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Original article

Chemical Composition, Antioxidant and Antimicrobial Activities of Essential Oil Extracted from waste of *Juniperus communis* L. Medicinal and Aromatic Plants Industry in Albania

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Abstract

The Medicinal and Aromatic Plants (MAPs) industry is relevant for Albania covering approximately 20% of agricultural exports. Mostly high quality dried parts of MAPs are traded, while products not fulfilling quality criteria and non-tradable plant parts are often regarded as waste products, even though they contain valuable bioactive substances. This study evaluates the composition and biological activity of the essential oil (EO) fraction of wastes generated from the MAPs industy of *Juniperus communis* L. in Albania.

Juniperus communis L. was collected from Korçë area, dried, screened for trade quality berries in an industrial plant for MAPs, and the waste parts underwent hidrodistilation in industrial distilators. Chemical composition was performed using Gas Chromatography coupled with Mass Spectrometry and identified 50 compounds, where main components were α-Pinene (24.47%), Sabinene (12.4%), Germacrene D (3.2%) and β-Myrcene (1.6%). The antioxidant capacity of *J. communis* L. EO was determined by 2,2-diphenyl-1-picrylhydrazil (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and expressed as Inhibitory Concentration of 50% of the free radical (IC50) where values were IC50= 155.4 µg/mL and IC50= 163.2µg/mL for DPPH and ABTS respectively. Antimicrobial activity of *J. communis* L. EO was determined against five bacteria, *Escherichia coli* ATCC 10535; *Salmonella enteritidis*, ATCC 49223, *Pseudomonas aeruginosa*, ATCC 9027; *Micrococcus luteus*, ATCC 10240; *Stenotrophomonas maltophilia*, ATCC 1363;and one yeast, *Candida albicans*, ATCC 10231 by microdilution method used to determine the minimum inhibitory concentration (MIC). The EO showed no antimicrobial activity against the first 3 bacterial strains, while it inhibited growth of *Micrococcus luteus* and *Candida albicans* at concentrations of 2.5 mg/mL and of *Stenotrophomonas maltophilia* at the maximum tested concentration of 5 mg/mL.

Keywords: *Juniperus communis* L. by-product, antioxidant, antimicrobial, MIC.

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INTRODUCTION

Albania has a long tradition in the production and export of medicinal and aromatic plants (MAPs), spanning more than 60 years, which is mainly based on the abundance of wild-grown MAPs. Compared to other agricultural subsectors, MAPs industry is primarily focused on exports, with over 95% of all MAPs exported, accounting for 20% of all agricultural exports (AASF, 2019). A considerable amount of post-harvest waste products are produced during the processing of MAPs, such as branches, leaves and fruits of lower quality (Routray. et al., 2017), that are not suitable for commercial use (FAO/WHO Codex Alimentarius Commission On Spices and Culinary Herbs, 2021). Since these residual biomasses have the same components and characteristics as the comercial product, they could be sources of bioactive chemicals (Navarrete et al., 2011; Wang et al., 2018). Most of these by-products are still treated as waste from the Albanian MAPs industry and discarted improperly or used as a burning material. Nevertheless MAPs by-products are a source of useful metabolites with significant biological characteristics that might give to finished products a unique quality (Sahaa. et al., 2020).

One of the most prized aromatic plants in the world is juniper (*Juniperus* spp.), found also in Albania in two main species, red juniper (*Juniperus oxycedrus* L.) and black juniper (*Juniperus communis* L.) which possess antioxidant, anti-inflammatory, and antimicrobial properties due to the occurrence of several secondary metabolites, such as phenolic constituents, terpenoids, and flavonoids, in their extracts (Mërtiri et al., 2024). Both spieces are employed for culinary and also essential oil (EO) uses, but there is a higher demand for the second, black juniper, in the Albanian MAPs industry. Also *J. communis* has been sold at higher prices in recent years in Albania due to domestic and international supply dynamics and higher domestic demand for essential oil production (Medicinal and Aromatic Plants Sector Study, 2021).

However, considerable quantities of *J. communis* herbal residues are formed during the post-harvest processing used to select the material to be marketed, and these residues can be used to extract its EO. While there are various ways to extract natural essential oils (EOs) from plants, steam distillation is the method most commonly employed in the industry today since it is easy to use and doesn't require the use of chemicals like solvents, which are often utilized in other extraction processes (Esteban et al., 2023).

EOs, many of which known for their antimicrobial activity, are attracting attention on the current issue with pathogenic bacteria whose resistance to antibiotics has grown over time. This is one of the main problems facing the pharmaceutical, food, and veterinary industries which aim discovering new bioactive compounds to be employed as bactericides (Mancuso et al., 2021). As many active organic compounds make up plant EOs, which act on microbial cells through different mechanisms, they do not induce microbial resistance (Rao et al., 2019). Additionally, since customers are increasingly requesting natural products as alternatives to synthetic preservatives, it is necessary to look for antioxidant and anti-

inflammatory active principles of natural origin. In this context, the US Code of Federal Regulation has classified juniper berries, essential oils, solvent-free oleoresins and natural extractives as Generally Recognized as Safe (GRAS) for their intended application (Code of Federal Regulation. Substances Generally Recognized as Safe, 2023).

The aim of this study was to characterize the chemical composition and assess the biological activity of the EO extracted from the by-products of *J. communis* MAPs industry, as a strong source of bioactive components for the creation of new products in food and pharmaceutical industry.

MATERIALS and METHODS

Plant material and EO extraction

Juniperus communis L. was collected from Korçë district, Albania, from 10th of August until the end of September 2021, Albania, dried, screened for trade quality berries which are mainly intended for gin spirit and teas production in an industrial plant for MAPs (PETKUS Technologie GmbH), and the remaining by-product underwent hidrodistilation in industrial distilators at BioBes sh.p.k. company (Sopëz, Albania).

Reagents and microorganisms

Antioxidant radical screening reagents [20-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium and potassium persulfate (ABTS)] were purchased from Alfa Aesar (Massachusetts, United States). Methanol was secured from VWR International (Fontenay-sous-Bois, France) and ethanol from Merk KGaA (Darmstadt, Germany).

The bacterial American type culture collection strains (ATCC) *Salmonella enteritidis* (ATCC:49223), *Escherichia coli* (ATCC:10535), *Pseudomonas aeruginosa* (ATCC:9027), *Stenotrophomonas maltophilia* (ATCC:13637). *Micrococcus luteus* (ATCC:10240), along with one fungal isolate *Candida albicans* (ATCC:10231) were procured from Microbiologics, Inc., (MinnesotaUnited States). 96-well plates were secured from Corning Inc. (New York, United States).

Blood agar medium and Muller Hinton Broth were procured from Remel Inc, (California, United States), 0.5 Polymer McFarland Standard from Thermo Fisher Scientific (Massachusetts, United States) and Dimethylsulfoxide (DMSO) from Sigma-Aldrich (Missouri, United States).

Gas Chromatography-Mass Spectrometry

Gas Chromatography-Mass Spectrometry essential oil analyses were performed on a Shimadzu GC-2010-GCMSQP2010 system operating at 70 eV. The temperature program was from 60^{o}C to 250^{o}C, at a rate of 5^{o}C/min. Helium was used as a carrier gas at a flow rate of 1.0 ml/min. Injection volume of each sample was 1 μL. Retention indices for all compounds were determined according to Van den Dool and Kratz, 1963, using n-alkanes as standards. The identification of the components was based on

comparison of their mass spectra with those of NIST21 and NIST107, Massada, 1976 and by comparison of their retention indices with literature data Adams, 2007. Component relative concentrations were calculated based on GC peak areas without using correction factors. Essential oils were often subjected to co-chromatography with authentic compounds (Fluka, Sigma).

Antioxidant activity

The free radical scavenging activity of the essential oils was measured *in vitro* by the 2,20-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium and potassium persulfate (ABTS) assay according to Brand-Williams *et al.*, (1995) and to Re *et al.*, (1998) respectively. The stock solution for the DPPH assay was prepared by dissolving 24 mg of DPPH with 100 mL of methanol and stored at 20°C. The working solution was obtained by diluting the DPPH stock solution with methanol to achieve an absorbance of about 0.98±0.02 at 517 nm using a spectrophotometer (Biochrom Ltd. Libra S22). The stock solution for the ABTS assay was prepared by dissolving ABTS in water at a concentration of 7 mM. The ABTS radical cation (ABTS•1) was obtained by combining ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and thus allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The working solution was obtained by diluting the ABTS stock solution with ethanol to achieve an absorbance of about 0.7±0.02 at 734 nm using a spectrophotometer.

Three ml portions of these solutions were mixed with 77 µL of the sample at 6 concentrations: 0.5, 1, 2, 5, 10, 20 mg/mL. After the mixture was shaken well for the DPPH assay it was incubated in the dark for 30 min at room temperature and then the absorbance was measured at 517 nm. For the ABTS assay the absorbance was measured at 734 nm after 5-6 minutes. The controls were prepared as above but without essential oil. The activity was assessed based on the percentage of the DPPH and ABTS radical removed as the following equation:

Scavenging activity in % = {(absorbance of control - absorbance of sample) / (absorbance of control)} x 100.

Result was calculated as the concentration of essential oil that inhibits 50% of the free radical (Inhibition Concentration IC_{50}).

Evaluation of antimicrobial activity by Micro-dilution Broth Experiment

The Essential Oil (EO) antimicrobial activities were investigated against different clinical and food-borne pathogens using standard American Type Tulture Collection strains, five bacteria along with one fungal isolate. The bacterial and fungal ATCC strains were stored at 4°C and sub-cultured for the experimental setup. Prior to the inoculation of the strains with EOs, the microorganisms were grown at 28 or 37 °C (for fungus and for bacteria) for 18-20 h on blood agar medium.

The MIC (Minimum Inhibitory Concentration) of each EO was determined using a broth microdilution method in 96-well plate in accordance with the Clinical & Laboratory Standards Institute (CLSI) protocols (M100 Ed34, 2024). In brief, bacterial suspensions were adjusted to a final concentration of 10^5 CFU/mL cells standardized by 0.5 McFarland in Muller Hinton Broth (MHB) media. EO stock concentration of 100 mg/ml was prepared by dissolving in DMSO. From this stock, a working concentration of 5 mg/ml was prepared (in MHB media) to be used in plate susceptibility testing, ensuring DMSO concentration less than 5%. This resulted in EO's concentration range of 5mg/ml to 0.0097 mg/ml on the plate. One hundred microliters of bacterial suspension were finally added to each well. The plate setup included the 11th column as the media control (negative control), and the 12th column containing bacteria and media (positive control). Additionally, rows D and E were designated for solvent controls, with row D with only EO (solvent control) and row E for DMSO (conc. used to dissolve EO) and bacteria served as DMSO control. The plates were incubated at 37°C for 24 h. MIC was determined using Tecan I-control software (Infinite M Plex TECAN) to measure the OD (600nm) compared with positive control. Everything was kept constant for determining antifungal activity except incubation was done for 45-48h, with OD determined at 530nm. MIC was determined as the lowest concentration of EO that inhibited visible growth of the tested microorganism (Puškárová A *et al.* 2017).

RESULTS AND DISCUSSION

Plant material and EO extraction

The amount of by-product from different batches ranged from 10 to 20% of the total mass of *J. communis* L., with a mean value of 13%. The by-product was mainly composed of niddles and few berries on the top of the branch. The industrial hydrodistillation employed to extract the EO from *J. communis* L. by-product yielded 0.8% of the total mass of the plant material and the EO resulted in white-yellow colour.

Quantitative and qualitative analysis of the EO

GC-MS analysis (Figure 1) identified in total 49 different components, counting for 86.4% of the EO. Results are given in Table 1, where compound are listed according to their Arithmetic Index. The oil was composed mainly by the monoterpene fraction (62.3%) with α-Pinene (24.47%) being the most abundant compound, followed by Sabinene (12.4%), γ -Terpinene (4.6%), Limonene (4.1%). The sesquiterpene compounds constituted 24.1% of the essential oil with β-Caryophyllene (3.9%) and Germacrene B (3.8%) as main compounds. In general the EO composition was in compliance with ISO 8879 and The European Pharmacopoeia 10th edition (Eur.Ph. 10th) specifications, exept for α-Pinene being slightly lower than the specification of ISO 8879, and α-Phellandrene being above the maximum level set by Eur.Ph. 10th.

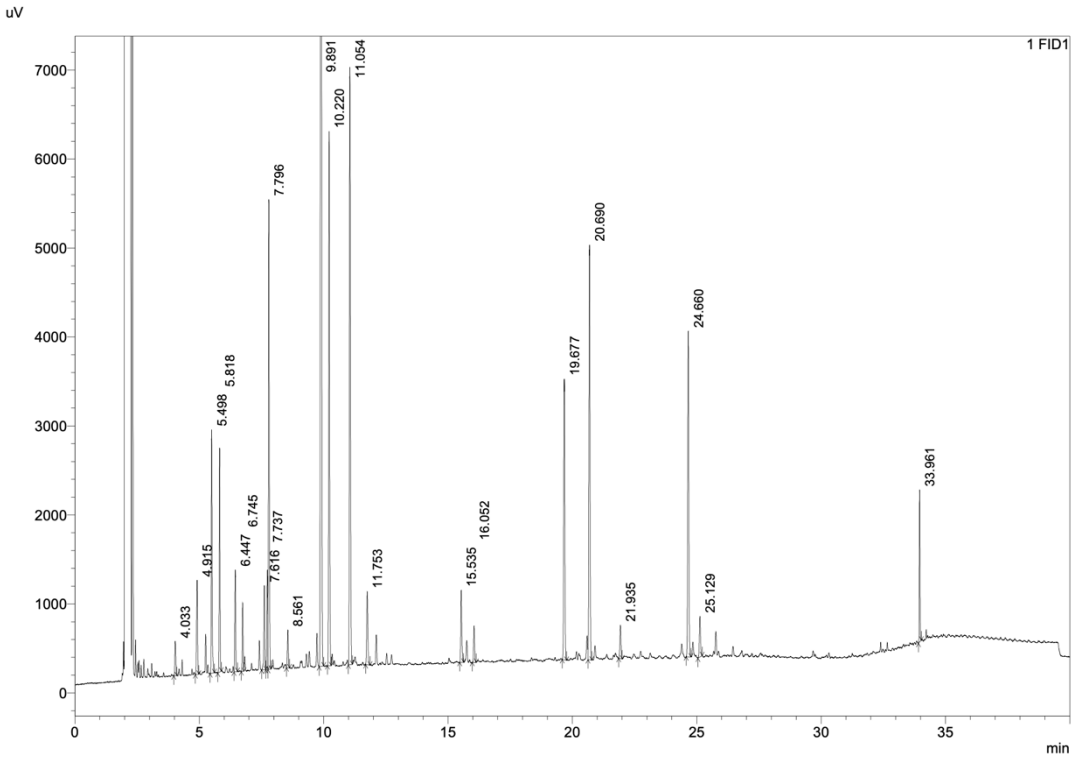

Figure 1. GC-MS chromatogram of EO from *J. communis* L. by-product.

Main components were in agreement with other reports from Albania (Salamon *et al.*, 2014; Buci *et al.*, 2018, Agastra *et al.*, 2021) and from other countries (Angioni *et al.*, 2003; Höferl *et al.*, 2014; Koukos *et al.*,1999) except for the concentration of β-Myrcene, where it is generally reported in higher concentrations as one of the main components. Similar β-Myrcene levels were found by Ainan *et al.*, (2022) with β-Myrcene *J. communis* L. harvested in Maroco. Probably this is due to high levels of leaves (niddles) in the by-product from *J. communis* L. as the primary component that distinguishes berry oil from needle oil is myrcene, which may be found in berry oil at levels as high as 20% whereas it can only be found in needle oil at levels as low as 5% (Shamir *et al.*, 2003).

Table 1. Composition of the essential oil from *J.communis* L. by-product and reference values obtained for specific compounds considered in ISO 8879 and Eur.Ph. 10th edition.

Compounds [a]	**AI** [b]	**Percentage (%)**	**ISO 8897**	**Eur. Ph. 10th**	**ID** [c]
Tricyclene	919	1.1			AI, MS
α-Thujene	926	3.3			AI, MS
α-Pinene	931	24.47	25-45	20-50	AI, MS, Co-GC
Camphene	945	0.3			AI, MS
Thuja-2,4(10)-diene	952	0.2			AI, MS
Sabinene	972	12.4	4-20	max. 20	AI, MS
β-Myrcene	992	1.6	3-22	1-35	AI, MS, Co-GC
α-Phellandrene	1003	1.5		max. 1	AI, MS
p-Cymene	1024	2.7			AI, MS, Co-GC
Limonene	1027	4.1	2-8	2-12	AI, MS
γ-Terpinene	1059	4.6			AI, MS, Co-GC
Terpinolene	1087	3.1			AI, MS
Monoterpene hydrocarbons		59.37 %			
Linalool	1101	0.08			AI, MS, Co-GC
α-Campholenal	1126	0.08			AI, MS
trans-Pinocarveol	1138	0.07			AI, MS
Borneol	1164	0.2			AI, MS, Co-GC
Terpinene-4-ol	1176	1.6	1-6	0.5-10	AI, MS, Co-GC
p-Cymen-8-ol	1187	0.1			AI, MS
α-Terpineol	1191	0.06			AI, MS
Citronellol	1232	0.15			AI, MS
Bornyl acetate	1286	0.15	n.d-0.6	max. 2	AI, MS, Co-GC
Undecanone	1297	0.14			AI, MS
α-Cubebene	1349	0.1			AI, MS
Citronellyl acetate	1357	0.2			AI, MS
Oxygenated Monoterpenes		2.93 %			
α-Ylangene	1371	0.1			AI, MS
α-Copaene	1375	0.1			AI, MS
β-Caryophyllene	1419	3.9		max. 7	AI, MS, Co-GC
β-Copaene	1428	0.3			AI, MS
γ-Elemene	1434	0.6			AI, MS
Aromadendrene	1438	0.5			AI, MS
Cis-Muurola-3,5-diene	1450	0.1			AI, MS
α-Carryophyllene	1453	1.4			AI, MS, Co-GC
trans-Cadina-1 (6), 4-diene	1473	0.2			AI, MS
γ-Muurolene	1477	1.4			AI, MS
Germacrene D	1481	3.2	1-5		AI, MS
β-Selinene	1486	0.4			AI, MS
trans-Muurola-4(14),5-diene	1492	0.1			AI, MS

Viridiflorene	1495	0.9		AI, MS
α-Muurolene	1500	0.7		AI, MS
δ-Amorphene	1510	0.1		AI, MS
γ-Cadinene	1514	1.6		AI, MS
δ-Cadinene	1524	1.2	1-3.5	AI, MS
Germacrene B	1557	3.8		AI, MS
Sesquiterpene Hydrocarbons	20.6 %			
Spathulenol	1578	0.3		AI, MS
Humulene epoxide II	1610	0.4		AI, MS
1-epi-Cubenol	1629	0.6		AI, MS
α-Muurolol (Torreyol)	1643	0.8		AI, MS
Cubenol	1648	0.6		AI, MS
α-Cadinol	1656	0.8		AI, MS
Oxygenated Sesquiterpenes	3.5 %			
Total		86.4 %		

[a]Compounds listed in order of elution from an HP-5 MS capillary column; [b] AI: Arithmetic indices as determined on a HP-5 MS capillary column using a homologous series of n-alkanes (C9-C23); [c] Identification method: AI=Arithmetic Index, MS=mass spectrum, Co-GC=Coinjection with authentic compound. Concentrations below 0.05% are marked as tr (traces).

Antioxidant activity

DPPH assay was one of the *in vitro* tests used in this study to determine the ability of juniper berry oil components to act as hydrogen atom donors. The EO form juniper by-product was a weak DPPH radical reducer with IC_{50} value of 155.4 µg/mL. This was expected as the main component in the EO, α-pinene, was found by Emami *et al.*, (2007) to show no antioxidant activity at all in relation to DPPH radicals. The same work established that γ-terpinene (17.74%) showed antiradical activity in relation to DPPH radicals. Also limonene in 10–50 µg/mL concentrations causes DPPH inhibition from 16% to 25% (Roberto *et al.*, 2010).

In relation with the ABTS radicals the result was similar with an IC_{50} value of 163.2µg/mL. The results are shown in Table 2.

Table 2. Antioxidant activity of essential oil from *J. communis* L. by-product by DPPH and ABTS assays.

Assay	**IC_{50}* *J. communis* L. EO**	***St. Dev***
DPPH	155.4 µg/mL	±7,04
ABTS	163.2 µg/mL	±8,78

Antimicrobial activity

Table 3 shows the antibacterial activity of essential oil from *J. communis* L. by-product as Minimum Inhibitory Concentration. In tested concentrations the oil was not active against *E. coli, S. enteritidis* and *P. aeruginosa* bacterial strains while it resulted active against *M. luteus* and *S. maltophilia* with MIC 2.5 and 5mg/ml respectively. Similar results were reported from Sela *et al.*, (2013) where *J. communis* leaves and berries EO using broth dilution method showed no activity against *E. coli*, *S. enteritidis* and *P. aeruginosa*. The oil showed antifungal activity against *C. albicans* yeast with MIC 2.5 mg/ml. However According to Sela *et al.*, (2013) no MIC could be measured against *C. albicans* yeast from *J. communis* leaves and berries EO. Similar results were obtained from Angioni *et al.*, (2003) were *J. communis* EO tested was not active against *C. albicans*. To our knowledge this is the first report that shows the antibacterial activity of essential oil from *J. communis* on *S. maltophilia.*

Table 3. Minimum Inhibitory Concentration of essential oil from *J. communis* L. by-product.

Microorganism	**MIC** ***Juniperus communis* L. antimicrobial activity**
E. coli ATCC 10535	NO MIC
S. enteritidis ATCC 49223	NO MIC
P. aeruginosa ATCC 9027	NO MIC
M. luteus ATCC 10240	2.5 mg/ml
S. maltophilia ATCC 13637	5 mg/ml
C. albicans ATCC 10231	2.5 mg/ml

CONCLUSIONS

The purpose of this study was to assess the essential oil that was industrially extracted from *J. communis* L. by-product produced by the MAPs industry by analyzing its chemical content and biological activity, in order to determine the potential value of this by-product.

The chemicals' presence is consistent with findings from the literature. Furthermore, proof of these compounds' existence in the EO supports the moderate free scavenging activity ascertained by the DPPH and ABTS assay, which is similar to EO isolated from *J. communis* L. primary material. Therefore, this industry by-product is appropriate to be used to produce EO that meets the general requirements for *J. communis* L. EO as established by ISO 8879 and Eur. Ph. 10th.

The terpenes and other volatile constituents found in the by-product plant material could be further utilized for the production of enriched aromatic beverages in which *J. communis* L finds wide applications (Enescu *et al.* 2016).

Considering all these findings, it is clear that the by-product plant material is a valuable source of bioactive compounds and that it could be used to produce novel nutritional products by as antioxidant, antimicrobial and flavoring additives to food.

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