal use of *Juniperus excelsa* in hyperactive gastrointestinal and respiratory disorders. J Nat Med. 2012;66(2):292-301.
6. Leporatti ML, Ivancheva S. Preliminary comparative analysis of medicinal plants used in traditional medicine of Bulgaria and Italy. J Ethnopharmacol. 2003;87(2-3):123-42.
7. Elmastaş M, Gülçin İ, Beydemir Ş, İrfan Küfrevioğlu Ö, Aboul-Enein HY. A study on the in vitro antioxidant activity of juniper (*Juniperus communis* L.) fruit extracts. Anal Lett. 2006;39(1):47-65.
8. Kurti L, Jovanova B, Kelmendi A, Hamidi M, Kadifkova-Panovska T, Kulevanova S. Antioxidant activity of Macedonian Juniper (*Juniperus communis* L.) fruit extracts. Toxicol Lett. 2015;238(2):S89.
9. Orhan N, Aslan M, Demirci B, Ergun F. A bioactivity guided study on the antidiabetic activity of Juniperus oxycedrus subsp. oxycedrus L. leaves. J Ethnopharmacol. 2012;140(2):409-15.
10. Zheljazkov VD, Semerdjieva IB, Dincheva I, Kacaniova M, Astatkie T, Radoukova T, Schlegel V. Antimicrobial and antioxidant activity of Juniper galbuli essential oil constituents eluted at different times. Ind Crops Prod. 2017;109:529-37.
11. Golebiowski M, Paszkiewicz M, Halinski L, Malinski E, Stepnowski P. Chemical composition of commercially available essential oils from Eucalyptus, Pine, Ylang, and Juniper. Chem Nat Compd. 2009;45(2):278-9.
12. Ciesla WM. Non-wood forest products from conifers. 1st ed. Rome: Food and Agriculture Organization of the United Nations; 1998. 124 p.
13. Lesjak MM, Beara IN, Orčić DZ, Ristić JD, Anačkov GT, Božin BN, Mimica-Dukić NM. Chemical characterisation and biological effects of *Juniperus foetidissima* Willd. 1806. LWT-Food Sci Technol. 2013;53(2):530-9.
14. Uríčková V, Sádecká J, Májek P. Classification of Slovak juniper-flavoured spirit drinks. J Food Nutr Res. 2015;54(4):298-307.
15. Brindza J, Toth D, Ostrovsky R, Kucelova L. Traditional Foods in Slovakia. In: Kristbergsson K, Oliveira J, editors. Traditional Foods. General and Consumer Aspects. New York, USA: Springer; 2016. p. 71-84.
16. Nybe EV, Mini Raj N, Peter KV. Spices. 1st ed. New Delhi: New India Publishing Agency; 2007. 316 p. (Horticulture science series; vol. 5).
17. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010;4(8):118-26.
18. Wilson BG, Bahna SL. Adverse reactions to food additives. Ann Allergy Asthma Immunol. 2005;95(6):499-507.
19. Si W, Gong J, Tsao R, Zhou T, Yu H, Poppe C, Johnson R, Du Z. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. J Appl Microbiol. 2006;100(2):296-305.
20. Gavarić N, Kovač J, Kretschmer N, Kladar N, Možina SS, Bucar F, Bauer R, Božin B. Natural Products as Antibacterial Agents — Antibacterial Potential and Safety of Post-distillation and Waste Material from *Thymus vulgaris* L., *Lamiaceae*. In: Bobbarala V, editor. Concepts, Immunology and Microbiology: Compounds and the Alternatives of Antibacterials. Rijeka, Croatia: InTech; 2015. p. 123-52.
21. Vasilijević B, Knežević-Vukčević J, Mitić-Ćulafić D, Orčić D, Franciškovič M, Srdic-Rajic T, Jovanović M, Nikolić B. Chemical characterization, antioxidant, genotoxic and in vitro cytotoxic activity assessment of *Juniperus communis* var. saxatilis. Food Chem Toxicol. 2018;112:118-25.
22. Sarker SD, Nahar L, Kumarasamy Y. 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, 2007;42:321-4.
23. Džamić AM, Nikolić BJ, Giweli AA, Mitić-Ćulafić DS, Soković MD, Ristić MS, Knežević-Vukčević JB, Marin PD. Libyan Thymus capitatus essential oil: antioxidant, antimicrobial, cytotoxic and colon pathogen adhesion-inhibition properties. J Appl Microbiol. 2015;119(2):389-99.
24. Mulyaningsih S, Sporer F, Zimmermann S, Reichling J, Wink M. Synergistic properties of the terpenoids aromadendrene

and 1, 8-cineole from the essential oil of *Eucalyptus globulus* against antibiotic-susceptible and antibiotic-resistant pathogens. Phytomedicine. 2010;17(13):1061-6.
25. Nunes BC, Martins MM, Chang R, Morais SA, Nascimento EA, de Oliveira A, Cunha LCS, da Silva CV, Teixeira TL, Ambrósio MALV, Martins CHG, de Aquino FJT. Antimicrobial activity, cytotoxicity and selectivity index of *Banisteriopsis laevifolia* (A. Juss.) B. Gates leaves. Ind Crops Prod. 2016;92:277-89.
26. Cavaleiro C, Pinto E, Gonçalves MJ, Salgueiro L. Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. J Appl Microbiol. 2006;100(6):1333-8.
27. Taviano MF, Marino A, Trovato A, Bellinghieri V, Melchini A, Dugo P, Cacciola F, Donato P, Mondello L, Güvenç A, De Pasquale R, Miceli N. *Juniperus oxycedrus* L. subsp. oxycedrus and *Juniperus oxycedrus* L. subsp. macrocarpa (Sibth. & Sm.) Ball."berries" from Turkey: Comparative evaluation of phenolic profile, antioxidant, cytotoxic and antimicrobial activities. Food Chem Toxicol. 2013;58:22-9.
28. Glišić SB, Milojević SŽ, Dimitrijević SI, Orlović AM, Skala DU. Antimicrobial activity of the essential oil and different fractions of *Juniperus communis* L. and a comparison with some commercial antibiotics. J Serb Chem Soc. 2007;72(4):311-20.
29. Cabral C, Francisco V, Cavaleiro C, Gonçalves MJ, Cruz MT, Sales F, Batista MT, Salgueiro L. Essential oil of *Juniperus communis* subsp. alpina (Suter) Čelak needles: chemical composition, antifungal activity and cytotoxicity. Phytother Res. 2012;26(9):1352-7.
30. Fierascu I, Ungureanu C, Avramescu SM, Cimpeanu C, Georgescu MI, Fierascu RC, Ortan A, Sutan AN, Anuta V, Zanfirescu A, Dinu-Pirvu CE, Valescu BS. Genoprotective, antioxidant, antifungal and anti-inflammatory evaluation of hydroalcoholic extract of wild-growing *Juniperus communis* L. (Cupressaceae) native to Romanian southern sub-Carpathian hills. BMC Complement Altern Med. 2018;18(1):3.
31. Holetz FB, Pessini GL, Sanches NR, Cortez DAG, Nakamura CV, Dias Filho BP. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem Inst Oswaldo Cruz. 2002;97(7):1027-31.
32. Adeyeye SA. Fungal mycotoxins in foods: A review. Cogent Food Agric. 2016;2:1213127.
33. Ashiq S. Natural occurrence of mycotoxins in food and feed: Pakistan perspective. Compr Rev Food Sci Food Saf. 2015;14(2):159-75
34. Ahmed D, Eide PW, Eilertsen IA, Danielsen SA, Eknaes M, Hektoen M, Lind GE, Lothe RA. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis. 2013;2(9):e71.
35. Filipowicz N, Kamiński M, Kurlenda J, Asztemborska M, Ochocka JR. Antibacterial and antifungal activity of juniper berry oil and its selected components. Phytother Res. 2003;17(3):227-31.
36. Griffin SG, Wyllie SG, Markham JL, Leach DN. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour Frag J. 1999;14(5):322-32.
37. Daglia M. Polyphenols as antimicrobial agents. Curr Opin Biotechnol. 2012;23(2):174-81.
38. Milenković M, Stošović J, Slavkovska V. Synergy between Essential Oils of *Calamintha* Mill. Species (Lamiaceae) and Antibiotics. Nat Prod Commun. 2018;13(3):371-4.
39. Cunha BA. Antibiotic side effects. Med Clin North Am. 2001;85(1):149-85.
40. Lee JH, Shim JS, Chung MS, Lim ST, Kim KH. In vitro antiadhesive activity of green tea extract against pathogen adhesion. Phytother Res. 2009;23(4):460-6.
41. Klančnik A, Zorko Š, Toplak N, Kovač M, Bucar F, Jeršek B, Smole Možina S. Antiadhesion activity of juniper (Juniperus communis L.) preparations against Campylobacter jejuni evaluated with PCR-based methods. Phytother Res. 2017; 32(3):542-50.