Antilisterial effect of juniper (Juniperus communis) and its mixed application with winter savory (Satureja montana) in beef protection

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Abstract. Juniperus communis essential oil and post-distillation waste were tested for their antibacterial potential against common food contaminants. Results of microdilution assay directed further study of Listeria monocytogenes. Checkerboard assay showed synergistic antilisterial effect of both substances with conventional antibiotics. By evaluating of their cytotoxicity on human colon cells, post-distillation waste was denoted as selective against L. monocytogenes, being able to interfere with its in vitro adhesion on colon cells. On the other hand, the food preservative potential of J. communis essential oil was studied and compared with the activity of Satureja montana essential oil. In a microdilution assay, both substances induced antilisterial effect against reference ATCC 19111 strain and three wild isolates. A checkerboard assay showed synergism against isolates. An in vitro time-kill assay was used to confirm the types of interaction, and to estimate curve MIC values. Finally, in situ antilisterial efficacies of the individual essential oils and their mixture on red wine-marinated beef, previously inoculated with ATCC 19111 strain or primoisolates from beef carcasses, were determined. All treatments enhanced the antilisterial potential of wine marinade. In conclusion, derivatives of J. communis possess significant antilisterial potential both in in vitro and in situ conditions, so further research is advised.

1. Introduction
In recent years, interest in plant-derived food additives and preservatives has expanded for numerous reasons. Synthetic additives and preservatives can induce different side effects, including altering biochemical parameters and inducing oxidative stress [1,2], allergic and hypersensitivity reactions [3,4], as well as inducing genotoxic and/or carcinogenic effects [5]. On the other hand, growing data confirming different health-promoting properties of natural flavors and preservatives is additionally strengthening this area [6]. Among aromatic and medicinal plants that are used in food preparations, common juniper (Juniperus communis L.) is important for food and beverage preparations. Juniper is a common meat seasoning, used to impart a sharp, clear flavor to meat, especially of game, wild birds and pork [7,8]. Furthermore, it is used to aromatize gin and the local juniper brandies manufactured in Slovenia, Slovakia and Serbia [9,10]. Juniperus species are also used in traditional medicine. Scientific studies have revealed juniper’s numerous biological activities, including antioxidant and anti-inflammatory [11], nephroprotective and hepatoprotective effects [12,13], as well as adjuvant cytotoxicity against cancer cells [14]. Moreover, the antimicrobial effect of Juniperus plants has also been reported [15-17]. Although antimicrobial effects were partly investigated previously, the effects of juniper had not been specifically directed toward foodborne pathogenic and spoilage bacteria, and had not been investigated in in situ conditions. That directed our research, and we studied the selective toxicity of J. communis derivatives against food contaminants [18], as well as in situ antibacterial effects of J. communis essential oil (EO) applied in wine-marinated beef individually and in combination with Satureja montana EO [19]. The aim of this paper is to report the results of our recent studies concerning the antibacterial potential of J. communis derivatives.
2. Antibacterial effect of *J. communis* derivatives and their synergistic potential to enhance conventional antibiotics activity

Juniper berries were collected from Stara Planina Mountain in July 2014. Distillation of EO was performed in Clevenger-type apparatus, while the aqueous solution remaining after distillation was used to prepare post-distillation waste (PDW), both as described by Vasilijević et al. [14]. Since juniper EO production is extensive, mainly due to the beverage, cosmetic and perfumery industries [20], the potential use of PDW as a source of biologically active substances seem to be of great importance. Chemical characterization of EO and PDW was provided by GC-MS and LC-MS/MS, respectively. Dominant constituents, amounting to more than 1% of EO and more than 0.1 mg/g of PDW, are listed in Table 1.

**Table 1.** Chemical composition of *Juniperus communis* essential oil and post-distillation waste

| Essential oil compound | Content (%) | Post-distillation waste | Content (mg/g of dw) |
|------------------------|-------------|-------------------------|----------------------|
| α-Pinene               | 23.61       | Quercetin 3-O-rhamnosylglucoside | 12.25 ± 0.37         |
| δ-Cadinene             | 10.71       | Quinic acid             | 11.09 ± 1.11         |
| Sabinene               | 9.53        | Catechin                | 5.534 ± 0.553        |
| Germacrene D           | 7.25        | Epicatechin             | 1.738 ± 0.174        |
| α-Muurolene            | 6.58        | Amentoflavone           | 0.392 ± 0.012        |
| γ-Cadinene             | 5.87        | Umbiliferone            | 0.253 ± 0.025        |
| Germacrene B           | 4.56        | Quercetin 3-O-glucoside | 0.232 ± 0.007        |
| β-Elemene              | 4.37        | Protocatechuic acid     | 0.145 ± 0.012        |
| α-Humulene             | 3.08        | Apigenin 7-O-glucoside  | 0.140 ± 0.007        |
| β-Caryophyllene        | 2.94        | Quercetin 3-O-rhamnoside| 0.139 ± 0.008        |
| Vidrene (thujopsene)   | 2.66        |                         |                      |
| Limonene               | 2.07        |                         |                      |
| α-Cadinene             | 1.83        |                         |                      |
| β-Myrcene              | 1.36        |                         |                      |
| 4-Terpineol            | 1.26        |                         |                      |
| γ-Elemene              | 1.31        |                         |                      |
| cis-Muurola-4(14),5-diene | 1.15     |                         |                      |
| β-Pinene               | 1.10        |                         |                      |

The antibacterial potential of *J. communis* EO and PDW against several reference strains of common food contaminants, monitored in microdilution assay as previously explained [21], revealed that *Listeria monocytogenes* was the most sensitive, especially in the case of PDW (minimal inhibitory concentration - MIC value 0.39 mg/mL, Table 2). Since the intention was to determine the adjuvant potential of *J. communis* derivatives to enhance the activity of common antibiotics, their effectiveness was also determined in a microdilution assay.

Effects of combinations of EO/PDW with ampicillin, azithromycin and streptomycin were estimated in the checkerboard assay. Type of interaction was determined by calculating fractional inhibitory concentration index (FICI) for two antimicrobial agents applied in combination, as in Mulyaningsih et al. [22]. The results obtained pointed to the synergistic enhancement of antibiotic activity against *L. monocytogenes* and *Staphylococcus aureus* (Table 3), while the best type of interaction between co-tested compounds against *Shigella flexneri* and *Pseudomonas aeruginosa* was additivism (data not shown). PDW synergistically increased the antimicrobial potential of all three
antibiotics against \( L. \ monocytogenes \), as well as of azithromycin against \( S. \ aureus \), while EO induced synergistic effects with ampicillin and streptomycin. Since antibiotic resistance is considered to be a serious global public health problem, and because antibiotic use can have side effects both on human health and the environment [23-25], this result is important.

Table 2. Antibacterial effect of \( Juniperus \ communis \) derivatives and conventional antibiotics

| Bacterial strains (ATCC) | PDW (mg/ml) | EO (mg/ml) | Amp\(^{1}\) (µg/ml) | Azm\(^{2}\) (µg/ml) | Str\(^{3}\) (µg/ml) |
|-------------------------|------------|------------|-------------------|-----------------|--------------|
|                         | MIC\(^{4}\) | MIC        | MBC\(^{5}\)       | MIC             | MBC          |
| Salmonella Enteritidis  | 12.5       | >25        | >50               | 800             | 50           |
|                         |            |            | >50               | 800             | 100          |
|                         |            |            |                   | 50              | 12.5         |
|                         |            |            |                   |                 | 12.5         |
| Escherichia coli        | 6.25       | >25        | >50               | 160             | >160         |
|                         |            |            | >50               | >160            | 50           |
|                         |            |            |                   |                 | 6.25         |
|                         |            |            |                   |                 | 6.25         |
| Shigella flexneri       | 3.125      | 25         | 12.5              | 50              | 0            |
|                         |            |            | 25                | 100             | 0            |
|                         |            |            |                   | 6.25            | 12.5         |
|                         |            |            |                   |                 | 1.56         |
|                         |            |            |                   |                 | 1.56         |
| Pseudomonas aeruginosa  | 3.125      | >25        | >50               | 160             | 1600         |
|                         |            |            | >50               | 1600            | 50           |
|                         |            |            |                   |                 | 100          |
|                         |            |            |                   |                 | >100         |
|                         |            |            |                   |                 | 12.5         |
|                         |            |            |                   |                 | 50           |
| Staphylococcus aureus   | 3.125      | 12.5       | 6.25              | 12.5            | 12.5         |
|                         |            |            | 12.5              | 25              | 1.56         |
|                         |            |            |                   |                 | 25           |
|                         |            |            |                   |                 | 6.25         |
|                         |            |            |                   |                 | 12.5         |
| Listeria monocytogenes  | 0.39       | 0.78       | 3.125             | 6.25            | 50           |
|                         |            |            | 3.125             | 50              | 3.125        |
|                         |            |            |                   |                 | 6.25         |
|                         |            |            |                   |                 | 12.5         |
|                         |            |            |                   |                 | 50           |
| Enterococcus faecalis   | 12.5       | 25         | 12.5              | 160             | 1600         |
|                         |            |            | 25                | 25              | 25           |
|                         |            |            |                   |                 | 200          |
|                         |            |            |                   |                 | 200          |

1Amp – ampicillin; 2Azm – azithromycin; 3Str – streptomycin; 4MIC – minimal inhibitory concentration; 5MBC – minimal bactericidal concentration

3. Antilisterial effect of \( J. \ communis \) derivatives – selectivity and antiadhesive potential

Accordingly to results of microdilution assay, further investigation was focused on \( L. \ monocytogenes \), as it was the most sensitive of the examined bacteria to EO and especially PDW. Bearing in mind that \( L. \ monocytogenes \) is an invasive food-borne pathogen that enters the host mainly in the intestine [26], we considered study of \( J. \ communis \) derivatives on human intestinal cells to be important. We used in vitro colon cell line models and determined the cytotoxicity against human cells, which enabled us to estimate if they were able to selectively kill only bacterial cells. Cytotoxicity was monitored in the MTT assay and IC\(_{50}\) values were determined [27]. Calculation of selectivity index (SI) values was performed as previously described [28]. The results obtained indicated that PDW induced lower cytotoxicity against both used colon cell lines, and therefore, PDW was selectively toxic to \( L. \ monocytogenes \) (Table 4). PDW, as a highly selective antilisterial agent, was further screened in in vitro adhesion-inhibition assay, performed as previously described [29]. This assay determined PDW’s potential to inhibit adhesion of \( L. \ monocytogenes \) to human intestinal host cells. Results revealed that a sub-inhibitory concentration (1/2 MIC) of PDW notably prevented \( L. \ monocytogenes \) adhesion to intestinal cells. The inhibition of adhesion for HT-29 and HCT116 cell line was 29% and 62%, respectively.

Table 4. \( J. \ communis \) EO and PDW: selective toxicity against \( L. \ monocytogenes \) and reductive potential of its adhesion on human colon cell lines HCT-116 and HT-29

|                   | PDW+amp | PDW+azm | PDW+str | EO+amp | EO+str | PDW+azm |
|-------------------|---------|---------|---------|--------|--------|---------|
| \( L. \ monocytogenes \) | 0.281   | 0.281   | 0.156   | 0.187  | 0.312  | 0.250   |
| \( S. \ aureus \)    |         |         |         |        |        |         |

3FICI = (MIC\(_{EO/PDW \text{ in comb.}}\)/MIC\(_{EO/PDW \text{ alone}}\)) + (MIC\(_{antibiotic \text{ in comb.}}\)/MIC\(_{antibiotic \text{ alone}}\)); Type of interaction is considered synergistic if FICI≤0.5
4. Antilisterial effect of *J. communis* and *S. montana* EOs - *in vitro* screening of primoisolates susceptibility

In further work, we focused on possible applications of *J. communis* EO in biocontrol of *L. monocytogenes* in meat. Taking into account that *J. communis* could be used individually or in combinations with EOs of other spices/herbs, *Satureja montana* EO was involved in this part of the research. It was selected in accordance with preliminary sensory evaluation (data not shown). In addition, the antilisterial effect of this oil could be used as reference value, since it has previously been well documented [30]. The EOs used in this part of the study were commercially provided and chemically characterized by GC-MS (Table 5), as previously described by Vasilijević et al. [19].

Comparative analysis of the EOs’ antilisterial effects was performed in microdilution assay, applied to two primoisolates from foods (isolates LMB and LMS, from beef carcass and salmon, respectively), one slaughterhouse environmental isolate (isolate LMT, from water drainage tunnel), and one reference strain ATCC 19111. MIC values were determined to be 0.5-1% (Figure 1).

**Table 5.** Chemical composition* of commercially provided *J. communis* and *S. montana* EOs

| Compound                      | Content (%) | Compound                      | Content (%) |
|-------------------------------|-------------|-------------------------------|-------------|
| **Juniperus communis EO**     |             | **Satureja montana EO**       |             |
| α-Pinene                      | 47.8        | Carvacrol                     | 30.7        |
| Sabinene                      | 11.0        | Thymol                        | 18.0        |
| β-Pinene                      | 8.5         | para-Cymene                   | 15.6        |
| Limonene                      | 5.8         | Borneol                       | 5.9         |
| Myrcene                       | 3.4         | γ-Terpinene                   | 5.5         |
| para-Cymene                   | 2.9         | cis-Caryophyllene             | 3.7         |
| α-Thujene                     | 2.7         | Linalool                      | 3.4         |
| cis-Sabinene hydrate          | 2.5         | trans-Murola-4(14),5-diene    | 1.5         |
| trans-Verbenol                | 1.6         | Camphor                       | 1.3         |
| Caryophyllene oxide           | 1.6         | α-Terpinene                   | 1.3         |
| trans-Pinocarvyl acetate      | 1.3         | α-Phellandrene                | 1.2         |
| α-Campholenal                 | 1.2         | Camphene                      | 1.0         |
|                               |             | Myrcene                       | 1.0         |

*Only dominant constituents comprising more than 1% of EO are presented.
1% concentration corresponds to 10 µl/ml EOs; i.e. to 8.58 mg/ml and 9.16 mg/ml for J. communis and S. montana EO, respectively.

**Figure 1.** MIC and MBC values of J. communis and S. montana EOs determined in microdilution assay performed on L. monocytogenes ATCC 19111 reference strain, and on isolates from salmon (LMS), slaughterhouse water drainage tunnel (LMT), and beef carcass (LMB).

The combined effect of the EOs was monitored in checkerboard and in time-kill assays, both applied as previously explained [19]. Screening in the checkerboard assay was performed with all tested isolates, while the time-kill assay was applied only to LMB isolate and ATCC 19111 strain. Results obtained in the checkerboard assay showed that synergism was determined for some oil combinations in the case of all isolates, while interaction of EOs active against ATCC 19111 was, at best, additive (Table 6).

**Table 6.** Synergistic and additive interaction between J. communis and S. montana oils determined by FICI values

| conc. J. communis [%] | conc. S. montana [%] | FICI* |
|------------------------|-----------------------|-------|
| Interpretation          |                       |       |
| LMS                    |                       |       |
| 0.25                   | 0.125                 | 0.625 | Additive |
| 0.125                  | 0.125                 | 0.375 | Synergistic |
| 0.0625                 | 0.25                  | 0.375 | Synergistic |
| 0.03125                | 0.25                  | 0.3125| Synergistic |
| 0.015625               | 0.25                  | 0.281 | Synergistic |
| 0.0078125              | 0.25                  | 0.266 | Synergistic |
| LMT                    |                       |       |
| 0.25                   | 0.25                  | 0.75  | Additive |
| 0.125                  | 0.25                  | 0.5   | Additive |
| 0.0625                 | 0.25                  | 0.375 | Synergistic |
| 0.03125                | 0.25                  | 0.3125| Synergistic |
| 0.015625               | 0.25                  | 0.281 | Synergistic |
| 0.0078125              | 0.25                  | 0.266 | Synergistic |
| LMB                    |                       |       |
| 0.25                   | 0.015625              | 0.516 | Additive |
| **0.25**               | **0.125**             | **0.375** | **Synergistic** |
| 0.125                  | 0.25                  | 0.375 | Synergistic |
| 0.0625                 | 0.25                  | 0.3125| Synergistic |
| 0.03125                | 0.25                  | 0.281 | Synergistic |
| 0.015625               | 0.5                   | 0.515 | Additive |
| ATCC 19111             |                       |       |
| **0.25**               | **0.125**             | **0.75** | **Additive** |

\^FICI = (MIC_{J.c.in comb}/MIC_{J.c.,alone}) + (MIC_{S.m.in comb}/MIC_{S.m.,alone}); Type of interaction is: synergistic if FICI ≤ 0.5, additive if 0.5 < FICI ≤ 1. The combination tested in time-kill assay is bolded.

Whilst for the checkerboard assay, combinations of EOs were prepared in different ratios, for the time-kill assay, only combinations containing EOs of J. communis and S. montana in the ratio 2:1 were used, in accordance with their effectiveness against LMB and ATCC 19111 in the checkerboard assay and with preliminary sensory evaluation (data not shown). Results of the time-kill assay...
confirmed the additivism observed in the case of ATCC 19111 (data not shown), and synergism detected in the case of LMB (Figure 2).

Interestingly, the time-kill assay revealed higher susceptibility of bacteria than was observed in the checkerboard and microdilution assays. This was attributed to differences in oxygen availability, which consequently induced metabolic changes. As a facultative anaerobe, *L. monocytogenes* performs aerobic respiration and possesses intensive metabolism in aerobic conditions, which leads to an increase of bacterial sensitivity to different stressors, including antimicrobials [31,32]. To quantify the observed differences in sensitivity, we further used data obtained from time-kill curves and calculated the curve MIC values (cMICs). This involved plotting the growth/inhibition rates during the first 12 hours of incubation vs. concentrations of tested substances, as previously explained [19]. Determined cMICs were approximately 0.03-0.04 for *S. montana* EO, and 0.10-0.14 for *J. communis* EO, both being remarkably lower than MICs determined in the microdilution assay.

Figure 2. Time dynamics of antilisterial effects against LMB isolate of essential oils of *J. communis*
5. Antilisterial potential of *J. communis* and *S. montana* EOs - *in situ* screening on wine-marinated beef

In further work, we monitored *in situ* antilisterial effect of *J. communis* and *S. montana* EOs on red wine-marinated beef. The EOs were tested individually and in a mixture prepared in the same ratio (*J. communis*: *S. montana* = 2:1) as in the previously explained *in vitro* time-kill assay. The preparation of wine marinades for the beef sirloin steaks and the marination process are explained in detail elsewhere [19]. Certainly, the sensory acceptability of the concentrations/combinations of the EOs used was previously confirmed (data not shown). To monitor the antilisterial effects, beef steaks were inoculated with *L. monocytogenes* (ATCC 19111 and LMB, final concentration 5 log CFU/g) prior to the marination process. Antilisterial effects on the marinated meat were determined periodically during 15 days of refrigerated storage. Analysis of results in a time dependent manner (monitoring the time dynamics for each marinade treatment) showed a bactericidal effect was obtained during the marination period (24 h), while subsequent meat storage was mainly accompanied by bacteriostatic effects (Tables 7 and 8).

Comparison of the different marinades with saline showed the antilisterial efficacy of *J. communis* EO was almost the same as that of *S. montana* EO, while the effect of the EO mixture was even higher in the case of *L. monocytogenes* ATCC 19111. Due to their antimicrobial active substances, such as thymol and carvacrol in *S. montana* EO, and pinene, limonene and sabinene in *J. communis* EO, the overall antimicrobial potential of red wine marinades was strengthened [33]. Taking into account that literature indicates the preservative potential of *S. montana* EO [30,34], our result showing the *in situ* antilisterial activities of *J. communis* and *S. montana* EOs are comparable, is of notable importance.

**Table 7.** Effect of red wine marinades containing EOs of *J. communis*, *S. montana* and their mixture on growth inhibition of *L. monocytogenes* ATCC 19111

| Day | S1 log CFU/g | W2 log CFU/g | W+JC-EO log CFU/g | W+SM-EO log CFU/g | W+Mix log CFU/g |
|-----|--------------|--------------|-------------------|-------------------|----------------|
| 0   | 5.24±0.15    | 3.61±0.20    | 3.71±0.18         | 3.55±0.21         | 3.32±0.19      |
| 1   | 5.44±0.19    | 3.82±0.15    | 3.47±0.22         | 3.22±0.20         | 3.14±0.17      |
| 3   | 5.65±0.27    | 3.59±0.19    | 3.30±0.22         | 3.49±0.28         | 2.83±0.14      |
| 5   | 6.09±0.22    | 4.19±0.21    | 3.46±0.25         | 3.22±0.27         | 2.43±0.15      |
| 7   | 6.59±0.24    | 4.14±0.16    | 3.32±0.18         | 3.10±0.16         | 2.15±0.16      |
| 9   | 6.77±0.25    | 4.25±0.25    | 3.28±0.21         | 3.36±0.24         | 2.70±0.11      |
| 11  | 6.84±0.26    | 4.07±0.21    | 3.44±0.18         | 2.98±0.18         | 2.68±0.15      |
| 13  | 7.11±0.33    | 4.73±0.18    | 3.57±0.23         | 3.42±0.21         | 2.55±0.14      |
| 15  | 7.04±0.31    | 4.59±0.22    | 3.04±0.26         | 2.80±0.24         | 2.45±0.17      |

*S* – saline; it was prepared as sterile solution of 0.85% w/v table salt and used as negative control, in order to estimate effects of marinades

**Table 8.** Effect of red wine marinades containing EOs of *J. communis* or *S. montana* EOs and their mixture on growth inhibition of *L. monocytogenes* LMB isolate

| Day | S1 log CFU/g | W2 log CFU/g | W+JC-EO log CFU/g | W+SM-EO log CFU/g | W+Mix log CFU/g |
|-----|--------------|--------------|-------------------|-------------------|----------------|
| 0   | 5.12±0.25    | 3.72±0.28    | 3.27±0.26         | 3.43±0.19         | 3.62±0.23      |
| 1   | 5.87±0.27    | 4.21±0.23    | 4.13±0.18         | 4.10±0.18         | 3.92±0.19      |
| 3   | 6.03±0.26    | 4.08±0.20    | 3.81±0.22         | 4.07±0.19         | 3.58±0.26      |
6. Conclusion and future perspectives

Taken together, the results obtained show the antilisterial effect of *J. communis* derivatives. They have potential to synergistically enhance the activities of other antimicrobials, such as conventional antibiotics and *S. montana* EO. Furthermore, selectivity and potential to reduce pathogen adhesion to intestinal cells, which leads to disturbance of the initial steps of host colonization, was shown for *J. communis* PDW. On the other hand, *J. communis* EO possesses preservative potential and could be used as a vehicle to control *L. monocytogenes* contamination in beef.

The data obtained indicate further investigation of the antimicrobial potential of *J. communis* could be developed in several directions. Searching for active substances in both EO and PDW, as well as elucidating their underlying mechanisms responsible for the observed effects is advised. In addition, taking into account the low cytotoxicity for human cells, determining the sensory properties and food preservative potential of PDW seems to be interesting. Finally, this work indicates investigations should be performed in other food matrices, as well as against different microbial contaminants.

Acknowledgement

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. 172058. We thank Branko Velebit, Institute of Meat Hygiene and Technology, for providing *L. monocytogenes* isolates.

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