Comprehensive Analysis of the Herbal Mixture Made of *Juniperus oxycedrus* L. Berries, Inner Bark of *Betula pendula* Roth., and Grains of *Avena sativa* L.

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Abstract

This is the first report of the high-performance liquid chromatography and gas chromatography–mass spectrometry profile of a herbal mixture (HM) made of *Juniperus oxycedrus* L. (redberry juniper) berries, inner bark of *Betula pendula* Roth. (silver birch), and grains of *Avena sativa* L. (oat), and its effect on the No. of micronuclei (MN) in human lymphocytes and toxicity toward *Artemia salina*. Constituents represented by over 1000 µg per g of methanol dry extract were gallic acid, protocatechuic acid, and ameto-flavone. The methanol extract of the HM at a concentration of 2.0 µg/mL decreased MN frequency by 38.3%, which was more than 3 times greater than that of the radioprotectant amifostine. The essential oil isolated from the HM was composed mainly of β-myrcene (32%) and showed weaker toxicity toward *Artemia salina* than the positive control after both incubation periods (24 h and 48 h). These findings suggest that the examined HM, beside its ethnopharmacological relevance on the elimination of renal calculi, also significantly reduces the No. of MN in human lymphocytes.

Keywords

herbal mixture, *Juniperus oxycedrus* L., *Betula pendula* Roth., *Avena sativa* L., HPLC, GC-MS, methanol extract, essential oil, micronucleus test, *Artemia salina* toxicity

Introduction

Nephrolithiasis is a condition caused by the formation and displacement of crystal agglomerates, also known as stones or calculi, in the urinary tract.¹ The incidence of kidney stone formation is increasing across the world,²³ in order to eliminate renal colic and calculi many supplements have emerged. Development of new drug formulations from well-known folk remedies may decrease the side effects of drugs, while maintaining the therapeutic effect.³ A decoction made of the berries of *Juniperus oxycedrus* L., inner bark of *Betula pendula* Roth., and grains of *Avena sativa* L. is used in the Balkan peninsula to eliminate kidney stones. Although there are numerous studies that validate the medicinal relevance of the individual mixture components⁴–⁷ there are no data for the evaluation of this herbal mixture (HM). Therefore, in the present study, high-performance liquid chromatography (HPLC) analysis of the methanol extracts of the HM and individual mixture components was performed, as well as determination of the protective effect of the methanol extracts on human peripheral blood lymphocytes by cytokinesis-block micronucleus assay (CBMN). Furthermore, gas chromatography–mass spectrometry (GC-MS) analysis and acute toxicity to *Artemia salina* (brine shrimps) were evaluated for the essential oils of *J. oxycedrus* berries and HM. The headspace (HS) volatiles of the individual herbal materials and the mixture were also determined. The ratio of HM constituents was the same as in the HM traditionally used to treat kidney stones.

Results and Discussion

HPLC Analysis

The chemical composition of the methanol extracts of *J. oxycedrus* berries, inner bark of *B. pendula*, grains of *A. sativa*, and the

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HM were determined using HPLC-UV analysis. Phenolic compounds were identified according to their retention times and UV spectra, which were compared with the available commercial standards. Quantification was performed using calibration curves obtained for phenolic acids (gallic acid), flavonoids (rutin and quercetin), and procyanidin amounts. It was found that oat products possess an impact on kaempferol, apigenin, and luteolin were detected in lower μg/g of dry extracts, respectively), while several derivatives of kaempferol, apigenin, and luteolin were detected in lower amounts. It was found that oat products possess an impact on uric acid excretion. In the conducted experiments, patients with elevated uric acid levels were treated by giving them an herbal tea containing 75% of A. sativa, which reduced uric acid levels.8

The most significant difference between the methanol extracts was the presence of bioflavonoids, detected only in the methanol extract of J. oxycedrus berries and HM.

Kaempferol-3-O-glycoside and gallic acid were the most abundant compounds of the A. sativa methanol extract (1080 and 2060 μg/g of dry extracts, respectively), while several derivatives of kaempferol, apigenin, and luteolin were detected in lower amounts. It was found that oat products possess an impact on uric acid excretion. In the conducted experiments, patients with elevated uric acid levels were treated by giving them an herbal tea containing 75% of A. sativa, which reduced uric acid levels.8

Birch bark consists of brown inner bark or cambium (approximately 75%) and white outer bark (approximately 25%). In the sample of inner bark examined, cyanidin and a procyanidin derivative were detected, as well as catechin, epicatechin, and syringic acid.

Since standards of all identified compounds were not available, their identification was achieved by comparison with available standards as follows: compounds 14 and 15 were identified as kaempferol derivatives, compounds 17 and 18 as apigenin and luteolin derivatives, while compounds 19, 20, and 21 were identified according to literature data.9 Also, compounds 2, 8, and 10 exhibited characteristic UV absorption bands indicating the presence of procyanidin B2 and cyanidin-3-O-glucoside derivatives, according to available standards.

Our results are in agreement with those previously published. Emmons and Peterson10 have reported the following phenolic compounds in fractions of oat (groats and hulls): gallic acid, protocatechuic acid, p-hydroxybenzaldehyde, p-coumaric acid, vanillic acid, caffee acid, vanillin, ferulic acid, sinapic acid, and avenanthramide. Apigenin, luteolin, and tricin are 3 major flavonones found in oat flour and were identified in the vegetative plant only as glycosides.11 Kaempferol glycosides were isolated from the bran of A. sativa.12

Bioflavonoids were detected as the main compounds of J. oxycedrus berries methanol extract, which is in agreement with previously published results.9 Phenolic acids (syringic acid 4-β-glucopyranoside), flavonols {(+)-catechin 7-O-β-D-xylpyranoside and (+)-catechin} and procyanidin dimers and trimers were determined in the inner bark using LC/MS analysis.13,14 It was demonstrated that the inner bark contains high amounts of flavonoids, arylbutanoids, diarylheptanoids, simple phenolic compounds, phenolic acids, lignans, and procyanidins.15,16

Chemical Composition of HS Volatiles and Essential Oils of J. oxycedrus Berries and HM

The qualitative composition and relative abundance of the HS volatile compounds of the berries of J. oxycedrus (HS1), inner bark of B. pendula (HS2), grains of A. sativa (HS3), HM (HS4), essential oils of J. oxycedrus berries (EO1), and HM (EO2) are given in the Table 2.

The most dominant component of sample HS1 was β-myrcene (65.4%), accompanied by α-pinene (23.9%) and limonene (6.8%). The dominant component of the essential oil of the berries was β-myrcene (37%), accompanied by α-pinene (13%), β-caryophyllene (8.7%), α-humulene (6%), and germacrene D (7.7%). The amount of β-myrcene was twofold smaller than that of the HS sample. Similarly, the essential oil isolated from the HM was composed mainly of β-myrcene (32%), but the amount of monoterpenes (43.2%) was twofold smaller than that of the HS volatiles of the HM (96.8%). The main monoterpene hydrocarbons of the HM (HS4) were β-myrcene (71.8%), α-pinene (17.2%), and β-pinene (1.2%), followed by a small amount of the sesquiterpene hydrocarbon, germacrene D (1.1%). Dominant HS volatiles of the inner bark of B. pendula (HS2) were primary aldehyde and alcohols n-hexanal (51.0%), n-hexanol (17.9%), and n-pentanol (6.7%), while monoterpenes were detected in significantly lower amounts compared to the other 2 samples (6.6%). The only detected monoterpene was β-pinene (6.6%).

Sesquiterpenes composed the major part of the HS volatiles of A. sativa grains, amounting to 51.6% of the total HS volatiles. The most abundant compounds (HS3) were the sesquiterpenes, germacrene D (46.5%), and α-humulene (5.1%), followed by the monoterpenes α-pinene (9.4%) and β-myrcene (5.6%).

Table 1. Chemical Composition of Methanol Extracts of Juniperus oxycedrus Berries (M1), Inner Bark of Betula pendula (M2), Grains of Avena sativa (M3), and Herbal Mixture (M4) (μg/g of dry Extract).

| S. No. | Compound                        | M1     | M2     | M3     | M4     |
|--------|--------------------------------|--------|--------|--------|--------|
| 1      | Tartaric acid                  | -      | -      | 232    | 210    |
| 2      | Procyanidin derivative         | -      | 159    | -      | 136    |
| 3      | Gallic acid                    | -      | 2060   | -      | 1555   |
| 4      | Catechin derivative            | -      | 32.9   | -      | 15     |
| 5      | Protocatechuic acid            | 5360   | -      | -      | 3407   |
| 6      | Catechin                       | -      | 52.3   | -      | 38     |
| 7      | Epicatechin                    | -      | 6.9    | -      | 3      |
| 8      | Cyanidin-derivative            | -      | 84.7   | -      | 78     |
| 9      | Syringic acid                  | -      | 2038   | -      | 956    |
| 10     | Cyanidin derivative            | -      | 227    | -      | 110    |
| 11     | Rutin                          | -      | 730    | -      | 580    |
| 12     | Kaempferol-3-O-glycoside       | -      | 1080   | -      | 880    |
| 13     | Quercetin                      | -      | 100    | -      | 72     |
| 14     | Kaempferol derivative          | -      | 100    | -      | 72     |
| 15     | Kaempferol derivative          | -      | 670    | -      | 480    |
| 16     | Apigenin                       | 310    | -      | -      | 210    |
| 17     | Apigenin derivative            | -      | 460    | -      | 251    |
| 18     | Luteolin derivative            | -      | 221    | -      | 150    |
| 19     | Cupressosilvone                | 3250   | -      | -      | 670    |
| 20     | Amentotropane                  | 3700   | -      | -      | 1030   |
| 21     | Bioflavonoid                    | 1400   | -      | -      | 590    |
The essential oil of the HM contained similar amounts of monoterpenoids and sesquiterpenoids (43.2% and 37.0%, respectively). Higher amounts of β-caryophyllene (12.7%) and germacrene D (12.1%) were detected in the essential oil of the HM than in the essential oil of the berries (8.7% and 7.7%, respectively), as shown in Figure 1.

Biological Activities of *J. oxycedrus* Berries, Inner Bark of *B. pendula*, Grains of *A. sativa*, and HM

**Cytokinesis-Block Micronucleus Assay.** The analysis of micronuclei (MN) in cultured lymphocytes is applied as a method for genotoxic studies. The methanol extracts of *J. oxycedrus* berries (MJO), inner bark of *B. pendula* (MBP), grains of *A. sativa* (MAS), HM, and pure compounds at concentrations of 1.0, 2.0, and 4.0 µg/mL were subjected to CBMN assay. The frequencies and distribution of MN in human lymphocytes were scored. The results are presented in Table 3 and Figure 2.

All tested compounds (in minimal doses of 1 µg/mL) exerted a beneficial effect by decreasing DNA damage of human lymphocytes in the range of 18.2% to 31.1%. Among the tested extracts, the most prominent effect was exhibited by the methanol extract of the HM at a concentration of 2.0 µg/mL, which gave a significant decrease (*P* < 0.01) in the MN frequency of 38.3%, which was threefold higher than the effect of amifostine (MN frequency of 11.4%). Also, treatment
of the cell cultures with the methanol extract of the HM at concentrations of 1 μg/mL and 4.0 μg/mL affected a significant decrease (\(P<0.01\)) in the frequency of MN (31.1% and 32.8%, respectively) compared with the control cell cultures (Figure 2). The methanol extracts of individual samples (M1, M2, and M3) at a concentration of 2.0 μg/mL exhibited a twofold higher activity than that of amifostine, while concentrations of 1 μg/mL and 4.0 μg/mL showed slightly lower activity, but were still more effective than amifostine.

The methanol extracts of the HM and mixture constituents showed the most significant reduction in the MN frequency at a concentration of 2.0 μg/mL, whereas concentrations of 1 μg/mL and 4 μg/mL were less effective, but still more effective than amifostine.

**Acute Toxicity—Artemia Salina Test**

Acute toxicity against *Artemia salina* is used for the preliminary assessment of the safety and potential pharmacological application of natural products. The results of the bioassay could provide information about the cytotoxic properties of the tested extract considering good correlation between the assay and cytotoxic activity against some human solid tumors.\(^{20,21}\) The results of the acute toxicity assay of the tested essential oils (berries—EO1 and HM—EO2) are summarized in Table 4. The final concentrations of the tested EO1 and EO2 in the Petri dishes were in the range of 4.57 to 73.125 μg/mL and 16.25 to 127.5 μg/mL (respectively), while the final concentration of Dimethyl sulfoxide (DMSO) was less than 1% (v/v).

To the best of our knowledge, there is no study regarding the acute toxicity of the essential oils of *J. oxycedrus* berries and the HM in the *Artemia salina* test. Based on the survival of nauplii after 24 h of incubation, EO1 showed higher toxicity than both the remaining samples, EO1/EO2 (almost twofold stronger) and EO1/SDS (1.2 times higher). Higher amounts of α-pinene and β-myrcene were detected in the essential oil of the berries, EO1 (13% and 37%, respectively) than in the essential oil of the HM, EO2 (4.1% and 32%), while the amounts of β-caryophyllene and germacrene D (8.7% and 7.7%, respectively for EO1) were smaller (12.7% and 12.1%, respectively for EO2). Previously, the methanol extract of the berries of *Juniperus drupacea* from Turkey was found to be moderately toxic against brine shrimps, with a Lethal concentration to 50% of nauplii (LC₅₀) value of 489.47 ± 27.8 μg/mL.\(^{20}\) For the incubation period of 48 h, both oils exhibited lower toxicity than SDS, EO1 being about 3 times less and EO2 about 5 times lower.

**Experimental**

**Plant Materials**

Berries of *J. oxycedrus* were collected from Klobuk, province of Trebinje (coordinates 42° 42′ 28.19″ N and 18° 31′ 35.59″ E, R. Srpska). Red-brown mature fruits were air-dried, lyophilized, and stored in a dark place. Grains of *Avena sativa* were purchased at a health food store. Inner bark of *Betula pendula* was collected in city park Vranje (coordinates 42.5521° N and 21.8989° E, Serbia). The voucher specimens have been deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš under the acquisition numbers: 14611-F, 14612-F, 14613-F, respectively.
Table 3. Incidence of Micronuclei (MN), Cytokinesis-Block Proliferation Index (CBPI), Distribution of MN per Cells, and Frequency of MN in Cell Cultures of Human Lymphocytes Treated With Different Concentrations of Methanol Extracts of Juniperus oxycedrus Berries (M1), Inner Bark of Betula pendula (M2), Grains of Avena sativa (M3), Herbal Mixture (M4), and Pure Compounds.

| Sample          | Conc. µg/mL | MN/1000 Bn cell | % Bn cell with MN | MN/Bn cell | CBPI | Frequency of MN |
|-----------------|-------------|-----------------|-------------------|------------|------|-----------------|
| Control         | 1.0         | 26.26 ± 0.34    | 2.12 ± 0.11       | 1.17 ± 0.06 | 1.66 ± 0.02 | 100%            |
| AM              | 1.0         | 21.93 ± 0.62    | 1.80 ± 0.12       | 1.22 ± 0.07 | 1.61 ± 0.04 | 83.51%          |
| MMC             | 0.2         | 34.59 ± 0.95    | 3.05 ± 0.17       | 1.13 ± 0.03 | 1.66 ± 0.05 | 131.72%         |
| M1              | 1.00        | 19.13 ± 0.41    | 1.58 ± 0.10       | 1.17 ± 0.07 | 1.64 ± 0.02 | 72.85%          |
| M1              | 2.00        | 18.15 ± 0.30    | 1.58 ± 0.05       | 1.15 ± 0.04 | 1.64 ± 0.03 | 69.12%          |
| M1              | 4.00        | 18.59 ± 0.98    | 1.64 ± 0.11       | 1.08 ± 0.04 | 1.64 ± 0.01 | 70.79%          |
| M2              | 1.00        | 20.17 ± 0.49    | 1.64 ± 0.04       | 1.16 ± 0.05 | 1.66 ± 0.01 | 76.80%          |
| M2              | 2.00        | 18.87 ± 0.85    | 1.64 ± 0.10       | 1.15 ± 0.04 | 1.64 ± 0.02 | 71.86%          |
| M2              | 4.00        | 19.73 ± 0.80    | 1.61 ± 0.03       | 1.22 ± 0.06 | 1.63 ± 0.02 | 75.10%          |
| M3              | 1.00        | 21.48 ± 0.71    | 1.85 ± 0.05       | 1.15 ± 0.02 | 1.71 ± 0.05 | 81.80%          |
| M3              | 2.00        | 20.42 ± 0.70    | 1.70 ± 0.08       | 1.20 ± 0.06 | 1.61 ± 0.01 | 77.76%          |
| M3              | 4.00        | 21.00 ± 0.48    | 1.76 ± 0.06       | 1.20 ± 0.06 | 1.65 ± 0.01 | 80.00%          |
| M4              | 1.00        | 18.09 ± 0.67    | 1.54 ± 0.06       | 1.11 ± 0.04 | 1.66 ± 0.03 | 68.90%          |
| M4              | 2.00        | 16.21 ± 0.42    | 1.50 ± 0.03       | 1.10 ± 0.03 | 1.61 ± 0.02 | 61.73%          |
| Hesperidin      | 1.00        | 17.98 ± 0.27    | 1.41 ± 0.05       | 1.27 ± 0.05 | 1.61 ± 0.01 | 63.18%          |
| Hesperidin      | 2.00        | 17.44 ± 0.36    | 1.45 ± 0.07       | 1.20 ± 0.04 | 1.65 ± 0.03 | 61.28%          |
| Hesperidin      | 4.00        | 17.66 ± 0.53    | 1.60 ± 0.07       | 1.10 ± 0.02 | 1.68 ± 0.04 | 62.05%          |
| Naringin        | 1.00        | 19.86 ± 0.24    | 1.60 ± 0.10       | 1.26 ± 0.09 | 1.66 ± 0.00 | 69.78%          |
| Naringin        | 2.00        | 19.94 ± 0.76    | 1.67 ± 0.14       | 1.18 ± 0.11 | 1.64 ± 0.01 | 68.31%          |
| Naringin        | 4.00        | 19.68 ± 0.57    | 1.59 ± 0.06       | 1.29 ± 0.07 | 1.63 ± 0.02 | 69.15%          |
| Rutin           | 1.00        | 17.41 ± 0.37    | 1.54 ± 0.10       | 1.14 ± 0.08 | 1.61 ± 0.07 | 61.17%          |
| Rutin           | 2.00        | 16.58 ± 0.43    | 1.54 ± 0.08       | 1.14 ± 0.05 | 1.70 ± 0.05 | 58.26%          |
| Rutin           | 4.00        | 16.98 ± 0.58    | 1.54 ± 0.10       | 1.17 ± 0.08 | 1.62 ± 0.03 | 39.66%          |

Note: MN/1000 Bn cells: incidence of MN in 1000 binucleated cells (examined for each concentration); % Bn cells with MN: % binucleated cells; Frequency of MN: incidence of MN present like % from control groups in cell cultures of human lymphocytes treated with different concentrations of extracts.

*a*—compared with control groups, statistically significant difference \( P<0.01\); \( a^*\)—compared with control groups, statistically significant difference \( P<0.05\); \( b\)—compared with amifostine—WR 2721; statistically significant difference \( P<0.01\); \( b^*\)—compared with amifostine—WR 2721, statistically significant difference \( P<0.05\); \( c\)—compared with mitomycin C; statistically significant difference \( P<0.01\); \( c^*\)—compared with mitomycin C, statistically significant difference \( P<0.05\).

The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at \( P<0.01\); \( <0.05\).

Abbreviations: AM, amifostine; MMC, mitomycin C.

Preparation of Methanol Extracts of Berries, Inner Bark, Grains, and HM

The extractions of individual species were performed in triplicate. Two g of each sample was chopped and subjected to ultrasound-assisted extraction with 20 mL of methanol (Sigma-Aldrich; purity \( \geq 99.9\%\)) using an ultrasound bath (UZK 8; Maget,) for 30 min; after that, the extracts were left in the dark (room temperature) overnight. Dry residues of the extracts were obtained using a rotary evaporator (KNF Laboxact) with the water bath set at 40 °C. The mixture of chopped dried berries (1.1 g), inner bark of B. pendula (0.5 g) and grains of A. sativa (1.6 g) was extracted with 30 mL of methanol in the same way as the individual components of the mixture. The extract yields were 17.4 ± 0.11% for J. oxycedrus berries, 1.33 ± 0.05% for B. pendula inner bark, 1.78 ± 0.04% for grains of A. sativa, and 18.01 ± 0.1% for mixture. The dry methanol extracts were prepared prior to HPLC analysis and MN assay.

Isolation of Essential Oils

The air-dried samples (11 g J. oxycedrus berries, 5 g B. pendula inner bark, and 16 g A. sativa grains) were cut into small pieces and subjected to hydrodistillation using a Clevenger-type apparatus for 2 h. The obtained essential oil was extracted with diethyl ether and dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure and stored at +4 °C prior to analysis.

The essential oil of J. oxycedrus berries was obtained in the same way as the HM. The yield of essential oils was 0.66% and 0.61%, respectively, based on the dried weight of the samples.

Sample Preparation for HS Analysis

For HS analysis of the HM, chopped berries of J. oxycedrus (0.5 g), inner bark of B. pendula (0.25 g), and grains of A. sativa (0.8 g) were placed in a vial for HS analysis and moistened with 1 mL.
of distilled water. Samples of the individual ingredients of the mixture were prepared in the same way using 1 g of each ingredient.

**GC-MS Analysis**

The samples were analyzed by a 7890/7000B GC/MS/MS triple quadrupole system in MS1 scan mode (Agilent Technologies) equipped with a Combi PAL sampler and HS for G6501B/G6509B. Details about GC-MS analyses, identification, and calculation of relative amounts of volatile compounds have been reported previously.

In short, a fused silica capillary column HP-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm) was used. The injector and interface operated at 250 and 300 °C, respectively. Temp. program: from 50 to 290 °C at a heating rate of 4 °C/min. The carrier gas was helium with a flow of 1.0 mL/min. The 500 μL HS volatile components and 1 μL essential oil solutions in hexane (1:100), respectively, were used. The amount of identified compounds was expressed as μg per g of dry extract weight (μg/g).

**Cytokinesis-Block Micronucleus Assay**

CBMN was performed as previously described. The cell culture lymphocytes were treated with 1.0, 2.0, and 4.0 μg/mL of the examined methanol extracts and available pure compounds (hesperidin, naringin, and rutin; Sigma-Aldrich).

Amifostine WR-2721 (98% S-[3-aminopropylamino] ethylphosphorothioic acid; Marligen-Biosciences) at a concentration of 1 μg/mL was used as a positive control. The alkylation agent mitomycin-C (MMC, Bristol-Myers Squibb), prepared by diluting the drug in phosphate buffer (PBS), was added at a final concentration of 0.2 μg/mL to the lymphocytes cultures and used as a negative control. Three experiments were performed for each sample. The results are expressed as the means ± standard deviation (SD). The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA), followed by the Tukey test. Statistical difference was considered significant at P < .05 and P < .01.

**Acute Toxicity—Artemia Salina Test**

Acute toxicity in *Artemia salina* (brine shrimps) was evaluated using the method previously described by Radulović and colleagues. Two teaspoons of lyophilized cysts of *Artemia salina* were added to 1 L of the artificial seawater. The suspension was thermostated at 28 °C, aerated with a strong airflow, and kept under constant illumination for 48 h, after which most of the cyst hatched. Freshly hatched nauplii (20 individuals) were transferred into Petri dishes containing 20 mL of

### Table 4. Acute Toxicity of Examined Samples and SDS (Positive Control) on *Artemia salina*, Expressed as LC50 in μg/mL After 24 h and After 48 h.

| Sample | LC50 24 h (95% confidence interval) | LC50 48 h (95% confidence interval) |
|--------|-----------------------------------|-----------------------------------|
| EO1    | 27.63 ± 0.0224 (±0.09%)           | 25.78 ± 0.0838 (±0.3%)            |
| EO2    | 47.45 ± 0.0733 (±0.2%)            | 37.31 ± 0.00924 (±0.02%)          |
| SDS    | 33.82 ± 0.0141 (±0.04%)           | 8.24 ± 0.00924 (±0.1%)            |

Abbreviations: EO1, essential oil of the berries; EO2, essential oil of the herbal mixture; LC50, lethal concentration to 50% of nauplii; SDS, sodium dodecyl sulfate.

The statistical significance of difference between the data pairs was evaluated by analysis of variance (ANOVA), followed by the Tukey test. Statistical difference was considered significant at P < 0.0001. There is a statistically significant difference (P < 0.0001) in activity between each of the oils and the SDS, as well as between the oils, SDS, and control sample (dimethyl sulfoxide [DMSO]).
artificial seawater. One hundred microliters of tested oil of each concentration (14.62; 7.31; 3.65; 1.83; 0.91 mg/mL for EO1 and 25.5; 13; 6.5; 3.25; mg/mL for and EO2) was added into Petri dishes as well. So, the final concentrations of the tested EO1 and EO2 in the Petri dishes were in the range of 4.57 of _A. salina_ (brine shrimps) test. Ivana Zrnzević block micronucleus test. Jovana Ickovski: acute toxicity in Artemia results; and Writing the paper. Dr Miroslava Stanković Experimental work (plant collecting, HPLC analysis); Analysis of the results; and Supervision of all of the work. Gordana Stojanović: Analysis of the results; Writing the paper; And

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

Not applicable, because this article does not contain any studies with human or animal subjects.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Ministry of Education, Science and Technological Development of Serbia (grant number No. 451-03-68/2022-14/200124).

**Acknowledgments**

The authors acknowledge the Ministry of Education, Science and Technological Development of Serbia for financial support (Agreement No. 451-03-9/2022-14/200124).

**Author’s Contribution**

Dr Ivana Zlatanović: Literature research; Designing a study; Experimental work (plant collecting, HPLC analysis); Analysis of the results; and Writing the paper. Dr Miroslava Stanković: cytokinesis block micronucleus test. Jovana Ickovski: acute toxicity in _Artemia salina_ (brine shrimps) test. Ivana Zrnzević: GC/MS analysis. Dr

**References**

1. Ramello A, Vitale C, Marangella M. Epidemiology of nephrolithiasis. _J Nephrol_. 2000;13(3):S45-S50. https://www.researchgate.net/publication/12196979_Epidemiology_of_nephrolithiasis

2. Stamatelou KK, Francis ME, Jones CA, Nyberg LM, Curhan GC. Time trends in reported prevalence of kidney stones in the United States: 1976–1994. _Kidney Int_. 2003;63(5):1817-1823. doi.org/10.1046/j.1523-1755.2003.00917.x

3. Worcester EM, Coe FL. Nephrolithiasis. _Prim Care_. 2008;35(2):369-391. doi.org/10.1016/j.pop.2008.01.005

4. Rizwan F, Yesmine S, Banu SG, Chowdhury IA, Hasan R, Chatterjee TK. Renoprotective effects of stevia (_Stevia rebaudiana_ Bertoni), amlodipine, valsartan, and losartan in gentamycin-induced nephrotoxicity in the rat model: biochemical, hematological and histological approaches. _Toxicol Rep_. 2019;6:683-691. doi.org/10.1016/j.toxrep.2019.07.003

5. Moreno L, Bello R, Primo-Yúfera E, Esplugues J. In vitro studies of methanol and dichloromethanol extracts of _Juniperus oxycedrus_ L. _Phytother Res_. 1997;11(4):309-311. doi.org/10.1002/(SICI)1099-1573(199706)114:3<309::AID-TPR873>3.0.CO;2-6

6. Šarić-Kundalić B, Dobeš C, Klátte-Asselmeyer V, Saukel J. Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina. _J Ethnopharmacol_. 2010;131(1):33-55. doi.org/10.1016/j.jep.2010.05.061

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7. Orhan N, Aslan M, Pekcan M, Orhan DD, Bedir E, Ergun F. Identification of hypoglycaemic compounds from berries of *Juniperus oxycedrus subsp. oxycedrus* through bioactivity guided isolation technique. *J Ethnopharmacol*. 2012;139(1):110-118. doi.org/10.1016/j.jep.2011.10.027

8. Singh R, De S, Belkheir A. Avena sativa (oat), a potential nutraceutical and therapeutic agent: an overview. *Crit Rev Food Sci Nutr*. 2013;53(2):126-144. doi.org/10.1080/10408398.2010.526725

9. Taviano MF, Marino A, Trovato A, et al. *Juniperus oxycedrus* L. Subsp. *oxycedrus* and *Juniperus oxycedrus* L. Subsp. *macnarcap* (Sibth. & Sm.) Ball. “berries” from Turkey: comparative evaluation of phenolic profile, antioxidant, cytotoxic and antimicrobial activities. *Food Chem Toxicol*. 2013;58:22-29. doi.org/10.1016/j.fct.2011.06.016

10. Emmons CL, Peterson DM. Antioxidant activity and phenolic contents of oat groats and hulls. *Cereal Chem*. 1999;76(6):902-906. doi.org/10.2135/cropsci2001.1676.

11. Peterson DM. Oat antioxidants. *J Cereal Sci*. 2001;33(2):115-129. doi.org/10.1006/jcrs.2000.0349.

12. Zhang WK, Jie-Kun XU, Zhang L, Guan-Hua DU. Flavonoids from the bran of Avena sativa. *Chin J Nat Med*. 2012;10(2):110-114. doi.org/10.3724/SP.J.1009.2012.00110.

13. Liimatainen J, Karonen M, Sinkkonen J, Helander M, Salminen JP. Characterization of phenolic compounds from inner bark of *Betula pendula*. *Holzforschung*. 2012;66(2):171-181. doi.org/10.1515/HF.2011.146

14. Liimatainen J, Karonen M, Sinkkonen J. Procyanidin xylosides from the bark of *Betula pendula*. *Phytochemistry*. 2012;76:178-183. doi.org/10.1016/j.phytochem.2012.01.008

15. Liimatainen J, Karonen M, Sinkkonen J, et al. Phenolic compounds of the inner bark of *Betula pendula*: seasonal and genetic variation and induction by wounding. *J Chem Ecol*. 2012;38(11):1410-1418. doi.org/10.1007/s10886-012-0199-2

16. Liimatainen J, Sinkkonen J, Karonen M, Pihlaja K. Two new phenylbutanoids from inner bark of *Betula pendula*. *Magn Reson Chem*. 2008;46(2):195-198. doi.org/10.1002/mrc.2163

17. Adams RP. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. 4th ed. Allured Publishing Corporation; 2007.

18. Salido S, Alarejos J, Nogueras M, et al. Chemical studies of essential oils of *Juniperus oxycedrus ssp. badia*. *J Ethnopharmacol*. 2002;81(1):129-134. doi.org/10.1016/s0378-8741(02)00045-4

19. Fenech M, Morley AA. The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations. *Mutat Res*. 1993;285(1):35-44. doi.org/10.1016/0027-5107(93)90049-L.

20. dos Santos JHM, Oliveira DF, de Carvalho DA, et al. Evaluation of native and exotic Brazilian plants for anticancer activity. *J Nat Med*. 2010;64(2):231-238. doi.org/10.1007/s11418-010-0390-0

21. Miceli N, Trovato A, Marino A, et al. Phenolic composition and biological activities of *Juniperus drupacea* Labill. Berries from Turkey. *Food Chem Toxicol*. 2011;49(10):2600-2608. doi.org/10.1016/j.fct.2011.07.004

22. Petrović G, Stamenković J, Kostevski IR, Stojanović GS, Mitić VD, Zlaković BK. Chemical composition of volatiles; antimicrobial, antioxidant and cholinesterase inhibitory activity of *Chaerophyllum aromaticum* L. (Apiaceae) essential oils and extracts. *Chem Biodivers*. 2017;14(5):e1600367. doi.org/10.1002/cbdv.201600367

23. Stojanović IŽ, Stanković M, Jovanović O, Petrović G, Šmelcerovic A, Stojanović GS. Effect of Hypogymnia physodes extracts and their depsidones on micronuclear distribution in human lymphocytes. *Nat Prod Commun*. 2013;8(1):109-112. doi.org/10.1177/1934578X1300800125.

24. Radulović NS, Sladenović MZ, Blagojević PD, et al. Toxic essential oils. Part III: identification and biological activity of new allyl-methoxyphenyl esters from a Chamomile species (*Anthemis segetalis* Ten.). *Food Chem Toxicol*. 2013;62:554-565. doi.org/10.1016/j.fct.2013.09.017

25. Hamilton MA, Russo RC, Thurston RV. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol*. 1977;11(7):714-719. doi.org/10.1021/es60130a004