



Article

Effects of Geographical Area and Harvest Times on Chemical Composition and Antibacterial Activity of *Juniperus communis* L. Pseudo-Fruits Extracts: A Statistical Approach

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Abstract: The beneficial effects of *Juniperus communis* L. extracts have been known for a long time. Therefore a scientific knowledge of the chemical profile leading to this bioactivity is required. The aim of this paper is to highlight the influence of geographical origin and harvest time on compositional elements of hydroalcoholic extracts of *J. communis* pseudo-fruits, but also on their antibacterial properties. The samples were collected from three mountainous area of Romania, during three consecutive months. The total polyphenols were determined by the Folin-Ciocalteu method, ranging between 12.67 and 14.77 mg GAE/g DW. High-performance liquid chromatography (HPLC) analysis was applied to identify fifteen phenolic compounds from the group of phenolic acids and flavonoids. The antibacterial effect was assessed on Gram-positive and Gram-negative bacterial strains. A good antimicrobial activity was achieved by the extracts of pseudo-fruits harvested during October and November from the Iezerlele Cindrelului Botanical Nature Reserve. The obtained results denote a diverse composition of active principles in common juniper pseudo-fruits and moderate antibacterial activity dependent on the harvest time and geographical area.

Keywords: *Juniperus communis*; polyphenols; antibacterial activity



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1. Introduction

Scientific research on the chemical composition of herbal-derived products should be the essential evidence for the efficacy and safety that traditional therapies claim, but also for their integration in pharmacological studies and clinical practices [1]. Both plant extracts and essential oils have been shown to be effective in prevention and/or treatment of various human diseases. Due to their highly complex composition, these natural products and their derivatives are of significant importance as sources of readily accessible and affordable remedies. One of the most well-known plants about which traditional medicine provides information from ancient times is *Juniperus communis* L. [2]. *J. communis* is a dioeciously conifer in the Pinales order, Cupressaceae family, frequently found in mountainous areas, with evergreen leaves in acicular form, with edible fruit-like seeds. They are wrapped in a protective, juicy, dark-colored layer, as berry-like female cones, hence the name pseudo-fruits [3]. In *J. communis*, berries formation, ripening and maturation occur during three years. In the first year of reproduction cycle, the cones are small and green, in the second year the mass doubles and the color becomes blue-black matte, and in the third year ripening occur from September to October [4–7].

Both leaves and pseudo-fruits of *J. communis* are rich in polyphenols [8,9], volatile components [10], bitter principles, carbohydrates, and various organic acids [11–16].

Leaves of common juniper with specific geographical origin showed a significant accumulation of polyphenols that can reach up to $3.43 \pm 0.17\%$ pyrogallol equivalents, and the predominant volatile compounds are δ -3-carene, α -pinene, sabinene, β -pinene, myrcene, limonene, β -phellandrene and D-germacrene [8,10]. Using LC-ESI-QTOF/MS methods, Tang et al. [17] established the existence of 148 phenolic compounds. Significant values of polyphenols were quantified by Höferl et al. [13], Olech et al. [18], Gu et al. [19], the results being extremely useful in the subsequent research that target efficient plant utilization. Volatile organic compounds have been investigated by Antonelli et al. [20] and Meneguzzo et al. [21] in terms of favorable impact on the human body. The studies showed that terpenoids and other volatile compounds are responsible for about 50–70% of beneficial emissions from coniferous forests, with positive health effects.

J. communis contain highly bioactive compounds, especially polyphenols, with a positive impact on various conditions such as cancer, respiratory, rheumatic and circulatory disorders, diabetes, acne infections and hypertension [22–33]. The antioxidant activity of essential oils and extracts of *J. communis* was noted by Manvi [29], Elmastaş et al. [34], Emami et al. [35], Fernandez and Edwin [36], Fierascu et al. [37]. A seasonal fluctuation of phenolic content and antioxidant activity were reported for other herbaceous and woody plants as well [38,39]. Adams et al. [40] found significant differences in the chemical composition of essential oils obtained from leaves of *Juniperus excelsa* and *Juniperus polycarpus* depending on the plant's origin (area and altitude).

The antimicrobial action assessed on gram-positive and gram-negative bacterial strains, but also on fungi was reported by a substantial number of studies [10,41–52]. The most important results were observed in bacterial strains such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*, while *Salmonella*, *Proteus*, *Pseudomonas* and *Klebsiella* strains were resistant to *J. communis* extracts. Some authors considered that the sensitivity of *Candida*, *Penicillium* and *Aspergillus* species to extracts obtained from the pseudo-fruits of *J. communis* could be due to their richness in polyphenols [12,14,43]. Given the antioxidant and nutraceutical potential, anti-carcinogenic, anti-inflammatory and antimicrobial properties of polyphenols, there is a growing interest to accurately assess the content of these bioactive compounds in *J. communis* [17,18,22,32,34].

In this study, we focused on identifying the optimum harvesting time of *J. communis* pseudo-fruits, which would ensure an important content of target compounds, with a significant antioxidant and antimicrobial activity, for an efficient exploitation in the pharmaceutical and food industry. We conducted for the first time the comparative assessment of the chemical profile and bioactivity of *J. communis* pseudo-fruits extract from three mountain natural populations in Romania, harvested in three consecutive months, the results being important from economic and ecological perspectives.

2. Materials and Methods

2.1. *J. communis* Samples

Samples of *J. communis* pseudo-fruits were harvested from Iezerele Cindrelului Botanical Nature Reserve (ICR), Bălea Lake Alpine Tundra (BLA), and Cozia National Park (CNP). The three harvesting areas were chosen as parts of the Southern Carpathians, located in south-central part of Romania, as follow: ICR is a protected area located on the northern side of the Frumoasa Plateau, in the Cindrelul Mountains, at 1999 m altitude ($45^{\circ}35'55''$ N $23^{\circ}47'51''$ E); BLA is located in the Făgăraș Mountains, at 2034 m altitude (45.60335° N 24.61714° E); PNC is located in the north-east part of Vâlcea County, at 1600 m altitude ($45^{\circ}04''$ N $24^{\circ}18'02''$ E). The choice of geographical areas was also made according to the dominance of *J. communis* species, so that in ICR, BLA and CNP juniper is the representative species of the Cupressaceae family [53–55].

Appreciating that in the autumn months the pseudo-fruits reach ripeness and their content in secondary metabolites is higher, ripe berries were carefully selected and hand-picked in September, October and November of 2021.

Voucher specimens of the samples were deposited at the Biotechnologies and Food Engineers Research Center within Lucian Blaga University of Sibiu and recorded with numbers 325/1–325/3.

2.2. Chemical, Reagents, Bacterial Strains and Culture Media

All chemicals and reagents used were of analytical grade. Ethanol 96% was obtained from Carl Roth GmbH, Karlsruhe, Germany). Folin-Ciocalteu reagent, sodium carbonate 7.5%, gallic acid, caffeic acid, ellagic acid, syringic acid, coumaric acid, caftaric acid, chlorogenic acid, vanillic acid, cinnamic acid, ferulic acid, catechin, epicatechin gallate, quercetin, kaempferol p-hydroxybenzoic acid, acetic acid, acetonitrile, dimethylsulphoxide were from Sigma-Aldrich GmbH, Steinheim, Germany.

Antibacterial activity of *J. communis* pseudo-fruits were assessed on six standard bacterial strains: three Gram-positive (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC29212, *Bacillus subtilis* ATCC23857) and three Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC13076, *Citrobacter freundii* ATCC43864).

The bacterial strains are deposited and preserved in the microbial culture bank from Microbiology Department of Biotechnologies and Food Engineers Research Center (CCBIA) within the Agricultural Sciences, Food Industry and Environmental Protection Faculty/Lucian Blaga University of Sibiu, Romania. Cultures are grown on agar plates and maintained at 4 °C for short period of time, avoiding contamination. An inoculum density of 0.5 McF = 1.5×10^8 CFU/mL was used to evaluate inhibition.

Bacteria were cultured on Mueller Hinton agar and Mueller Hinton broth supplied by Sigma-Aldrich GmbH, Steinheim, Germany.

2.3. Extraction Procedure

J. communis samples consisting of 50 g of pseudo-fruits for each geographical location and each month, respectively, were dried at 50 °C in a Memmert incubator to constant mass, then grounded and stored at 12 °C. Powdered plant material was extracted with ethyl alcohol: distilled water (1:1 v/v), in a plant material: solvent ratio of 1:50 (w/v), for 24 h at a temperature of 15 °C. The crude extracts were filtered with the vacuum pump through Whatman filter paper no. 54, concentrated and dried in a rotary evaporator. The resulted dry extract was weighed and dissolved in 10 mL of distilled water, specific concentrations of the samples being obtained, depending on the harvesting period and the geographical location.

2.4. Determination of Total Phenolic Content (TPC)

Total polyphenols were determined by the spectrophotometric method using the modified Folin-Ciocalteu method [56]. A volume of 0.20 mL extract was homogenized with 0.80 mL Folin Ciocalteu reagent (10% v/v) and 1 mL sodium carbonate (7.5% m/v). The samples were incubated in the dark at room temperature for 60 min. The absorbance was read with a UV-1900 SHIMADZU spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at a wavelength of 750 nm, the expression being in milligrams equivalent to gallic acid/gram dry weight (mg GAE/g DW). The determinations were performed in triplicate for a more accurate picture of the results.

2.5. HPLC Analysis

Phenolic compounds were identified following the HPLC method proposed by Gu et al. [19] slightly modified and using HPLC Smartline, KNAUER GmbH (Germany) equipment provided with a PDA detector, set to the following λ wavelengths: 280 nm, 320 nm, 360 nm, quaternary pump and automatic injection system. The mobile phase, eluent A consisted of a water-acetic acid solution (95/5 v/v) and eluent B from acetonitrile/water/acetic acid (100/95/5 v/v/v), using a C18 chromatographic column (Zorbax SB-Aq: 250 mm \times 4.6 mm i.d., 5.0 μ m p.s.). The volume of sample injected was 50 μ L, the flow rate being set at 1 mL/minute. The mobile phases were degassed for

20 min at 20 °C, the gradient profile being established by mixing both phases according to the scheme: 0–25 min, 15% B; 25–35 min, 25% B; 35–45 min, 35% B; 45–70 min, 45% B; 70–75 min, 55% B; 75–80 min, 100% B; 80–85 min, 10% B. The identification and quantification of phenolic compounds was achieved by comparison with selected standards, using calibration curves for each individual compound. The experiments were performed in triplicate and the values were expressed as averaged mg/g extract. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follows: $LOD = 3 \times (S/N)$ and $LOQ = 10 \times (S/N)$, where S/N is the signal-to-noise ratio.

2.6. Assessment of Antibacterial Activity and Minimum Inhibitory Concentration

The antimicrobial activity of *J. communis* pseudo-fruits hydroalcoholic extracts was determined using the modified Kirby-Bauer diffusion method. 100 µL of the test bacteria were activated in 10 mL of Mueller Hinton broth culture medium for 24 h. Prepared Petri dishes with Mueller Hinton agar culture medium were inoculated with 100 µL bacterial suspension with a concentration of 0.5 McFarland (density 0.5 McF = 1.5×10^8 CFU/mL). The extracts were tested using 6 mm diameter sterilized filter paper discs. The discs were impregnated with 10 µL of the test sample, incubated at 37 °C for 24 h and the diameters of the inhibition zone were measured with a ruler (mm). Each antimicrobial test was performed in triplicate and the mean obtained was reported. Standard disks of ampicillin (2 µg) and chloramphenicol (10 µg) were used as controls.

Disk diffusion method with successive dilutions was applied to assess the minimum inhibition concentration (MIC) of *J. communis* pseudo-fruits extracts. Plant extracts were diluted in distilled water over a range of 5 mg/mL to 0.1 mg/mL and 10 µL of each test dilution was pipetted onto a disk placed in Petri dishes containing solid Mueller Hinton culture medium coated with the test microorganism. After 24 h of incubation at 37 °C the areas of inhibition were measured. Depending on the concentration and the size of the inhibition zone, graphs are made, the MIC being calculated using linear regression.

2.7. Statistical Analysis

All experiments were performed in triplicate. Results are presented as the Mean \pm standard deviation (SD) of more independent experiments. Analysis of variance (one-way ANOVA and two-way ANOVA) was applied to analyze the data for statistical significance and to determine the effects of categorical variables on the chemical composition of the extracts. The significant differences among means were determined using Duncan test. Significant differences were set at $p \leq 0.05$. Principal component analysis (PCA) and discriminant analysis (DA) were applied to reduce the dimensionality of the data and to establish the relations between the studied variables. Pearson correlations ($p < 0.05$ and $p < 0.01$) were used to identify correlations between variables. The data were mean-centered by subtracting the mean of each variable from its values, in order not to achieve a small variance of the coefficients of the principal components. This was done to remove any potential bias in the data and to ensure that the first principal component was the direction of maximum variance in the data, the data being further displayed as single point for each variable. IBM SPSS Statistics 20 and Addinsoft XLSTAT version 2014.5.03 software (Addinsoft Inc.; New York, NY, USA) were used for statistical analysis of the data.

3. Results

3.1. Dry Weight and Final Concentration of the Extracts

Dried extracts of *J. communis* pseudo-fruits were reconstituted in distilled water and the final concentration obtained from each sample is presented in Table 1. The extraction yield increased on average by 15–20% during the three months of harvesting. Data obtained in our study revealed that the highest extraction yield was characteristic of pseudo-fruits harvested at the end of autumn, suggesting harvest time is a critical factor in the industrial use of *J. communis* pseudo-fruits.

Table 1. Extraction yield and final concentrations of *J. communis* pseudo-fruit extracts during the three months of harvesting (September, October and November) from the Iezerle Cindrelului Botanical Nature Reserve, Bâlea Lake Alpine Tundra and Cozia National Park.

Month of Harvesting	ICR		BLA		CNP	
	Extraction Yield (mg) *	Final Concentrations (mg/mL) *	Extraction Yield (mg) *	Final Concentrations (mg/mL) *	Extraction Yield (mg) *	Final Concentrations (mg/mL) *
September	129.3 ± 0.04 ^f	12.93 ± 0.01 ^f	135.2 ± 0.26 ^e	13.52 ± 0.03 ^e	106.7 ± 0.14 ^h	10.67 ± 0.01 ^h
October	144.5 ± 0.9 ^d	14.45 ± 0.09 ^d	151.7 ± 0.4 ^b	15.17 ± 0.04 ^b	111.1 ± 0.19 ^g	11.11 ± 0.02 ^g
November	149.1 ± 0.08 ^c	14.91 ± 0.01 ^c	167.3 ± 0.17 ^a	16.73 ± 0.02 ^a	129.4 ± 0.06 ^f	12.94 ± 0.01 ^f

* Each value represents mean ± SD; a, b, c, d, e, f, g, h—interpretation of the significance of the differences, by means of the Duncan test, $p \leq 0.05$.

3.2. Determination of Total Phenolic Content (TPC)

The ICR samples showed an accumulation of polyphenols between 13.69 ± 0.01 mg GAE/g DW and 24.01 ± 0.02 mg GAE/g DW, the value increasing with the maturation of the pseudo-fruits. For the BLA samples the values varied between 13.52 ± 0.01 mg GAE/g DW and 24.77 ± 0.02 mg GAE/g DW. For the CNP samples the values of polyphenols ranged between 12.67 ± 0.01 mg GAE/g DW and 19.83 ± 0.01 mg GAE/g DW registering an increased percentage of 58.2% in the last month of pseudo-fruits harvesting (Figure 1).

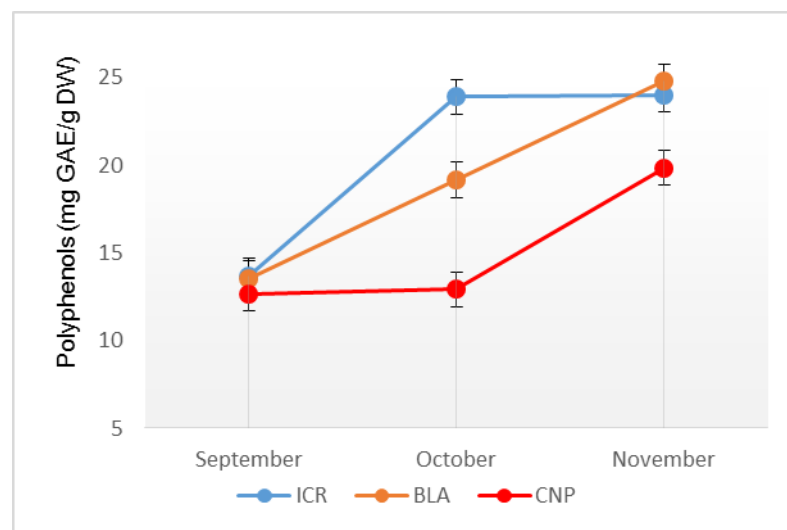


Figure 1. Total polyphenols quantified in the samples of *J. communis* pseudo-fruits harvested during three months (September, October and November) from the Iezerle Cindrelului Botanical Nature Reserve, Bâlea Lake Alpine Tundra and Cozia National Park.

3.3. HPLC Profile of the Extracts

As noted in Table 2, the targeted phenolic compounds were from the group of phenolic acids and flavonoids.

In *J. communis* pseudo-fruits harvested from Southern Carpathians, caffeic acid was found between 0.092 and 0.0945 mg/g DW in the ICR samples, 0.0883 and 0.0903 mg/g DW in the BLA samples, and lower values were recorded for CNP samples where the maximum did not exceed 0.0775 mg/g DW.

The lowest content of caftaric acid reached a minimum value of 0.0013 mg/g DW in the samples collected from CNP in September and a maximum of 0.0027 mg/g DW in those collected in October from the ICR. It is important to emphasize, caftaric acid was not found in the samples collected in November from the Cindrel massif, in September from the Bâlea area and in October from CNP.

Table 2. Phenolic compounds identified and quantified in pseudo-fruits of *J. communis* harvested during three months (September, October and November) from the Iezerele Cindrelului Botanical Nature Reserve, Bălea Lake Alpine Tundra and Cozia National Park.

Compound	ICR Sample (mg/g DW) *			BLA Sample (mg/g DW) *			CNP Sample (mg/g DW) *			LOD/LOQ (mg/g)
Harvest Time	September	October	November	September	October	November	September	October	November	
Caffeic acid	0.0923 ± 0.0009 ^b	0.0944 ± 0.0005 ^a	0.0945 ± 0.0004 ^a	0.0883 ± 0.0003 ^d	0.0901 ± 0.0005 ^c	0.0903 ± 0.0003 ^c	0.0728 ± 0.0002 ^f	0.0723 ± 0.0003 ^f	0.0775 ± 0.0005 ^e	0.0173/0.0523
Caftaric acid	0.0026 ± 0.0002 ^a	0.0027 ± 0.0002 ^a	0.0013 ± 0.0003 ^c	0.0013 ± 0.0003 ^c	0.0016 ± 0.0002 ^c	0.002 ± 0.0003 ^b	0.0013 ± 0.0003 ^c	0.0013 ± 0.0001 ^c	0.0016 ± 0.0003 ^c	0.0003/0.0008
Cinnamic acid	1.0245 ± 0.0003 ^a	1.0247 ± 0.0003 ^a	1.0248 ± 0.0003 ^a	1.0234 ± 0.0004 ^b	1.0235 ± 0.0003 ^b	1.0235 ± 0.0003 ^b	1.0175 ± 0.0004 ^c	1.0175 ± 0.0004 ^c	1.0175 ± 0.0002 ^c	0.0194/0.0588
Chlorogenic acid	0.0102 ± 0.0002 ^c	0.0102 ± 0.0001 ^c	0.0106 ± 0.0002 ^{bc}	0.0109 ± 0.0002 ^{ab}	0.0111 ± 0.0002 ^a	0.0102 ± 0.0004 ^c	0.0102 ± 0.0003 ^c	0.0105 ± 0.0002 ^{bc}	0.0102 ± 0.0004 ^c	0.0029/0.0089
Coumaric acid	0.4671 ± 0.0003 ^d	0.467 ± 0.0001 ^d	0.4677 ± 0.0003 ^c	0.4701 ± 0.0004 ^b	0.4701 ± 0.0002 ^b	0.4711 ± 0.0004 ^a	0.4271 ± 0.0002 ^f	0.4301 ± 0.0002 ^e	0.43 ± 0.0003 ^e	0.0118/0.0358
Ellagic acid	0.0006 ± 0.0001 ^b	0.0006 ± 0.0001 ^b	0.0006 ± 0.0001 ^b	0.0006 ± 0.0001 ^b	0.0006 ± 0.0001 ^b	0.0023 ± 0.0003 ^a	0.0006 ± 0.0002 ^b	0.0006 ± 0.0001 ^b	0.0006 ± 0.0001 ^b	0.0002/0.0005
Ferulic acid	0.0001 ± 0.0001 ^c	0.0001 ± 0.0001 ^c	0.0006 ± 0.0001 ^b	0.0008 ± 0.0001 ^a	0.0006 ± 0.0001 ^b	0.0001 ± 0.0001 ^c	0.0001 ± 0.0001 ^c	0.0002 ± 0.0001 ^c	0.0001 ± 0.0001 ^c	0.0000/0.0001
Gallic acid	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0001 ± 0 ^a	0.0000/0.0001
Syringic acid	0.0021 ± 0.0002 ^a	0.0021 ± 0.0003 ^a	0.0022 ± 0.0002 ^a	0.0009 ± 0.0001 ^b	0.0009 ± 0.0002 ^b	0.0009 ± 0.0002 ^b	0.0009 ± 0.0001 ^b	0.0009 ± 0.0001 ^b	0.0009 ± 0.0006 ^b	0.0003/0.0008
Vanillic acid	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0001/0.0002
P-hydroxybenzoic acid	5.2987 ± 0.0003 ^c	5.2988 ± 0.0003 ^c	5.2991 ± 0.0004 ^c	5.3907 ± 0.0003 ^b	5.3908 ± 0.0003 ^b	5.3917 ± 0.0003 ^a	4.1297 ± 0.0004 ^f	4.1385 ± 0.0001 ^e	4.200 ± 0.0003 ^d	0.0341/0.1033
Catechins	7.2987 ± 0.0002 ^b	7.3481 ± 0.0002 ^b	7.35 ± 0.0002 ^b	7.4907 ± 0.0003 ^b	7.4987 ± 0.0003 ^b	7.4988 ± 0.0002 ^b	8.8981 ± 0.0003 ^a	8.8987 ± 0.0004 ^a	9.8989 ± 0.005 ^a	0.0351/0.1063
Epicatechin gallate	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0002 ± 0 ^a	0.0001/0.0002
Quercetin	1.0245 ± 0.0001 ^b	1.0251 ± 0.0002 ^b	1.026 ± 0.0003 ^b	1.1005 ± 0.0002 ^a	1.1001 ± 0.0002 ^a	1.0999 ± 0.0002 ^a	0.9245 ± 0.0001 ^e	0.9335 ± 0.0001 ^d	1.002 ± 0.00033 ^c	0.0239/0.0725
Kaempferol	4.0012 ± 0.0001 ^h	4.0111 ± 0.0002 ^f	4.0115 ± 0.0001 ^e	4.1013 ± 0.0002 ^d	4.1012 ± 0.0002 ^d	4.1016 ± 0.0002 ^c	4.0082 ± 0.0003 ^g	5.9913 ± 0.0001 ^a	5.8992 ± 0.0002 ^b	0.0284/0.07860

* Each value represents mean ± SD; a, b, c, d, e, f, g, h—interpretation of the significance of the differences, by means of the Duncan test, $p \leq 0.05$.

Cinnamic acid was identified at values exceeding one unit, being within the range 1.0245 and 1.0248 mg/g DW in the Cindrel area, 1.0234 and 1.0235 mg/g DW in the Bâlea area and decreased by an average of 0.7% compared to the maximum recorded, in the Cozia area. Chlorogenic acid content was detected in a range of values between a minimum of 0.0100 mg/g DW in the samples collected in November in the Cozia massif to a maximum of 0.0111 mg/g DW in the samples collected in October in the Bâlea glacier circus. Coumaric acid accumulated in more significant quantities in all samples, maximum values of 0.4677 mg/g DW being identified in November ICR samples, and of 0.4711 mg/g DW and 0.4301 mg/g DW in October BLA and CNP samples respectively.

Elagic acid, ferulic acid, and epicatechin gallate were identified in very small quantities or were not detected in the analyzed samples. Thus, elagic acid was found only in three samples, October, November in Cindrel (0.0006 mg/g DW) and November in Bâlea area (0.0023 mg/g DW).

Among the derivatives of benzoic acid, gallic acid and vanilla acid were released in extremely small amounts. The HPLC analysis revealed the average values of 0.0001 mg/g DW and 0.0002 mg/g DW in all samples collected from the Cindrel and Cozia areas. These phenolic acids were missing in the BLA samples. Syringic acid content ranged from 0.0009 mg/g DW in the BLA samples to a more significant amount of 0.0022 mg/g DW in the samples collected from the Cindrel area. This acid was not identified in the samples collected from the CNP.

High levels of P-hydroxybenzoic acid were found in all samples, the quantified values reaching up to a maximum of 5.3917 mg/g DW in the BLA, values above 5 mg/g DW being found in all samples collected both from the BLA and ICR. The minimums of this compound were around 4.1297 mg/g DW in the CNP samples.

Running the two-way Anova test revealed that for nine of the fifteen phenolic compounds, respectively caffeic acid, caftaric acid, coumaric acid, ellagic acid, ferulic acids, p-hydroxybenzoic acid, quercetin, epicatechin gallate and chlorogenic acid were highly influenced by the interaction of the two independent categorical variables, location and harvest month, for $p \leq 0.05$. Over 90% of the variance of quercetin content and over 70% of the variance for the other acids content mentioned above (except epicatechin gallate and chlorogenic acid) is due to the interaction of the two variables (Table 3).

Table 3. The effect of independent categorical variables (location*month) on the chemical composition of the extracts. Data were subjected to two-way ANOVA and Duncan test for interpretation of the significance of the differences among means, $p \leq 0.05$.

Tests of Between-Subjects Effects			
Dependent Variable: Phenolic Compounds/Source Location*Month			
Phenolic Compounds	F	Sig.	Partial Eta Squared
Caffeic acid	40.236	0	0.856
Caftaric acid	24.932	0	0.787
Cinnamic acid	0.265	0.898	0.038
Chlorogenic acid	5.867	0.002	0.465
Coumaric acid	40.294	0	0.857
Ellagic acid	85.685	0	0.927
Ferulic acid	83.705	0	0.925
Gallic acid	0.274	0.892	0.039
Syringic acid	0.084	0.987	0.012
Vanillic acid	-	-	-
P-hydroxybenzoic acid	20.36	0	0.798
Catechins	0.916	0.469	0.119
Epicatechin gallate	3.071	0.033	0.313
Quercetin	18.777	0	0.996
Kaempferol	5.715	0.056	0.582

Flavonoids were identified in the pseudo-fruits of *J. communis* in significant amounts. In our study, the samples harvested in November from CNP showed a maximum content of catechins (9.8989 mg/g DW) and the ICR-September samples revealed the minimum value of these flavonoids (7.2987 mg/g DW). Epicatechin-3-gallate was not identified and quantified in September in any sample, with very small amounts visible in October and November when the values did not exceed 0.0002 mg/g DW.

Quercetin content was quantified around 1 mg/g DW, this compound being present in all samples analyzed. Kaempferol was noted for accumulations of over 4 units in all samples taken. In the ICR, the determined values were between 4.0012 mg/g DW and 4.0111 mg/g DW in September and November respectively. In the BLA samples these values were on average 2.2% higher, and in the CNP samples ranged between 4.0082 mg/g DW in September and 5.9913 mg/g DW in October. Lower values were obtained in November when the accumulation of Kaempferol did not exceed 5.8992 mg/g DW.

The analyzed data for the PCA are represented by the 12 phenolic compounds (caffeic acid, caftaric acid, cinnamic acid, chlorogenic acid, coumaric acid, ellagic acid, ferulic acid, syringic acid, p-hydroxybenzoic acid, catechins, quercetin and kaempferol) identified and quantified in pseudo-fruits of *J. communis* harvested during three months (September, October and November) from the Iezerlele Cindrelului Botanical Nature Reserve, Bâlea Lake Alpine Tundra and from the Cozia National Park. Three of the phenolic compounds were not included in the DA, as gallic acid, vanillic acid and epicatechin gallate were not identified in any of the samples.

The statistical approach, PCA and DA respectively, reduces the 12 measured variables to a two-dimension model without losing any information (Figure 2). The first two discriminant functions explained a 100% of the variation in the raw data, according to the preliminary findings; furthermore, their amounts were analyzed, representing phenolic compound concentration linear combinations. Both the absolute values and the signs of the coefficients (positive and negative) must be taken into account. Amounts near to zero, as in the case of cinnamic acid, indicate that this variable has limited significance in connection to the corresponding variable, while higher values, as in ellagic acid, indicate a considerable effect on the behavior of the discriminant factor. A positive or negative sign indicates that the specified discriminant variable is significant. The relative importance of the discriminant variables of the various compounds determines the positions of the points in the graphical representation. As it can be observed, samples harvest in September were clearly separated from the other two sampling periods, highlighting the fact that the concentration of phenolic compounds in that period is significantly different, the samples originating from 3 distinct regions showing a similar behavior. The other two harvest periods (October and November) had the same signs for several variables along the first discriminant function. However, an obvious separation between the latter was observed by their variable's contribution to the second discriminant function.

Principal component analysis (PCA) allowed the investigation of data variability and the differentiation of extract sources depending on the month of harvest, the geographical location and the compositional profile. PCA score plot showed that the first two principal components (PC1 and PC2) explained approximately 71% of the variance, with a clear grouping of samples (Figure 3). The BLA samples are located in the upper right quadrant, with a clear separation according to the harvest month. CNP samples are positioned on the left side of the score plot, while the ICR samples are positioned lower right quadrant. The loading plot presents the separation of the extracts based on their phenolic content. While ferulic acid was found in the highest concentration in BLA from September, quercetin, coumaric acid and p-hydroxybenzoic acid were detected at the highest values in BLA samples. The high content of cinnamic, caftaric, syringic and caffeic acids is specific to ICR samples, and CNP samples are distinguished by the content of kaempferol and catechins.

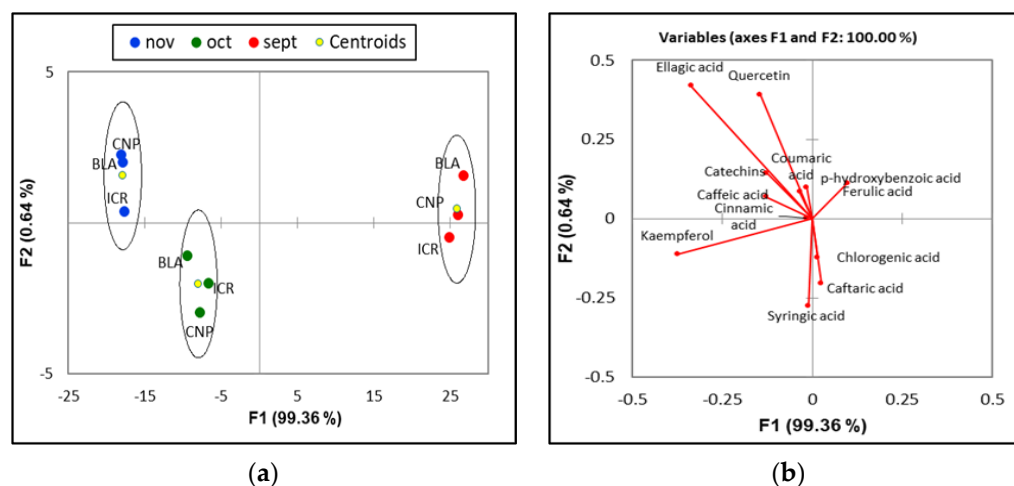


Figure 2. (a) Discriminant analysis of the *J. communis* pseudo-fruits based to their harvest month; (b) Variables/Factors correlations graphical representation.

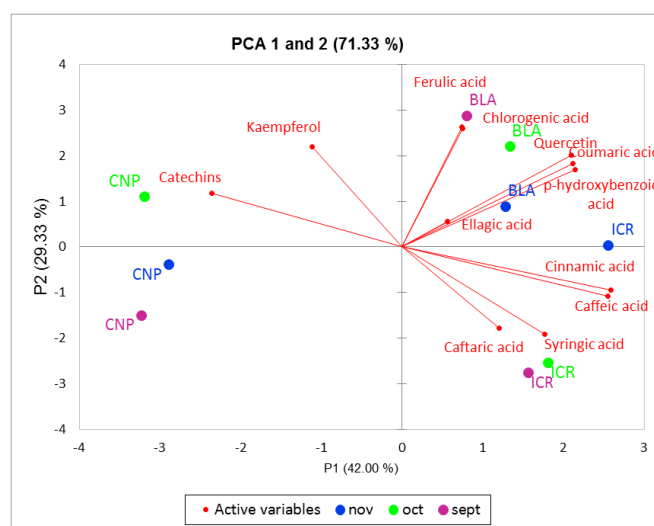


Figure 3. Differentiation of *J. communis* pseudo-fruits harvested during three consecutive months (September, October and November). Colored symbols correspond to the samples of *J. communis* pseudo-fruits harvested from from Iezerlele Cindrelului Botanical Nature Reserve, Bălea Lake Alpine Tundra and Cozia National Park.

The results were confirmed by the Pearson correlation index performed for the extracts of *J. communis* pseudo-fruits. The heatmap of Pearson correlation coefficient (Figure 4) revealed a strong positive correlation between caffeic acid, cinnamic acid, coumaric acid and p-hydroxybenzoic acid and strong negative correlations with catechins and kaempferol.

3.4. Antibacterial Activity and MIC of *J. communis* Pseudo-Fruits Extracts

In the tests performed, antibacterial activity was monitored by the diffusion method, implicitly achieving the MIC on Gram-positive and Gram-negative bacteria, and the results are presented in Table 4. Antibacterial activity is fully manifested in Gram-positive bacteria analyzed (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC29212, *Bacillus subtilis* ATCC23857), regardless of the harvest month, and 66.6% in gram-negative bacteria *Escherichia coli* ATCC 25922, ATCC, *Citrobacter freundii* strain ATCC43864 not showing sensitivity to *J. communis* extracts. The MIC values resulting from the linear regression calculation are less than 500 µg/mL in 21.05% of the samples (susceptible microorganism), between 500 µg/mL and 1000 µg/mL in 50.87% of the samples (moderate susceptible microorganism) and over 1000 µg/mL in 28.07% of the samples (resistant microorganism).

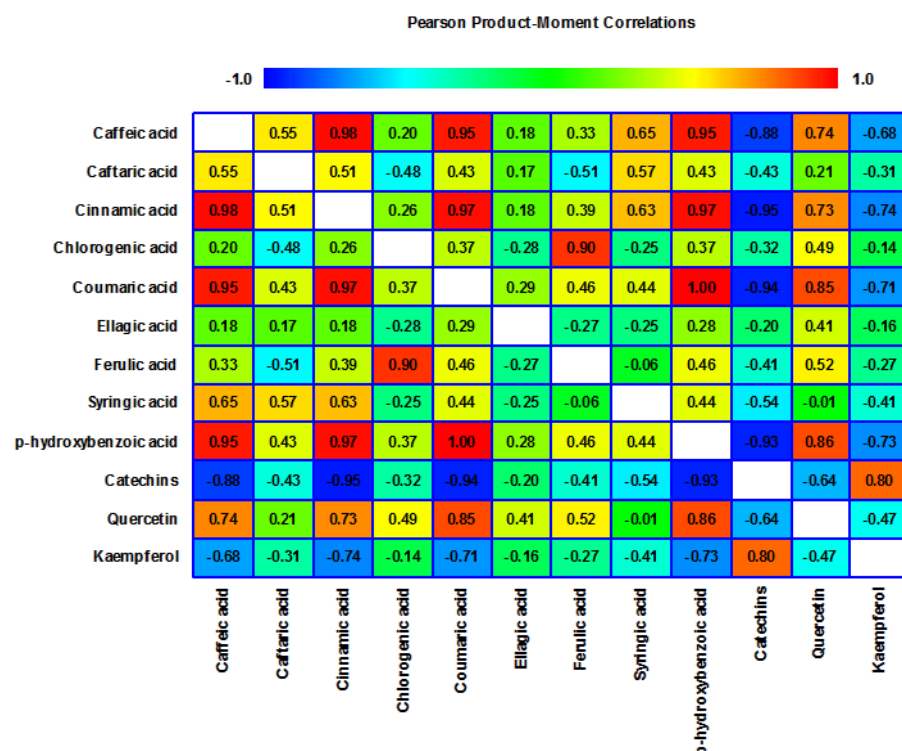


Figure 4. Heatmap of Pearson correlation coefficient obtained from chemical compositional variables analyzed from pseudo-fruits of *J. communis* harvested during three months (September, October and November) from the Iezerlele Cindrelului Botanical Nature Reserve, Bălea Lake Alpine Tundra and Cozia National Park.

Table 4. Antibacterial activity established for the extracts of *J. communis* pseudo-fruits harvested during the three consecutive months (September, October and November) from the Iezerlele Cindrelului Botanical Nature Reserve, Bălea Lake Alpine Tundra and Cozia National Park.

Bacterial Strains Samples		<i>S. aureus</i> ATCC29213		<i>E. faecalis</i> ATCC29212		<i>B. subtilis</i> ATCC23857		<i>E. coli</i> ATCC 25922		<i>S. enteritidis</i> ATCC13076		<i>C. freundii</i> ATCC43864	
		DD **	MIC *	DD **	MIC ***	DD **	MIC ***	DD *	MIC ***	DD *	MIC ***	DD *	MIC ***
ICR *	September	20.2 ± 0.1 _{def}	≤500	14.1 ± 0.1 _b	1000	12.3 ± 0.1 _c	1000	16.2 ± 0.1 _b	>500	8.2 ± 0.1 ^b	1000	-	
	October	20.6 ± 0.1 _g	≤500	15.1 ± 0.7 _b	>500	12.3 ± 0.2 _c	1000	16.2 ± 0.4 _b	>500	8.9 ± 0.4 ^b	1000	-	
	November	21.3 ± 0.3 _h	500	16.4 ± 0.8 _{ab}	>500	14.8 ± 2.1 _{bc}	>500	17.6 ± 1.4 _{ab}	>500	9.1 ± 0.1 ^b	>1000	-	
BLA *	September	19.5 ± 0.5 _d	≥500	14.1 ± 3.0 _b	>1000	15.6 ± 0.6 _{bc}	>500	17.8 ± 0.6 _b	>500	10.1 ± 0.8 _b	>1000	-	
	October	18.4 ± 0.5 _h	≥500	15.2 ± 2.6 _b	>500	16.7 ± 1.2 _{bc}	>500	19.0 ± 1.3 _b	>500	10.3 ± 0.3 _b	>1000	-	
	November	20.3 ± 0.3 _{fg}	500	17.1 ± 0.5 _b	>500	17.0 ± 0.1 _{bc}	>500	19.5 ± 0.7 _b	>500	10.2 ± 0.2 _b	>1000	-	
CNP *	September	18.1 ± 0.1 _c	≥500	16.4 ± 1.33 _{ab}	>500	13.9 ± 2.7 _{bc}	>1000	17.5 ± 1.3 _{ab}	>500	10.2 ± 0.2 _b	>1000	-	
	October	19.8 ± 0.2 _{de}	≥500	15.4 ± 0.4 _{ab}	>500	16.2 ± 3.2 _{bc}	>500	16.6 ± 0.1 _{ab}	>500	10.4 ± 0.3 _b	>1000	-	
	November	19.9 ± 0.1 _{efg}	≥500	16.5 ± 1.9 _{ab}	>500	18.5 ± 0.3 _b	>500	16.8 ± 0.1 _{ab}	>500	10.3 ± 0.2 _b	>1000	-	
A		24.6 ± 0 ^b	≤500	19.6 ± 1.5 _a	≤500	24.4 ± 5.1 _a	<500	22.8 ± 5.4 _a	<500	18.8 ± 7.3 _a	<500	6.3 ± 3.6 ^b	>1000
	C	27.1 ± 0 ^a	≤500	16.7 ± 3.3 _{ab}	>500	23.4 ± 3.5 _a	500	20.7 ± 4.5 _a	>500	21.8 ± 1.0 _a	500	17.0 ± 6.3 _a	>500

* Each value represents mean ± SD; a, b, c, d, e, f, g, h—interpretation of the significance of the differences, by means of the Duncan test, $p \leq 0.05$. ** DD—Disc diffusion (zone of inhibition including the diameter of disc 6 mm), 0–15 mm zone of inhibition = resistant microorganism; zone of inhibition 15–20 mm = moderate susceptible microorganism and zone of inhibition 20–30 mm zone of inhibition = susceptible microorganism. *** MIC expressed as µg/mL. A—ampicillin 2 µg, C—chloramphenicol 10 µg.

4. Discussion

It is known that the ripening cycle of the pseudo-fruits of *J. communis* covers a period of approx. 18 months [57] and berries can be harvested throughout the growing season [58]. Although the influence of the harvest period on the chemical profile of various extracts and essential oils has been addressed by several studies [59–61], there is nonetheless a need to deepen this topic. To obtain the best results, it has been suggested autumn is the best season for harvesting juniper berries or from January to April for harvesting juniper branches [59–63]. However, the data suggest that optimization of harvesting protocols is mandatory. Falasca et al. [62] found *J. communis* essential oil with a similar composition in July and November. The authors stated that the change of season could be a stress factor that leads to this unusual biochemical. Based on the previous results, in our study we choose to harvest the pseudo-fruits in the autumn months and November was the most favorable month of harvest. At the same time, it is also appreciated that the geographical distribution is responsible for the extraordinary quantitative and qualitative variability of secondary metabolites in juniper-based products [4,62,63]. Total polyphenols are a relevant indicator for the antioxidant quality of a product. Following the determinations performed, it was observed the values of total polyphenols oscillate both depending on the month of harvest and the area of origin. An increase dependent on the harvest month of polyphenols was noticed for the BLA samples. Tang et al. [13] set values of 9.08 ± 0.01 mg/mL in *J. communis* pseudo-fruits harvested from Ozspice Store, Melbourne, Australia, while Miceli et al. [49] set an amount of 48.06 ± 0.99 mg/g DW in *J. drupacea* fruit from Turkey. In addition to the influence of the species, these significantly different values can be attributed to the impact of sampling season and pedoclimatic conditions factors that were previously incriminate for variation of content and composition of essential oil/extracts from various juniper species [18,60,62–64].

Phenolic acids varied significantly depending on their membership, but also on the geographic area and harvest time. Identified as a ubiquitous phenolic compound in all plant species, caffeic acid is a secondary metabolite with numerous and remarkable physiological properties, from antimicrobial and antiviral effects to anti-hepatocellular carcinoma activity [65]. For each mountain area, the maximum content of caffeic acid was defined by the month of November. Similarly, the highest p-hydroxybenzoic acid content was noted in November. Although a statistically significant accumulation of caffeic acid and p-hydroxybenzoic acid in November was noted only for ICR sample and BLA sample respectively, the constant increase in values during the autumn season can be attributed to cold stress. A couple of studies shows that polyphenols, including caffeic acid and p-hydroxybenzoic acid cover a wide range of biological properties, including abiotic stress response in plants and plants protection against chilling stress through various mechanisms that maintain cellular redox homeostasis [66–69].

At the same time, our data suggests the geographical location and time of ripening influence accumulation of caftaric acid. Thus, the premature pseudo-fruits from the CNP represent a better source of caftaric acid, when compared with BLA and ICR. This valuable phenolic compound is a caffeic acid derivative, with important therapeutic effects, recently reviewed by Koriem [70].

Catechins are important secondary metabolites, which are found in variable amounts in the various parts of the plants. Green tea plants (*Camellia sinensis* (L.) Kuntze) are well known for their high content of catechins [71]. Catechins have notable health benefits, such as strong and stable antioxidant activity [72], protective capacity against UV radiation and anti-ageing effects [73], anti-microbial activity [74], multidrug resistance in cancer [75,76] and immunomodulatory effects [77]. In our study, catechins were found at significantly higher values in CNP samples, regardless of the harvest month. However, various amounts of total polyphenols were reported in *J. communis* leaves and pseudo-fruits with different geographical origin [17,18,49]. In a recently published review, Gonçalves et al. [78] present a comprehensive analysis of the bioactive compounds identified in *J. communis*, in conjunction with their potential applications.

Antibacterial activity characterized extracts from pseudo-fruits of *J. communis*. The value of the zones of inhibition varies in the samples analyzed according to the month of harvest and the degree of ripening, which suggest a significant variation in accumulation of compounds with antibacterial action. Statistical analysis revealed the CNP–October and November samples were more effective against *S. aureus*, *E. faecalis* and *E. coli*. These results could be attributed to the significantly higher content of catechins and kaempferol that marked these samples. Significant results in the field were also obtained by Angioni et al. [10], El-Sawi et al. [12], Filipowicz et al. [44], Zhang et al. [50].

The primary aim of this research was to provide evidence of the optimal period for harvesting *J. communis* pseudo-fruits by analyzing their phenolic profile and identifying target compounds. Therefore, statistical analysis by DA and PCA were performed in line with the pseudo-fruits origin and the harvest period. Clear separation of the defining samples for the month of September highlights the fact that the concentration of phenolic compounds in that period is significantly different, the samples originating from 3 distinct regions showing a similar behavior. The other two harvest periods (October and November) had the same signs for several variables along the first discriminant function. However, a clearer separation between the latter was observed by their variable's contribution to the second discriminant function. Statistical analysis revealed that the geographic location influences the phenolic content of pseudo-fruits. Therefore, the BLA samples were marked by higher content of chlorogenic, coumaric, ferulic, ellagic, p-hydroxybenzoic acids and quercetin, CNP samples were rich in kaempferol and catechins, while the ICR samples were distinguished by a higher content of caffeic, caftaric and cinnamic acids.

5. Conclusions

The variety of results obtained on extracts from the pseudo-fruits of *J. communis* demonstrates that they can accumulate valuable compounds in different amounts depending on the harvest period and area of origin. The total polyphenols were in a significant amount in areas with higher altitude and November was the most favorable month of harvest. The samples from Cozia National Park were dominated by kaempferol and catechins as phenolic content, while samples from Iezerlele Cindrelului Botanical Nature Reserve were distinguished by the high content in cinnamic, caftaric, syringic and caffeic acids. The determined phenolic compounds are present in amounts in which they have an antibacterial effect on Gram-positive and Gram-negative bacteria, with catechin predominating, followed by p-hydroxybenzoic acid and kaempferol. The strongest antibacterial effects were noted in the case of *J. communis* extracts from the Iezerlele Cindrelului Reserve and Bălea Lake Alpine Tundra areas on the *Staphylococcus aureus* strain ATCC 29213. Further investigations into the chemical composition of these extracts under local soil and climatic conditions are recommended in order to finalize aspects that may lead to the formation of a complete picture of the relationships between environmental factors and beneficial properties of *J. communis* pseudo-fruits.

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