Article
Volatile Compounds and Antioxidant and Antifungal Activity of Bud and Needle Extracts from Three Populations of Pinus mugo Turra Growing in Romania

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Abstract: Pinus mugo Turra is a shrub-like conifer with multiple healing properties. P. mugo extracts are rich in active ingredients such as volatile compounds, tannin, higher alcohols, vitamins, and minerals. In this study, we identified and quantified the secondary metabolites from buds and needles of P. mugo harvested from three different mountain areas in Romania. The main volatile compounds contained in the extracts were analyzed by gas chromatography coupled with mass spectrometry, and the most significant were pinene, germacrene, limonene, and caryophyllene. The total polyphenol content (TPC) was in the range of 46.77 ± 0.3 and 77.99 ± 0.5 mg GAE/g and the total flavonoid content (TFC) 24.90 ± 0.1 and 54.78 ± 0.3 mg QE/g. The content of ascorbic acid ranged between 12.21 mg/100 g and 27.34 mg/100 g, concentrations that are recommended for natural sources of ascorbic acid. Moderate antimicrobial activity on yeasts and molds was not dependent on plant origin. By highlighting the rich content of active compounds, and moderate antioxidant and antifungal activity, this study is an argument for the beneficial use of P. mugo bud and needle extracts, regardless of the habitat of origin on the Romanian territory, in the fields of medicine and the food industry or in the implementation of eco-friendly practices.

Keywords: Pinus mugo; volatile compounds; polyphenols; flavonoids; antioxidant activity; antifungal activity

1. Introduction
Pinus, the largest genus of conifers, with approximately 113 species, is widespread in the Northern Hemisphere, especially in the high massifs, where subalpine communities are formed [1-3].

Pinus mugo Turra, also known as dwarf mountain pine, is a small conifer, native to Central and Eastern Europe, that grows at altitudes between 200 m and 2700 m [4]. In Romania, it is found starting from an altitude of 1350 m in the Western Carpathians to 2100 m in the Southern Carpathians [5], as quoted by Roșca et al. [6]. P. mugo is a cold-tolerant xerophyte, light-loving, well adapted to harsh environmental conditions, surviving on nitrogen-poor soils, tolerating precipitation in varying amounts, and is perfectly adapted to petrophytic habitats, rich in basic rocks such as limestone, sandstone, granite, etc. Due to its long and extensive roots, dwarf mountain pine has an important role in consolidating wetlands, in protecting and fixing debris and weather-eroded coasts, and in regulating the...
hydrological regime, being an excellent buffer for avalanches [7,8]. At the same time, being an evergreen plant with an attractive shape, a large number of cultivars find dendrological applicability [9,10].

From the perspective of chemical composition, studies have shown that valuable plant secondary metabolites, including those from genus *Pinus*, possess significant health benefits [11–14]. More than 100 secondary metabolites have been identified in *P. mugo* essential oil by Venditti et al. [15], the most important accumulations being of valuable compounds such as oxygenated monoterpenes (32.7%) and oxygenated diterpenes (22.3%), but also sesquiterpene hydrocarbons (13.1%) and oxygenated sesquiterpenes (11.6%). The presence of monoterpenes, diterpenes, and other volatile compounds in the extracts or essential oils extracted from *Pinus* sp. was highlighted in several studies [16–24]. The needles of *Pinus* sp. are a rich source of ascorbic acid, flavonoids, and other phenolic compounds [25–27].

From the needles, bark, buds, and cones are obtained extracts or essential oils rich in secondary metabolites that are used against rheumatic and lung diseases, as anti-inflammatory and expectorant agents [15,28,29]. Moreover, due to its rich polyphenol composition, extracts of *P. mugo* have high antioxidant activity [25,30,31], which gives them antitumor and antiproliferative properties [29,32–36]. The pharmacological attribute is complemented by the studies carried out by Basholli-Salihu et al. [29], who highlighted the anti-inflammatory activity and cytotoxic effect of *P. mugo* oils on three tumor cell lines. Antibacterial and antifungal action have often been reported [21,25,31,37,38], but further studies are needed to validate the antimicrobial profile of this species.

The beneficial effects of various pine extracts on human health are often reported in the literature, but nevertheless the use of pine-based products is not greatly explored, especially as an ingredient in the food industry [3]. Lately, in Europe, there has been a growing trend regarding the use of supplements made from natural *Pinus* bark extracts that are highly rich in phytochemicals and known to help prevent some diseases. This idea led to the functionalization of foods and drinks with plant-based extracts. It also had a large impact on the supplement market, where the production and sale of dietary supplements, especially plant-based ones, has grown at an exponential rate. Moreover, some essential oils are acknowledged as stimulators of the nervous system. Both extracts and essential oils are used as preservatives and antioxidants in food and food packaging materials to prevent food spoilage and rancidity [21]. Having a diverse spectrum of applications, pine extracts represent a new alternative in the pharmaceutical, agricultural, and nutritional fields.

The main components of an extract or an oil from this conifer species do not depend only on its concentration, being also based on the specific odor threshold, which is essentially determined by its structure and volatility, or even by the minor components that derive from oxidation or degradation reactions and which have a strong impact on the perfume, so that the intensity of the aroma does not depend only on a single component [27]. For the aromatic constituents, the conditions during the processing and storage of plant material are very important because they are influenced by humidity and light, leading to a variation in their content that can ultimately affect the overall profile [29].

In traditional medicine, *P. mugo* syrup is used as an expectorant [39], in the treatment of coughs and throat inflammation [40]. In Romanian folk medicine, the leaf buds of *P. mugo*, harvested in February, are used in the form of a 3–10% infusion as a diuretic and antiseptic in cystitis and urethritis, and as an anti-inflammatory in respiratory diseases [41,42]. In the food industry, shoots are used for flavoring dishes of fish and meat or jam [40,43]. The use of essential oils, pine nuts, and pinewood oil was also mentioned in the manufacture of soaps, detergents, and household cleaning supplies [44]. An extensive review regarding the secondary metabolite content and the pharmacological properties, antimicrobial activity, and food applications of *Pinus* species was recently presented by Dziedziński et al. [14].

In this study, we aimed to evaluate the chemical composition and antioxidant and antifungal activity of extracts from the buds and needles of *P. mugo* harvested from three mountain massifs located in the southwestern, central–eastern, and central–southern parts
of the Southern Carpathians, in Romania. To our knowledge, this is the first study showing significant differences in yield and composition in valuable compounds from the buds and needles of *P. mugo* from three populations from the same mountain group in Romania. The results of this study are useful for identifying the geographical area that can be successfully exploited for the expansion of new plantations and thus increasing the productivity of target compounds for the pharmaceutical and food industries.

2. Materials and Methods

2.1. Pinus mugo Samples, Fungi Strains, and Culture Media

The dwarf mountain pine samples (buds and needles) were collected in May 2021 from three mountain areas (one representative sample for each location), as follows: Cindrel-Iujbea 45°34′45.9″ N, 23°48′43.3″ E; Făgăraș-Viștea 45°36′10.9″ N, 24°44′9.5″ E, and Cozia-Rotunda 45°17′49″ N, 24°13′26″ E. The characterization of the harvesting areas is presented in Table 1. The samples were identified, noted, and stored within CCBIA from L. Blaga University, Sibiu, Romania. The scoring was performed as follows: Cindrel-Iujbea/buds (CIB), Cindrel-Iujbea/needles (CIN); Făgăraș-Viștea/buds (FVB), Făgăraș-Viștea/needles (FVN), Cozia-Rotunda/buds (CRB), Cozia-Rotunda/needles (CRN).

<table>
<thead>
<tr>
<th>Local Harvest</th>
<th>Location in the Southern Carpathians</th>
<th>Altitude (m)</th>
<th>Climate</th>
<th>Average Annual Temperatures (°C)</th>
<th>Annual Average of Precipitation (mm³)</th>
<th>Geological Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cindrel-Iujbea</td>
<td>Southwestern</td>
<td>1750</td>
<td>Temperate moderate</td>
<td>4–6</td>
<td>800–1400</td>
<td>Crystalline shales</td>
</tr>
<tr>
<td>Făgăraș-Viștea</td>
<td>Central–eastern</td>
<td>1800</td>
<td>Temperate continental</td>
<td>6–8</td>
<td>1100</td>
<td>Crystalline shales</td>
</tr>
<tr>
<td>Cozia-Rotunda</td>
<td>Central–southern</td>
<td>1550</td>
<td>Temperate moderate</td>
<td>4+3</td>
<td>1200</td>
<td>Hard gneiss rocks</td>
</tr>
</tbody>
</table>

Stems and culture media used for antimicrobial assessment were: *Candida albicans* 2231—yeast, *Cryptococcus neoformans* 223, *Penicillium chrysogenum* 1004, *Aspergillus flavus* 1082 (CCBIA collection from L. Blaga University, Sibiu, Romania), Czapek Dox broth, Czapek Dox agar (Sigma-Aldrich GmbH, Taufkirchen, Germany).

The samples were stored at the Biotechnologies and Food Engineers Research Center (CCBIA) within the Agricultural Sciences, Food Industry, and Environmental Protection Faculty/ULB Sibiu, receiving the following registration number: 225/1-225/6.

2.2. Chemical and Reagents

The chemicals and reagents involved in the extraction process were: 99.8% methanol (CH₃OH), Folin–Ciocălăteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH—C₁₈H₁₂N₂O₆), Trolox (C₁₄H₁₇O₃), metaphosphoric acid (H₃PO₄), dimethyl sulfoxide (DMSO—C₇H₈O), 7.5% sodium carbonate (Na₂CO₃), Whatman filter paper with a pore size of 15 µm, gallic acid (C₇H₆O₅), and quercetin (C₁₅H₁₀O₇) from Sigma-Aldrich GmbH, Germany; sodium nitrite (NaNO₂), 10% aluminum chloride hexa-hydrate (AlCl₃·6H₂O), sodium hydroxide (NaOH) 1 M, glacial acetic acid (CH₃COOH), and ascorbic acid (C₆H₈O₆) from Honeywell Fluka.

2.3. Extraction Procedure

After harvest, the buds and needles of *P. mugo* were dried in the incubator at a temperature of 45 °C for 10 days (until constant mass). The samples were crushed to a granularity between 100 and 500 µm and stored in the dark at a temperature of 10 °C ± 1 °C. Accurately weighed, 10 g of each sample was refluxed three times in 100 mL of 80% methanol for 4 h. The samples were filtered through a filter paper with a porosity of 15 µm, using a vacuum pump, concentrated in a rotary evaporator, and stored at 4 °C for analysis.
In order to determine the total polyphenols, flavonoids, and antioxidant activity, methanol extracts were used (5 mg/mL methanol 80% (w/v)). For the assessment of the antifungal activity, aqueous extracts were prepared (0.25 mg extract/5 mL distilled water (w/v)).

Extraction Procedure for GC/MS

For GC/MS analysis, 500 mg of plant powder was subjected to extraction in an Erlenmeyer beaker fitted with a rodded stopper, 3 times, with 10 mL of methanol, for 15 min, at 500 rpm. The organic extract was filtered on filter paper and concentrated to 1 mL.

2.4. Identification and Quantification of Volatile Compounds by the GC/MS Method

The main volatile compounds present in P. mugo extracts were analyzed by gas chromatography coupled with an Agilent GC 6890-MS 5973N mass spectrometry device (Santa Clara, CA, USA) and a DB-5 capillary column (30 m × 0.25 mm, 0.25 µm thick, J&W Scientific, Santa Clara, CA, USA).

The obtained extracts were filtered through a 0.5 µm microporous polytetrafluoroethylene membrane and then 1 µL of the filtered extract was injected directly with a part ratio of 1:10. The carrier gas was helium (He, 99.999%) at a flow rate of 0.8 mL/min.

The column temperature range used for the separation of volatile compounds increased from 40 °C (maintained for 4 min) to 120 °C at a rate of 6 °C/min, then to 180 °C for 6 min with a rate of 8 °C/min, and finally to 220 °C for 6 min. Injector and detector temperatures were set at 230 °C and 250 °C.

The identification of the target compounds was performed by comparing their mass spectra with those listed in the Wiley 275 library. Furthermore, the experimental values of the retention indices (IR) were determined using the automated mass spectral deconvolution and identification system (AMDIS, ver. 2). Mass spectra were acquired in electron ionization (EI) mode (70 eV) in the range m/z 40–450. Samples were injected by an automatic liquid sampler (1 mL; split mode, 1:30).

2.5. Determination of Total Phenolic Content (TPC)

The total polyphenol concentration was measured by spectrophotometry using the modified Folin–Ciocâlteu method [45]. A volume of 1.58 mL of distilled water and 100 µL of Folin–Ciocâlteu reagent were added to 20 µL of methanol extract. Subsequently, 300 µL of 20% sodium carbonate was added, the samples being kept for 2 h in the dark at room temperature. The reading was performed on the spectrophotometer (UV-1900 SHIMADZU spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 765 nm, in the presence of a control sample defined by the same reagents, except the extract. The calibration curve was performed, and gallic acid was used as a standard. The total polyphenol content was expressed in gallic acid equivalent/g of dry extract (DE), the determinations being performed in triplicate.

2.6. Determination of Total Flavonoid Content (TFC)

The total flavonoid content was determined based on the method used by Al-Rifai et al. [46], slightly modified. Briefly, 5 mL aqueous extract was homogenized with 0.3 mL of 5% NaNO₂ solution and incubated in the dark. After 5 min, 0.5 mL of 10% AlCl₃·6H₂O solution was added and the reaction mixture was kept in the dark for another 15 min. After the complete reaction, 2 mL of 1 M NaOH and distilled water were added to a total volume of 10 mL. The absorbance was read at a wavelength of 510 nm, the total flavonoid content being expressed in mg equivalent to quercetin/gram of extract.

2.7. Assessment of Antioxidant Activity

Evaluation of antioxidant activity was performed using the slightly modified spectrophotometric method [47], which involves the use of 1,1-diphenyl-2-picrylhydrazyl (DPPH) to evaluate the properties of compounds to remove free radicals or their ability
to donate hydrogen. From the methanol stock solution of DPPH (24:100), the working solution was prepared in a ratio of 10 mL stock solution and 90 mL methanol. For 35 min, 25 µL of each sample was allowed to react with 175 µL DPPH working solution at 22 °C, in the dark.

The absorbance was read at a 515 nm wavelength with the UV-1900 SHIMADZU spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Distilled water was used as a control. The results were expressed as milligrams of Trolox equivalent per gram of DE (mg TE/g DE).

The inhibition percentage (I) was calculated according to the equation

\[
% I = \frac{Ab - Aa}{Ab} \times 100,
\]

where Ab is the absorbance of the control, and Aa is the absorbance of the reaction between the sample and the radicals.

### 2.8. Identification of Ascorbic Acid

For the assessment of the ascorbic acid (AsA) content in buds and needles of *P. mugo* by the slightly modified HPLC method proposed by Meos et al. [48], the equipment Smartline, KNAUER GmbH Germany, equipped with a PDA detector, quaternary pump, automatic injection system, and C18 chromatographic columns, was used. The mobile phase, eluent A, consisted of a solution of water/acetic acid (95/5 v/v), and eluent B of acetonitrile/water/acetic acid (100/95/5, v/v/v). For extraction, 10 g of needle and bud powder, respectively, was homogenized with metaphosphoric acid/distilled water (3:100 v/v) and glacial acetic acid/distilled water (8:100 v/v) for 45 min, in the dark. The supernatant obtained after settling and filtration was used in the determination of AsA. A volume of 10 µL of each sample was injected into the chromatographic column and eluted with the mobile phase for 15 min at 30 °C at a flow rate of 1 mL/min. The obtained values were expressed in mg/100 g DE.

### 2.9. Evaluation of Antifungal Activity

Antifungal activity was determined by the minimum inhibitory concentration (MIC) method using diffusion discs. *P. mugo* extracts were diluted to 25% in DMSO, followed by five successive dilutions, resulting in the following final concentrations: 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL. The strains of *C. albicans* 2231, *C. neoformans* 223, *P. chrysogenum* 1004, *A. flavus* 1082 were activated for 24 h in liquid culture medium, Czapek Dox broth, at a temperature of 25 °C, and then brought to a concentration of 0.5 McFarland by spectrophotometric reading. Pre-prepared fungal strains were inoculated into Petri dishes with Czapek Dox agar culture medium. Sterile discs of 6 mm in diameter were placed on the culture medium, and 10 µL of diluted extract was pipetted onto each disc. The cultures were incubated at 25 °C for 72 h. The lowest concentration of *P. mugo* extract that was able to inhibit fungal growth was considered the MIC. DMSO was used as a negative control and Posaconazole as an antifungal.

### 2.10. Multivariate Analysis

The main approach of multivariate statistical analysis was based on principal component analysis (PCA) to explain the significant associations between quality parameters, total phenolic compound content, and volatile organic compound (VOC) data. Pearson correlations (\( p < 0.05 \) and \( p < 0.01 \)) were used to identify correlations between all variables included in the dataset. All statistical analyzes were performed using Addinsoft XLSTAT software, version 2014.5.03 (Addinsoft Inc., New York, NY, USA).

### 3. Results

GC/MS analysis showed a varied accumulation of volatile compounds in *P. mugo* bud and needle extracts (Table 2). The most significant values were obtained in the case
of the compound δ-3-carene, which was identified in buds at values between 19.66% and 21.42%, and in needles at values between 18.28% and 20.94%, the highest percentage being specific to the CIB samples. Another valuable compound identified in the samples was α-pinene, which ranged from 14.35% to 16.05% in buds and from 15.27% to 16.31% in needles, the FVN samples being characterized by the highest percentage. The volatile compound germacrene D was found in values between 8.93% and 12.14% in buds and between 7.81% and 12.13% in needles, and the trans-caryophyllene compound was present in values between 5.66% and 8.44% in buds and between 6.32% and 8.12% in needles.

Table 2. Chemical composition of *Pinus mugo* bud and needle extracts from three mountainous areas in Romania (Cindrel-Iujbea, Făgăraș-Viștea, Cozia-Rotunda).

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>Ions (m/z)</th>
<th>Cindrel-Iujbea (%) Buds</th>
<th>Needles</th>
<th>Făgăraș-Viștea (%) Buds</th>
<th>Needles</th>
<th>Cozia-Rotunda (%) Buds</th>
<th>Needles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanal</td>
<td>6.369</td>
<td>44.56.100</td>
<td>0.01</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>7.146</td>
<td>31.59.60</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>8.236</td>
<td>93.121.136</td>
<td>14.77</td>
<td>15.27</td>
<td>14.35</td>
<td>15.63</td>
<td>16.05</td>
<td>15.99</td>
</tr>
<tr>
<td>Camphene</td>
<td>8.916</td>
<td>93.121.136</td>
<td>6.78</td>
<td>5.96</td>
<td>4.13</td>
<td>6.32</td>
<td>5.98</td>
<td>4.27</td>
</tr>
<tr>
<td>Sabinene</td>
<td>9.003</td>
<td>77.93.136</td>
<td>0.88</td>
<td>0.91</td>
<td>0.78</td>
<td>0.34</td>
<td>0.47</td>
<td>0.82</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>10.138</td>
<td>41.93.136</td>
<td>3.11</td>
<td>3.48</td>
<td>2.17</td>
<td>2.45</td>
<td>1.99</td>
<td>1.91</td>
</tr>
<tr>
<td>β-Mycene</td>
<td>11.005</td>
<td>69.93.136</td>
<td>1.45</td>
<td>1.76</td>
<td>2.12</td>
<td>2.31</td>
<td>2.17</td>
<td>1.99</td>
</tr>
<tr>
<td>Octanal</td>
<td>12.127</td>
<td>39.84.128</td>
<td>1.01</td>
<td>0.92</td>
<td>0.23</td>
<td>0.41</td>
<td>0.16</td>
<td>0.17</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>14.243</td>
<td>91.191.34</td>
<td>1.1</td>
<td>0.92</td>
<td>0.18</td>
<td>1.23</td>
<td>0.45</td>
<td>-</td>
</tr>
<tr>
<td>D-Limonene</td>
<td>15.891</td>
<td>43.67.168</td>
<td>6.78</td>
<td>5.96</td>
<td>4.13</td>
<td>6.32</td>
<td>5.98</td>
<td>4.27</td>
</tr>
<tr>
<td>Linalool acetate</td>
<td>16.008</td>
<td>93.121.169</td>
<td>-</td>
<td>-</td>
<td>0.12</td>
<td>0.11</td>
<td>-</td>
<td>0.17</td>
</tr>
<tr>
<td>2-Methyl-1-butanol nerolidol</td>
<td>17.087</td>
<td>57.70.88</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-Methyl-1-butanol</td>
<td>17.319</td>
<td>57.70.88</td>
<td>0.1</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-Terpenyl acetate</td>
<td>18.624</td>
<td>43.121.196</td>
<td>1.79</td>
<td>1.98</td>
<td>2.12</td>
<td>1.73</td>
<td>1.49</td>
<td>1.56</td>
</tr>
<tr>
<td>α-Terpine</td>
<td>18.718</td>
<td>93.121.136</td>
<td>1.01</td>
<td>0.99</td>
<td>0.19</td>
<td>1.13</td>
<td>0.45</td>
<td>0.38</td>
</tr>
<tr>
<td>Terpinene-4-ol</td>
<td>18.906</td>
<td>71.111.154</td>
<td>0.21</td>
<td>0.37</td>
<td>0.71</td>
<td>0.32</td>
<td>0.22</td>
<td>0.71</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>19.005</td>
<td>59.93.154</td>
<td>0.23</td>
<td>0.34</td>
<td>0.31</td>
<td>0.16</td>
<td>0.11</td>
<td>0.22</td>
</tr>
<tr>
<td>α-Terpinolene</td>
<td>19.136</td>
<td>79.121.136</td>
<td>5.87</td>
<td>6.02</td>
<td>5.23</td>
<td>4.99</td>
<td>6.12</td>
<td>4.57</td>
</tr>
<tr>
<td>Myrtenol</td>
<td>21.222</td>
<td>79.108.152</td>
<td>0.11</td>
<td>0.03</td>
<td>-</td>
<td>0.04</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>22.356</td>
<td>95.136.196</td>
<td>3.44</td>
<td>2.09</td>
<td>3.48</td>
<td>4.55</td>
<td>4.01</td>
<td>3.12</td>
</tr>
<tr>
<td>β-Copaene</td>
<td>23.118</td>
<td>119.161.204</td>
<td>0.22</td>
<td>0.31</td>
<td>0.31</td>
<td>0.44</td>
<td>0.38</td>
<td>0.35</td>
</tr>
<tr>
<td>a-Hummulene</td>
<td>24.936</td>
<td>93.121.204</td>
<td>2.19</td>
<td>2.31</td>
<td>2.45</td>
<td>3.11</td>
<td>3.27</td>
<td>3.44</td>
</tr>
<tr>
<td>α-Cadinene</td>
<td>25.224</td>
<td>161.189.204</td>
<td>0.22</td>
<td>0.24</td>
<td>0.16</td>
<td>0.19</td>
<td>0.32</td>
<td>0.29</td>
</tr>
<tr>
<td>δ-Cadinene</td>
<td>25.647</td>
<td>105.161.204</td>
<td>8.29</td>
<td>7.93</td>
<td>7.71</td>
<td>7.17</td>
<td>8.03</td>
<td>8.56</td>
</tr>
<tr>
<td>α-Cadinol</td>
<td>26.055</td>
<td>95.121.222</td>
<td>5.63</td>
<td>5.15</td>
<td>4.94</td>
<td>5.9</td>
<td>6.18</td>
<td>6.57</td>
</tr>
<tr>
<td>Trans-caryophyllene</td>
<td>28.164</td>
<td>93.133.204</td>
<td>7.88</td>
<td>7.92</td>
<td>8.44</td>
<td>8.12</td>
<td>5.66</td>
<td>6.32</td>
</tr>
</tbody>
</table>

Regarding the accumulation of other compounds, they were at subunit values (sabinene, camphene, octanal) or around 1–3%, with a definite accumulation of α-humulene (2.19–3.44%), β-myrcene (1.45–2.31%), or α-pinene (1.91–3.38%).

Terpenic compounds varied according to their chemical structure as follows: α-terpenyl acetate was identified in a variable percentage of 1.49–2.12% in buds and 1.56–1.98% in needles, α-terpinolene at 5.23–6.12% and 4.57–6.02% in buds and in needles, respectively. α-Terpinene was identified in bud extracts in an amount of 0.19–1.01% and in needles at 0.38–1.13%, α-terpineol was in the range of 0.11–0.31% in buds and between 0.16% and 0.34% in needles, and terpinene-4-ol was identified at values of 0.21–0.71%. Hexanal and 3-methyl-1-butanol were identified at a value of 0.01% only in samples from Cindrel-Iujbea, and 2-methyl-1-butanol nerolidol was identified only in FVN samples. Linalool acetate was not detected in the buds and needles of Cindrel-Iujbea and needles of Cozia-Rotunda. Myrtenol was absent in FVB and CRN samples. Bornyl acetate was
present in all samples, with values between 2.09% and 4.55% in needles and 3.44% and 4.01% in buds.

Multivariate analysis by Hotelling transformation (Karhunen–Loève transformation) was applied to reduce the number of variables to the most representative [49]. Based on the aromatic profile of the bud and needle extracts from the three mountainous areas, statistical models were built in which the main components were grouped, while maintaining the maximum possible variation. The analysis of the PCA from the bud and needle extracts (Figure 1) showed a separation of the samples depending on the area of origin, CIN, FVB, and CRN being located on the positive semiaxes (Figure 1a). Among these samples, defined by terpinene-4-ol, 1-propanol, δ-cadinene, β-myrcene, and phenolic compounds, CRN was located in the first quadrant, far apart from the other samples. The CIN sample was located near the intersection of the third and fourth quadrants, contributed by α-terpineol, sabinene, α-terpenyl acetate, and trans-caryophyllene. Nearby, in the third quadrant, was located CIB, richer in aromatic compounds such as 1-hexanol, β-pinene, δ-3-carene, α-terpinolene, etc. Germacrene D, α-humulene, α-pinene, α-cadinene, 2-methyl-1-butanol, nerolidol, and other compounds predominated in the needle extracts harvested from Făgăraș-Viștea and buds from Cozia-Rotunda, with samples located in the second quadrant, in the negative semiaxis (Figure 1b).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Differentiation of bud and needle sources based on the compositional profile; (a) PCA plot illustrating differentiation of bud and needle sources based on the compositional profile. Colored symbols correspond to the 3 regions defined in this study. The first two principal axes explained approximately 69 % of the variance; (b) PCA plot showing the multivariate variation among the 3 different regions in terms of chemical compositional variables. Vectors show the contribution of each compositional variable to the overall distribution as well as their strength.

Even though the investigated metabolites are qualitatively similar, there was a noticeable quantitative variation in VOCs across the different geographical locations. There was a wide variation in the quantity of several compounds in *P. mugo* across the three studied locations. Based on these observations, we used the Kaiser–Meyer–Olkin measure of sampling adequacy test (KMO), generated by PCA, to identify the first nine most important VOCs with greater differentiation power, with which we investigated the differences in the ratios of VOCs in the studied blends of *P. mugo* extracts (Figure 2).
Figure 2. Mean amounts of the nine most frequent VOCs for classifying *Pinus mugo* buds and needles harvested from the mountainous areas Cindrel-Iujbea, Făgăraș-Viștea, Cozia-Rotunda in Romania, by using KMO.

To identify a model for characterizing the overlapping bud and needle sources of *P. mugo* following PCA analysis, the multivariate analysis was supplemented by cluster analysis that minimized variation within a group and identified the set of homogeneous groups. Thus, the segmentation analysis highlighted two clusters, a similar chemical and isotopic composition being highlighted for the bud and needle samples from Cozia-Rotunda and Făgăraș-Viștea, compared to the buds and needles from Cindrel-Iujbea (Figure 3).

Figure 3. Distribution of the clusters for bud and needle samples from Cindrel-Iujbea, Făgăraș-Viștea, Cozia-Rotunda.

3.1. Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) and Evaluation of Antioxidant Activity

Following the determinations carried out, a series of significant values of polyphenols and flavonoids were established, which impress on the collected samples remarkable antioxidant qualities. According to the data presented in Table 3, the polyphenols ranged from 46.77 ± 0.3 mg GAE/g to 77.99 ± 0.5 mg GAE/g for *P. mugo* bud extracts and from 55.53 ± 0.3 mg GAE/g to 68.23 ± 0.4 mg GAE/g for needle extracts.
Table 3. Total polyphenols and flavonoids in bud and needle methanol extracts of Pinus mugo harvested from the mountainous areas Cindrel-Iujbea, Făgăraș-Vișteș, Cozia-Rotunda in Romania.

<table>
<thead>
<tr>
<th>Area of Origin</th>
<th>Extract</th>
<th>TPC * (mg GAE/g ± SD)</th>
<th>TFC * (mg QE/g ± SD)</th>
<th>TE * (mM/g DE ± SD) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cindrel-Iujbea</td>
<td>Buds</td>
<td>46.77 ± 0.3</td>
<td>24.90 ± 0.1</td>
<td>115.64 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>Needles</td>
<td>55.53 ± 0.3</td>
<td>36.34 ± 0.2</td>
<td>124.32 ± 11.3</td>
</tr>
<tr>
<td>Făgăraș-Vișteș</td>
<td>Buds</td>
<td>55.12 ± 0.3</td>
<td>37.10 ± 0.2</td>
<td>124.21 ± 13.2</td>
</tr>
<tr>
<td></td>
<td>Needles</td>
<td>68.23 ± 0.4</td>
<td>26.89 ± 0.2</td>
<td>164.93 ± 15.2</td>
</tr>
<tr>
<td>Cozia-Rotunda</td>
<td>Buds</td>
<td>77.99 ± 0.5</td>
<td>54.78 ± 0.3</td>
<td>194.54 ± 11.8</td>
</tr>
<tr>
<td></td>
<td>Needles</td>
<td>64.56 ± 0.2</td>
<td>45.55 ± 0.2</td>
<td>155.71 ± 23.6</td>
</tr>
</tbody>
</table>

* TPC—total polyphenol content; GAE—gallic acid equivalent; TFC—total flavonoid content; QE—quercetin equivalent; DPPH—2,2-diphenyl-1-picrylhydrazyl; TE—Trolox equivalent; SD—standard deviation.

The determinations were supplemented by results on the total flavonoid concentration, where values between 24.90 ± 0.1 mg QE/g and 54.78 ± 0.3 mg QE/g were identified for bud extracts and values between 26.89 ± 0.2 mg QE/g and 45.55 ± 0.2 mg QE/g for needle extracts.

3.2. Identification and Quantification of Ascorbic Acid and Evaluation of Antioxidant Activity

Following the determinations performed by the HPLC method, AsA was identified in the extracts derived from both buds and needles of P. mugo (Figure 4). AsA was found in lower amounts in buds, for which the values recorded ranged from 1.33 mg/100 g DW to 2.45 mg/100 g DW, while in needles, the values reached up to 27.34 mg/100 g DW. The most significant values of AsA were found in the FVN samples (27.34 mg/100 g DW), followed by CIN (25.67 mg/100 g DW), and the lowest was identified in samples from Cozia-Rotunda (12.21 mg/100 g DW). The antioxidant activity was at DPPH values (IC$_{50}$) between 10.7 ± 0.12 µg/mL and 13.2 ± 0.23 µg/mL, the most significant results being observed for CRB samples.

![Figure 4. Quantification of ascorbic acid and antioxidant activity of bud and needle methanol extracts of Pinus mugo from three mountainous areas of Romania (Cindrel-Iujbea, Făgăraș-Vișteș, Cozia-Rotunda).](image)

3.3. Antifungal Activity

In our study, the Posaconazole antifungal presented very good activity against C. albicans 2231, C. neoformans 223, and A. flavus 1082 (0.125 µL/mL), having no effect on the strain of P. chrysogenum. The assessment of antifungal activity revealed that the mold strain of P. chrysogenum showed sensitivity to extracts from P. mugo needles at a 0.125 µL/mL concentration, and bud extracts did not induce any inhibition of fungal growth. In comparison, A. flavus 1082 showed sensitivity to both bud extracts (0.25 µL/mL) and needle extracts (0.125 µL/mL).
The inhibitory effects of the tested extracts were null against *C. neoformans* strain 223, except for the needle extract from Făgărăș-Viștea, which showed a minimum level of inhibition at a maximum concentration of 1 µL/mL (Table 4).

Table 4. The minimum inhibitory concentration established for bud and needle aqueous extracts of *Pinus mugo* harvested from three mountainous areas of Romania (Cindrel-Iujbea, Făgărăș-Viștea, Cozia-Rotunda).

<table>
<thead>
<tr>
<th>Strains</th>
<th>CIB</th>
<th>CIN</th>
<th>FVB</th>
<th>MIC (µL/mL)</th>
<th>FVN</th>
<th>CRB</th>
<th>CRN</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> 2231</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em> 223</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>1.00</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>0.125</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>NE</td>
<td>0.125</td>
<td>NE</td>
<td>0.125</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>0.125</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em> 1082</td>
<td>0.25</td>
<td>0.125</td>
<td>0.25</td>
<td>0.125</td>
<td>0.25</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>DMSO negative control</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

NE—non-inhibitory effect.

4. Discussion  

Even if the geographical locations where the buds and needles of *P. mugo* were collected are located at a distance from urban settlements, the possibility of anthropogenic influences exists, triggering the synthesis of some volatile isoprenoids. The reasons for the heterogeneity of dwarf mountain pine monoterpene chemistry are not well known, and genetic and environmental variables might have different impacts on the composition of monoterpenes. For instance, individual monoterpenes, such as δ-3-carene (the dominant constituent), myrcene, limonene, and terpinolene, are known to have different degrees of heritability and are mainly determined by genetic factors, while α-pinene (the second most dominant constituent) and β-pinene are more dependent on environmental variables [21,50], these influences being observed in the variability of the present studied compounds. A higher value (27.8%) of δ-3-carene was identified in *P. mugo* needles from a natural population in “Bjeshkët e Nemuna” National Park, Kosovo [20]. However, it is relevant to mention in this context that the amount of monoterpenes varies depending on the plant part and the phenological stage [51]. Understanding the differences in VOC composition between closely related plant species could lead to a better understanding of the function of VOCs and could provide insights into the mechanisms underlying resistance.

Phenolic compounds have always been a benchmark in the chemical characterization of plant extracts, including conifers, with the aim of discovering alternative natural sources to commercial antioxidants. To date, the antioxidant activity of various pine species has been reported [25,33,37,52–55]. Grassmann et al. [30] reported the good antioxidative activity of the essential oil from *P. mugo* when evaluated in lipophilic environments. In another important study, among several conifer-derived essential oils, the best antioxidant activity was reported by Garzoli et al. [31] for *P. mugo*. In our study, the most significant values of TPC, TFC, and DPPH were obtained in the case of extracts from buds harvested from the Cozia-Rotunda area. Studies in other areas have shown lower [25] or higher values [33] than those shown by bud and needle extracts of *P. mugo* from Romanian populations.

AsA is a low-molecular-weight biochemical with essential antioxidant properties in humans. Although, until now, considerable differences in the bioavailability of synthetic and food-derived vitamin C have not been highlighted, an important number of epidemiological studies and meta-analyses indicate a decrease in the incidence of chronic diseases as a result of dietary vitamin C intake [56]. Raal et al. [57] determined, for several conifer species, a variation in AsA in the fresh samples between $0.1 \times 10^{-3}\%$ and $15 \times 10^{-3}\%$, values significantly lower compared to those determined in the samples collected from Făgărăș-Viștea and Cindrel-Iujbea. In our study, HPLC determination indicated that AsA are found in the largest amount in FVN samples, which indicates them as an emerging alternative in relieving oxidative stress. It is worth noting here that a recent study reported...
the higher antioxidant potential of the organic and hydroethanolic extracts obtained from needles of *Pinus nigra* J.F. Arnold in comparison with isolated phenolic compounds and diterpenes, suggesting the synergic antioxidant activity of phytochemicals [58].

The antifungal activity of the *P. mugo* extracts shown in Table 4 may be due to the high content of δ-3-carene. A relatively recent study showed that δ-3-carene effectively inhibited the growth of the postharvest pathogen *Penicillium expansum*. Moreover, the essential oil of *Pinus ponderosa* Douglas ex P. Lawson & C. Lawson, with content of 7.9% β-pinene, has been shown to be highly effective against *Fusarium culmorum*, *F. solani*, and *F. poae* [59]—hence the possibility of exploiting these biochemicals and plants with high content of this VOC for the development of environmentally friendly pathogen control practices [60]. In our study, CIN samples with the highest content of β-pinene inhibited the growth of *P. chrysogenum* and *A. flavus* 1082 at the lowest concentration of 125 µg/mL. The studies carried out by Scalas et al. [61] showed good inhibitory activity for *Pinus sylvestris* L., especially in combination with itroconazole, while Mačionienė et al. [62] showed that the 1% (v/v) essential oil of *P. sylvestris* has no antimicrobial activity. It is important to emphasize that the number of studies that evaluate the antifungal potential of *P. mugo* extracts is very limited [25,38]. Thus, the results reported in our study are significant and complete the picture of the antifungal potential of *P. mugo* extracts.

The differences determined regarding the content of volatile compounds and the antioxidant and antifungal capacity of the *P. mugo* buds and needles extracts can be attributed to the variety of biotic and abiotic factors in the three mountain massifs of the Southern Carpathians. On the other hand, this study can be an important starting point in the application of genetic engineering techniques and genome selection to improve economic characteristics, especially in the production of chemical compounds of interest.

5. Conclusions

The results reported by this study demonstrate that the geographical location influences the chemical composition of *P. mugo* extracts and present a new image of the bioactive qualities of plants that grow in various mountainous areas of Romania. The determinations performed via GC/MS methods identified a series of volatile compounds, the most significant amounts being represented by δ-3-carene, α-pinene, germacrene-D, D-limonene, and *trans*-caryophyllene, but also by terpene compounds. A similar chemical composition was highlighted for the bud and needle samples from Cozia-Rotunda and Făgăraș-Viștea. Polyphenols were found in amounts that support their use in the treatment of various diseases, the most important values being determined in the extracts from the buds harvested from Cozia-Rotunda. Flavonoids and vitamin C, in accordance with antioxidant activity, led to the establishment of a profile with important medicinal and nutritional properties. *P. mugo* could constitute an important reserve of dietary supplements and phytochemicals, useful in the treatment of inflammatory diseases and the respiratory system; it could be exploited for the identification of unique chemical compounds with antimicrobial activity and used in the food industry as a source of additives, flavorings, and preservatives. Lastly, these results suggest the importance of the growth/culture medium for the improvement of and capitalization on the chemical composition of *P. mugo* extracts and may constitute baseline data in subsequent research.


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