Essential oil composition of *Pinus heldreichii* Christ., *P. peuce* Griseb., and *P. mugo* Turra as a function of hydrodistillation time and evaluation of its antimicrobial activity

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Abstract

Pinus heldreichii and *P. peuce* are Balkan endemics, Tertiary relicts, while *Pinus mugo* is an edificator in the phytocenoses in the Alpine mountain range. The chemical profile and

essential oil (EO) yield and composition of these three species as a function of distillation time (DT) was the subject of this study. The working hypothesis was that EO of *P. heldreichii*, *P. peuce* and *P. mugo* would produce EO with dissimilar composition and antimicrobial activity at different DT. The EO yields in the controls were 0.4% for *P. heldreichii,* 1.3% for *P. peuce* and 1.08% for *P. mugo.* During the first 0-15 min interval of the distillation, up to 70% of the total EO was extracted in *P. heldreichii,* 53.85% in *P. peuce* and 54.63% in *P. mugo*. Overall, the EO of the three species had relatively constant compositional profile at the different DTs, which contradicts the working hypothesis. Most of the quantitative differences in the main components were determined. The EO obtained from 0-15 min DT interval had the following major compounds: limonene (76.9%-77.0%) and *α*-pinene (12.47%-15.54%) in *P. heldreichii; α*-pinene (34.26% - 43.75%), limonene (19.2%-22.2%), and *β*-pinene (9.24%-11.48%) in *P. peuce; α*-pinene (16.48%-17.84%), *α*-phellandrene (23.06-23.76%), and *β*-phellandrene (28.12%-28.79%) in *P. mugo;*. The obtained results of EO from the three studied species are a prerequisite for obtaining EO with a certain, desired composition while reducing the distillation duration and saving energy and time. The tested EOs demonstrated significant antimicrobial activity against *Escherichia coli*.

Keywords: Balkans endemic; distillation time; essential oils; *Pinus*.

1. Introduction

Pinus L. is the largest genus in the Pinaceae comprising over 100 conifers distributed in the northern hemisphere (Dziedziński et al., 2021; Gernandt et al., 2005). According to Flora Europaea there are 20 *Pinus* species (Gaussen et al., 1964). Six of them can be found in Bulgaria (*P. nigra* Arnold, *P. mugo* Turra, *P. sylvestris* L., *P. heldreichii* Christ, *P. brutia* Ten. and *P. peuce* Griseb.) (Assyov and Petrova, 2012; Yordanov, 1963).

Pinus heldreichii is a Tertiary relict, Balkan subendemic (Valchev and Rosakova, 2015). The species is distributed on the Apennine Peninsula (Southern Italy), in the western and in the southern part of the Balkan Peninsula, and in areas with sub-Mediterranean climate. In Bulgaria, *P. heldreichii* is distributed in Pirin and Slavyanka Mountains (1400 - 2200 m asl) (Panayotov et al., 2010; Rangelova and Panayotov, 2013; Valchev and Rusakova, 2015; Vasileva and Panayotov, 2016;).

Pinus peuce is a Balkan endemic, which is found only in Bulgaria, Albania, Greece, Macedonia, Montenegro, and Serbia (Rusakova, 2015b). The mountains Rila, Pirin and Stara Planina in Bulgaria are the eastern part of the area of *P. peuce* distributions (Rusakova, 2015b).

In Bulgaria, *Pinus mugo* is a dominant species (edificator) in the phytocenoses of the upper subalpine subrange (2500–2800 m asl) in Rila Mountains, Pirin Mountains, and the Western Stara Planina (The Balkan) Mountains (Rusakova, 2015a).

Pinus species are economically important as a source of wood, paper, resins, charcoal, EOs, food, etc. (Dziedziński et al., 2021; Karapandzova et al., 2015). Extracts of different *Pinus* species are characterized by a wide range of biological activity such as antiviral, antiseptic, antibacterial, fungicidal, antioxidant and antidiabetic (Ha et al., 2020; Karapandzova et al., 2015; Mitić et al., 2018; Xie et al., 2015; Zulfqar et al., 2020). Some of these extracts are widely used in the traditional medicine against diseases: for inhalation in case of inflammation of the upper respiratory tract, for colds and for rheumatic problems (Karapandzova et al., 2014).

Due to the limited distribution of *P. peuce* and *P. heldreichii* and the disjunctive distribution of *P. mugo*, there are only a few studies on their phytochemical composition (Table 1). Samples of the *P. peuce* and *P. mugo* from Bulgaria have not been studied so far while for *P. heldreichii* we found only one research (Table 1). The literature review shows that the

76 phytochemical composition of the three species was analyzed by different methods such as
77 steam distillation, hydrodistillation and extracted with solvents (Table 1). There were various
78 duration of DT from two hours (h), six h, 24 h and up to 20 days and different parts of plants
79 were studied (Table 1). All of this is a prerequisite for differences in the composition of the
80 EO of studied species. Overall, there were no previous studies to reveal how DT would alter
81 *P. mugo, P. heldreichii,* and *P. peuce* EO. Previous research revealed that the EO may have a
82 different qualitative composition and different biological activity as a result of different
83 distillation times (DT) (Cannon et al., 2013; Semerdjieva et al., 2019a, 2019b; Zheljazkov et
84 al., 2012a, 2012b, 2013a, 2013b, 2013c). On one hand, reducing the distillation time (DT)
85 saves time and reduces energy and resource consumption (Chemat et al., 2019) while on the
86 other hand it results in EO with a certain differential composition.

87 The objective of this study was to determine the effect of DT on the quantitative and
88 qualitative composition of EO of *P. heldreichii*, *P. peuce* and *P. mugo* distributed in Bulgaria
89 as a potential for obtaining EO with a specific composition. The working hypothesis was that
90 the EO extracted in different time intervals in the three species will have different
91 compositions. Furthermore, this study assessed the antimicrobial activity of the EOs of the
92 three *Pinus* species.

93

94 **Table 1.** The main compounds of essential oils and distillation type and distillation time (DT)
95 in literature reports on *P. mugo, P. peuce* and *P. heldreichii*

Author year, country	Plant species	DT[1]	Plant part	Chemical compounds
Simić et al. (1996), Serbia	*P.heldreichii*		needles	limonene (20.26–25.15%), germacrene D (42.64-42%), *β*-caryophyllene (10.58–13.32%);

Koukos et al., (2000), Greece	*P. peuce*	3h	twigs and needles	*α*-pinene (7.38%), *β*-pinene (12.46%), *β*-phellandrene (26.93%), *β*-caryophyllene (4.48%) citronellol (12.48%)(twigs); *α*-pinene (23.07%), camphene (5.52%), *β*-pinene (22.00%), *β*-phellandrene (6.78%), bornyl acetate (9.76%), citronellol (13.42%) (needle)
Naydenov et al. (2005), Bulgaria		20 days, diethyl and petrol ether	needles	*a*-pinene (16.92-18.60%), camphene (1.86-2.23%), *β*-pinene (5.07-6.49%), δ-3-carene (3.20-4.96%), limonene (36.90-48.20%), *β*-farnesene (4.73-7.64%), γ-muurolene (14.85-22.87%),
Nikolić et al. (2007), Serbia and Montenegro	*P.heldreichii*,	24h with pentane	needles	limonene (26.3%), alpha-pinene (17.5%), germacrene D (13.5%), beta-caryophyllene (10.4%);
Nikolić et al. (2008), Serbia/ Montenegro	*P. peuce*	24h with pentane	needles	α-pinene (36.5%), germacrene D (11.4%) camphene (8.5%), bornyl acetate (6.8%), *β*-pinene (6.8%), *β*-caryophyllene (5.2%), *β*-phellandrene (4.7%),
Nikolić et al. (2011), Serbia/ Montenegro	*P.peuce; P.heldreichii***,**	24h with pentane	needles	*α*-pinene (36.5%), *β*-pinene (6.8%) - *P. peuce*; germacrene D (13.5%) - *P.heldreichii*;
Nikolić et al. (2014), Serbia, Scardo-Pindic mountain	*P. peuce*	24h with pentane	needles	*α*-pinene (45.5%), germacrene D (11.1%), *β*-pinene (10.8%), camphene (10.3%); bornyl acetate (5.0%);
Nikolić et al. (2015), Serbia Scardo-Pindic mountain	*P.heldreichii*	24h with pentane	needles	germacrene D (28.7%), limonene (27.1%), *α*-pinene (16.2%). *β*-caryophyllene (6.9%), *β*-pinene (5.2%);

Karapandzova et al. (2010), R Macedonia, NP Pelister	*P. peuce*	4h	needles; branches with needles; branches without needles	*α*-pinene (12.89-23.77%), *β*-pinene (6.16-13.0%), limonene + β-phellandrene (3.08-13.94%), bornyl acetate (1.13-10.56%), *trans* (E)-caryophyllene (4.13-7.30%), germacrene D (8.75-19.90%);
Karapandzova et al. (2012), R Macedonia	*P. peuce*	4h	needles	*α*-pinene (12.89-27.34%), *β*-pinene (6.16-13.13%), limonene + β-phellandrene (2.09-6.64%), bornyl acetate (2.92-11.67%), *trans*-(*E*)-caryophyllene (4.63-7.13%), germacrene D (8.75-20.14%);
Karapandzova et al. (2014), R Macedonia	*P. peuce*	4h	twigs with needles, twigs without needles	*α*-pinene (23.8–39.9%, 21.2–23.3%), camphene (2.2–5.5%), *β*-pinene (10.1–17.1%, 8.2–16.4%), limonene+*β*-phellandrene (6.8–14.0%, 8.8–23.6%), bornyl acetate (2.3–6.9%, 1.1–3.4%), trans-(*E*)-caryophyllene (3.6–4.3%, 3.2–7.3%), germacrene D (7.1–9.5%, 5.0–10.3%);
Karapandzova et al (2015), R Macedonia	*P.peuce; P. mugo*	4h	needles	flavonoid glycosides, flavonols, methylated flavonols, acylated flavonol glycosides with ferulic and *p*-coumaric acid;
Karapandzova et al. (2019), R Macedonia	*P. mugo*	4h	needles	Δ^3-carene (12.11-18.74 %), α-pinene (7.21-12.92%), limonene+β-phellandrene (3.05-5.72%), germacrene D (2.38-11.81%), *trans* caryophyllene (5.65-6.44 %), δ-cadinene (4.03-6.58 %), bicyclogermacrene (3.03-6.84 %), α- cadinol (3.42-4.98 %);
Hajdari et al. (2015), Kosovo	*P. mugo*	3h	twigs, needles, cones	*α*-pinene (needles: 16.9–24.5%, twigs: 4.5–8.8%, cones: 3.1–5.6%), *β*-pinene (needles: 1.5–5.4%, twigs: 2.2–15.4%, cones: 1.3–14.2%), *δ*-3-carene (needles: 15.4–27.8%, twigs: 24.0–51.6%, cones: 10.5–31.5%), limonene + *β*-phellandrene (twigs: 12.6–24.2%; cones: 2.1–9.3%), (*E*)-caryophyllene (needles: 4.4–8.9%, twigs:

				4.0–10.8%, cones: 10.3–26.9%), germacrene D (needles: 4.0–8.3%, twigs: 0.2–6.19%, cones: 0.1–12.4%);
Hajdari et al. (2016), Kosovo	*P. peuce*	3h	needles and twigs	*α*-pinene (needles: 21.6–34.9%, twigs: 11.0–24%), *β*-phellandrene (needles: 4.1–27.7; twigs: 29.0–49.8%), *β*-pinene (needles: 10.0–16.1, twigs: 6.9–20.7%);
Basholli-Salihu et al. (2017), Kosovo	*P.heldreichii*, *P. peuce, P. mugo*	3h	needles, twigs, cones	*δ*-3-carene (15.8-28.05%), *α*-pinene (4.1-21.34%), *β*-pinene (10.99 twigs) (*P. mugo*); *α*-pinene (15.96-36.79%), camphene (8.04% needles), *β*-phellandrene (6.07-35.82%), *β*-pinene (13.00-21.48%) (*P. peuce*); limonene (43.93-64.22%), *α*-pinene (10.57-14.32%), germacrene D (17.7%) (*P. heldreichii*);
Mitić et al. (2018), Serbia	*P. peuce* *P. mugo*	2h	needles	*α*-pinene (18.0% *P. mugo*), (43.0% *P. peuce*); *β*-pinene (4.1% *P. mugo*), (13.0% *P. peuce*); *δ*-3-carene (21.3%); limonene + *β*-phellandrene (7.6% *P. mugo*), (5.5% *P. peuce*); bornyl acetate (5.1% *P. mugo*); (7.7% *P. peuce*); germacrene D (5.6% *P.mugo*), (6.5% *P. peuce*);
Graikou et al. (2012), Greece	*P.heldreichii,*	93.33 min	wood	*α*-pinene (6.43%); limonene (28.70%); longifolene (6.89%), cembrene (23.82%), kaurene (5.88%);
Bojović et al. (2011), Serbia	*P.heldreichii,*	24h with pentane	needles	limonene (12.47%), *α*-pinene (10.14%), Δ-carene (5.9%), germacrene D (25.65%), *β*-caryophyllene (11.69%);
Lis et al. (2019), Poland	*P. mugo*	3h	needles, twigs, bark, wood, cones, young shoots	twigs with needles - 3-carene (23.8%), myrcene (22.3%), *α*-pinene (10.3%); needles - *α*-pinene (18.6%), 3-carene (11.3%), bornyl acetate (8.3%); twigs without needles, young shoots, bark, wood - 3-carene (28.6%, 15.0%, 18.5%, 34.6%), myrcene (23.4%, 24.0%, 24.6%, 9.4%); cones oil (*E*)-*β*-caryophyllene (24.0 %);
Garzoli et al. (2021), Italy	*P. mugo*	6h	needles	*α*-pinene (16.6–44.0%), *β*-pinene (7.5–44.7%), limonene (9.5–32.5%), *γ*-terpinene (0.3–19.7%);

[1]Distillation time -DT

2. Materials and Methods

2.1. Materials

2.1.1. *Collection of the Plant Material for EO isolation of Pinus peuce, P. helderichii and P. mugo from Bulgaria*

The samples of*, P. heldreichii, P. peuce* and *P. mugo* were collected after an official permit (# 185/11.03.2020 of Pirin National Park; # 78008/24.03.2020 of Central Balkan National Park; Vitosha Park) from the directorate of the parks. The materials of target species were collected in July from natural populations as follows*: P. heldreichii* – Pirin National Park, locality Dzhamdzhievi rocks (41.76464N 023.41928E; 1929 masl) and Slavyanka mountain, locality Marina meadow (41.40000N 023.60950E) ; *P. peuce* – Central Balkan National Park (Stara planina), locality Marifa (42.73893N 024.46991E; 1396 masl); *P. mugo* – Vitosha mountain, locality above x. Aleko (42.58147N 023.28932E; 1928 masl) (Supll. Fig. 1). The materials (twigs with leaves) of the three species were deposited at the Agricultural University, Plovdiv, Bulgaria herbarium, SOA (Thiers 2012), under number 063033, 063047, and 063022.

2.2. Essential oil (EO) isolation of P. heldreichii, P. peuce and P. mugo

The collected materials of three species were dried in a well-aerated environment in the laboratory. The EO of 100 grams of air-dried needles with one-two year old twigs of *P. heldreichii, P. peuce* and *P. mugo* were extracted by Clevenger type hydrodistillation in 2-L distillation units (https://en.laborbio.com/) in 1.6 L of water, at the laboratory in the Botany and Agrometeorology Department at Agricultural University in Plovdiv. The samples of *P. peuce, P. heldreichii* were mixed in a blender with 0.800L water in order to disrupt the EO cavities (Fig. 1) to which 0.800 L water was added. In total, 1.600 L water was used.

120 For *P. mugo,* two different experiments were performed namely (1) grinded needles
121 with one year old twigs in a blender and (2) non-grinded needles with one year-old twigs.

122 The extraction of all samples was performed in two replicates. The distillation in each
123 repetitions started when the first drop of EO drops into the Florentina part of the appraturs.
124 The different EO fractions were captured in the following time ranges: for *P. heldreichii*
125 (from Slavyanka mountain) were 0-15, 15-30, 30-60, 60-120 min and a control of 0-120 min
126 (non stop). The timeframes for *P. peuce* were 0-15, 15-30, 30-60 min, and 0-360 min non-
127 stop control. The timeframes for *P. mugo* were 0-15, 15-30, 30-60, 60-120 min and 0-360 min
128 non-stop (control) for both (grinded and non-grinded).

129 The EO fractions within these timeframes were captured without interrupting the
130 hydrodistillation process. The EO was transferred into 4-mL vials and placed in a freezer and
131 after that it was separated from water. The EO was measured on an analytical scale and it was
132 stored at 4-6 °C until analyzed. The oil content (yield) was reported by weight.
133 *2.3. Gas Chromatography Mass Spectrometry Flame Ionization Detection (GC-MS-FID) of*
134 *essential oil (EO)*

135 The isolated EO from all *Pinus* samples were analyzed in gas chromatography with
136 simultaneous mass spectrometry and flame ionization detection (GC-MS-FID). Fifty
137 microliters of oil was transferred into a 10 mL volumetric flask and brought to volume in
138 $CHCl_3$. A 1 mL aliquot of each diluted oil sample was placed by glass pipet into a GC vial for
139 analysis. Oil samples were analyzed using a DB-5 column (30 m × 0.25 mm fused silica cap.
140 column, film thickness of 0.25 µm) on an Agilent 7890A GC with an Agilent 5975C inert XL
141 MSD. Chemical standards and oils were analyzed using the following conditions: injector
142 temp., 240 ◦C; column ramp temperature from 60 to 240 ◦C at 3 ◦C/min, followed by holding
143 at 240 °C for 5 min; He as the carrier gas injection volume of 1 µL with a split ratio of 25:1);
144 MS mass range from 50 to 550 *m/z*; FID temperature was 300 °C. Post-column splitting was

145 performed (50% FID/50% MS) and all compounds were identified by Kovat and/or Retention
146 Index analysis (Adams et. al., 2009), direct comparison of MS data and analyte retention time
147 to that of authentic standards and comparison of mass spectra with those reported in the NIST
148 mass spectral database. Commercial standards of α-pinene, camphene, β-pinene, myrcene, α-
149 phellandrene, β-phellandrene, terpinolene, limonene, bornyl acetate, β-caryophyllene,
150 germacrene D, bicyclogermacrene, and δ-cadinene were purchased from Sigma-Aldrich (St.
151 Louis, MO, USA). Spathulenol was obtained from our in-house collection of standards and
152 had been previously characterized. Compounds quantified by performing area percentage
153 calculations based on the total combined FID area.

154 *2.4. Antimicrobial Activity*

155 Essential oils from the following DT were used to evaluate the antimicrobial activity: *P.*
156 *heldreichii* - 0-360min(Pirin), 0-15min (Slavianka), and 0-360min (Slavianka); *P. peuce* - 0-
157 15min, and 0-360min; *P. mugo* - 0-15min, and 0-360min

158 *2.4.1. Microorganisms tested*

159 Nine microorganisms were tested for antimicrobial activity: Gram-positive bacteria
160 (*Staphylococcus aureus* subsp. *aureus* CCM 2461, *Listeria monocytogenes* CCM 4699,
161 *Bacillus cereus* CCM 2010), Gram-negative bacteria (*Salmonella enterica* susp. *enterica*
162 CCM 3807, *Pseudomonas aeruginosa* CCM 1959, *Escherichia coli* CCM 3988), and yeasts
163 (*Candida albicans* CCM 8186, *Candida glabrata* CCM 8270, *Candida tropicalis* CCM
164 8223), were used for the antimicrobial activity testing. The microorganisms were used from
165 the Czech collection of microorganisms (CCM, Brno, Czech Republic). There were used the
166 microorganisms get from the Czech collection of microorganisms (CCM, Brno, Czech
167 Republic). The Mueller–Hinton broth (MHB, Oxoid, Basingstoke, UK) at 37 °C, and yeasts
168 in Sabouraud Dextrose broth (SDB, Oxoid, Basingstoke, UK) was used for bacteria

cultivation. It was set at 25 °C overnight. The antimicrobial activity is based on inhibition halos diameters expressed in millimeters.

2.4.2. Disc Diffusion Method

One hundred microliters of bacterial suspension after incubation on the Mueller–Hinton agar (MHA, Oxoid, Basingstoke, UK) and yeast suspensions on the Sabouraud Dextrose agar (SDA, Oxoid, Banigstoked, UK) were spread for the agar disc diffusion method. There was 15 µL of the EO infused on the filter paper discs (6 mm diameter), tested and placed on the in-oculated MHA or SDA resp. The MHA and SDA were kept for 2 hours at the temperature 4 °C and then the temperature was set at 37 °C resp. 25 °C for 24 hours under aerobic conditions. Cefoxitin and Gentamicin as antibiotics (30 µg/disc, Oxoid, Basingstoke, UK) as well as Fluconazole (30 µg/disc, Oxoid, Basingstoke, UK) as antigungal were used as positive controls. Three replications were set for testing.

2.5. Statistical analyses

2.5.1. Effects on EO yield and composition

(A) *Pinus heldreichii*: the effect of DT (5 levels: 0-120 [control]), 0-15, 15-30, 30-60, and 60-120 min) on oil yield (%) and the concentration (%) of *α*-pinene, *β*-myrcene, *β*-pinene, limonene, bornyl acetate, germacrene D, *β*-caryophyllene, total, monoterpenes, and sesquiterpenes was determined using a one-way (with 5 DT treatments) analysis of variance (ANOVA);

(B) *Pinus peuce*: the effect of DT (4 levels: 0-360 [control]), 0-15, 15-30, and 30-60) on oil yield (%) and the concentration (%) of *α*-pinene, camphene, *β*-myrcene, *β*-pinene, limonene, bornyl acetate, germacrene D, *β*-caryophyllene, total, monoterpenes, and sesquiterpenes was determined using ANOVA of a CRD with 4 DT treatments.

193 (C) *Pinus mugo*: the effects of grinding (2 levels: grinded and non-grinded) and DT (5 levels:
194 0-360 [control]), 0-15, 15-30, 30-60, and 60-120 min) on oil yield (%) and the concentration
195 (%) of α-pinene, β-pinene, camphene, β-myrcene, α-phellandrene, β-phellandrene, bornyl
196 acetate, terpinolene, β-caryophyllene, germacrene D, bicyclogermacrene, δ-cadinene,
197 spathulenol, total, monoterpenes, and sesquiterpenes was determined using ANOVA of a 2 x
198 5 factorial design.

199 For each response variable of model assumptions on the error terms (the error terms have
200 constant variance and are distributed normally) were validated by using the residuals as
201 described in Montgomery (2020). When the effect of DT and/or grinding was significant at
202 the 5% level of significance or marginally significant at the 10% level of significance, the
203 means of the treatments or treatment combinations were compared using Tukey's studentized
204 range test at 5% level of significance. The statistical analysis was done completed using SAS
205 (SAS Institute Inc. 2014) software.

206 *2.5.2. Antimicrobial activity*

207 (A) The effect of the combination of DT and Population (3 levels: 0-360min Pirin, 0-15min
208 Slavianka, and 0-360min Slavianka) on 9 antimicrobial activities (*S. aureus* subsp. *aureus*, *L.*
209 *monocytogenes*, *S. enterica* subsp. *enterica*, *B. cereus*, *P. aeruginosa*, *E. coli*, *C. albicans*, *C.*
210 *glabrata*, and *Ca. tropicalis*) of *P. heldreichii* was determined using a one-way analysis of
211 variance with 3 DT and Population treatments;

212 (B) The effect of DT (2 levels: 0-15min, and 0-6h) on 9 antimicrobial activities *S. aureus*
213 subsp. *aureus*, *L. monocytogenes*, *S. enterica* subsp. *enterica*, *B. cereus*, *P. aeruginosa*, *E.*
214 *coli*, *C. albicans*, *C. glabrata*, and *Ca. tropicalis*) of *P. peuce* was determined using a one-
215 way analysis of variance with 2 DT treatments.

216 (C) The effects of Grinding (2 levels: Grinded, and Not Grinded) and DT (2 levels: 0-15min,
217 and 0-360min) on 9 antimicrobial activities *S. aureus* subsp. *aureus*, *L. monocytogenes*, *S.*

enterica subsp. *enterica*, *B. cereus*, *P. aeruginosa*, *E. coli*, *C. albicans*, *C. glabrata*, and *Ca. tropicalis*), of *P. mugo* was determined using analysis of variance of a 2 x 2 factorial design; In all these three analyses, the model assumptions were verified as described above.

3. Results and Discussion

3.1. Essential oil (EO) content (yield) of P. heldreichii, P. peuce and P. mugo in different distillation timeframes (DT) fractions

The effect of DT on EO yield of the three species (*P. heldreichii, P. peuce* and *P. mugo*) was significant and comparison of the means results are presented in Table 2 and Table 3. Our study found that in the different DT, the EO yield ranged from 0.07 to 0.4% for *P. heldreichii;* 0.09 to 1.3% for *P. peuce* (Table 2)*,* and from 0.14 to 1.08% in *P. mugo* (Table 3). The EO yields of the controls were 0.4% for *P. heldreichii,* 1.3% for *P. peuce*, 1.03% for *P. mugo* non grinded and 1.08% *P. mugo* grinded. However, the three *Pinus* species EO yields decrease with the increasing the duration of the DT (Table 2; Table 3). In the first 0-15 min of distillation, up to 70% of the total amount of EO was released in *P. heldreichii,* 53.85% for *P. peuce* and for 54.63% *P. mugo* (grinded). It is evident that in the grinded sample of *P. mugo* in the 0-15 min interval, a larger amount of EO was released (Table 3). Figure 1 shows that EO cavites in all three species are internal and the grinding of the raw material contributes to their destruction and easier EO extraction (Fig. 1). The analysis of variance *p*-values that show whether the main effects and/or the interaction effect of DT and/or grinding (Gr) on oil yield (%) of *P. mugo* is significant or not are shown in Table 3.

As mentioned above, there were no previous studies on the EO composition of *Pinus heldreichii*, *P. peuce* and *P. mugo* following different DT. This is the first study on the three *Pinus* species EO yield and composition at different DT. The means presented in Table 2 and Table 3 showed that with the increase in DT, the EO yield of the three species decreased

(Table 2; Table 3). Previous research demonstrated that maximum EO yields of different plant species occured at different time intervals (Semerdjieva et al., 2019a, 2019b; Zheljazkov et al., 2012a; 2013a, 2013b, 2013c; Cannon et al., 2013)). For example, DT of 20 min provided maximum EOs yields for *Mentha* × *piperita* L., *Cymbopogon flexuosus* Steud, and *Cymbopogon martinii* (Cannon et al., 2013). However, in *P. ponderosa,* the amount of EO increased with DT duration (Zheljazkov et al., 2012c). Apparently, the maximum EO release is specific for each plant species, and it depends on the type and location of the secretory structures, the way samples are processed, and the type of distillation performed.

According to literature reports, EO yield of *P. peuce* and *P. mugo* (leaves, twigs, twigs with leaves, cones) depends on the plant organs and on the plant origin (Hajdari et al., 2015, 2016) (Table 1). For example, when studying the different parts (needles, twigs, cones) from *P. mugo,* Hajdari et al., (2015) found EO in range of 0.3–0.8% in needles, 1.0–2.4% in twigs and 0.1–0.5% in cones. Large range of variability in EO yield was found also in *P. peuce* (0.7 to 3.3%) for the population of the species in Kosovo (Hajdari et al., 2016), in Macedonia (from 2.86-9.93 mL/kg; 7.5 to 17.3 mL/kg) (Karapandzova et al., 2010, 2014). Generally, the studied species (*P. heldreichii, P. peuce* and *P. mugo*) have been conducted with different distillation times (Table 1) and for some of them the yield was not reported (Bojović et al., 2011; Nikolić et al. 2007, 2008, 2011).

Table 2: Mean oil yield (%) of *P. heldreichii*and *Pinus peuce* in different distillation timeframes (DT) fractions

DT (min)	Oil yield (%)	
	P. heldreichii	*P. peuce*
0-15	0.28 a[1]	0.70 b

15-30	0.09 b	0.13 c
30-60	0.07 b	0.09 c
60-120	0.07 b	-
Control	0.39 a	1.26 a

[1]Within each column, means sharing the same letter is not significantly different

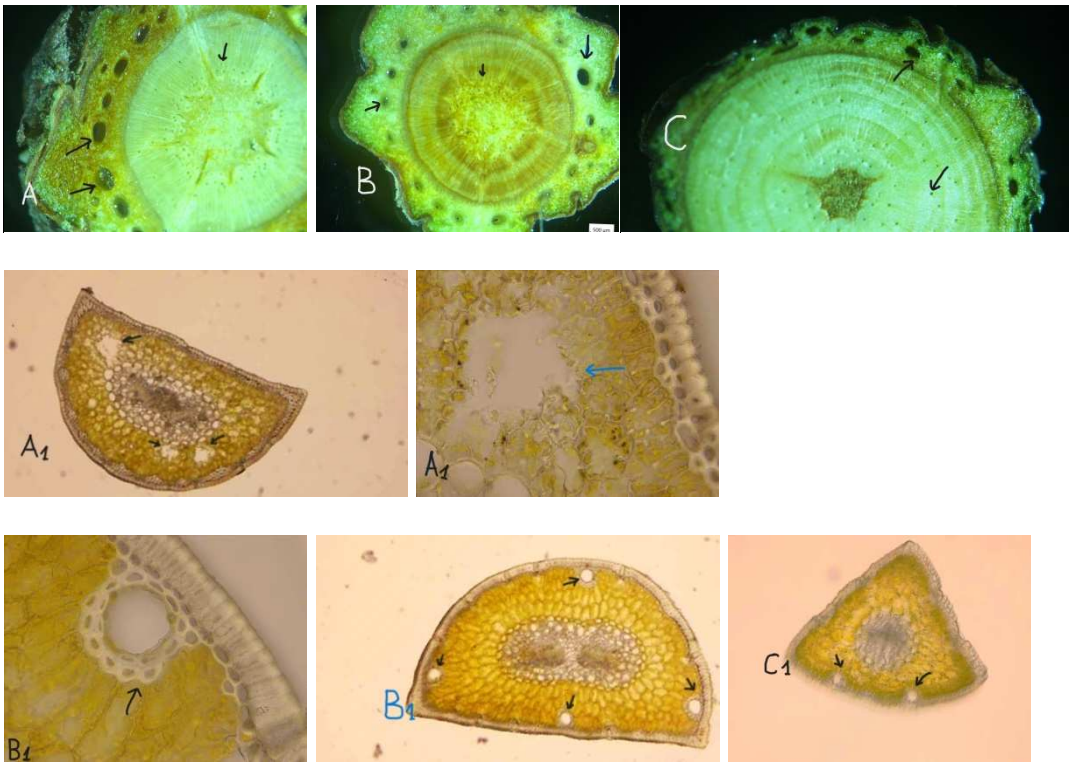

Fig. 1. Cross-section of twigs and leaves of *Pinus heldreichii* (A-twig; A1-leaf); *P. mugo* (B-twig; B1-leaf); *P. peuce* (C-twig; C1-leaf); images A, B, C were taken with a Stereo Microscope DM-143-FBGG, Motic Images Plus 3.0; images A_1, B_1, C_1 were taken with a Microscope Leica ICC 50W. Resin ducts and cavites are marked with arrows.

Table 3. ANOVA *p*-values that show the significance of the main and interaction effects of distillation time (DT) and grinding (Gr) on oil yield (%) (A) and mean oil yield (%) obtained from the 4 distillation times (DT) and control (straight 360 min) (B) and the 2 grindings (C)

of *Pinus mugo*. The *p*-values of significant effects that require multiple means comparison are shown in bold. $\sqrt{MSE}$ = square root of the Mean Square Error (MSE) estimates the common standard deviation (σ).

(A) Source of Variation	*p*-value	(B) DT	Oil yield	(C) Grinding	Oil yield
DT	**0.001**	0-15	0.48 b[1]	Grinded	0.42 a
Gr	**0.014**	15-30	0.14 c	Non-grinded	0.36 b
DT*Gr	0.295	30-60	0.14 c		
$\sqrt{MSE}$	0.039	60-120	0.15 c		
		Control	1.03 a		

[1]Within each column, means sharing the same letter is not significantly different.

3.2. Essential oil (EO) composition

Our study evaluated the effect of DT on EO yield, composition, and the antimicrobial activity on *P. heldreichii, P. peuce* and *P. mugo* from Bulgaria. The DT of the three species was 0-120 (control), 0-15, 15-30, 30-60, and 60-120 min in *P. heldreichii*; 0-360 (control), 0-15, 15-30, and 30-60 in *P. peuce* DT; and 0-360 (control), 0-15, 15-30, 30-60, and 60-120 min in *P. mugo.* The *P. peuce* EO fractions obtained in the DT intervals 60-120; 120-180; 180-240; and 240-360 min, and the *P. mugo* fractions obtained in the DT intervals 120-180; 180-240; 240-360 min were insufficient for handling and therefore they were not analyzed.

3.2.1. The composition of essential oil (EO) of P. heldreichii in different distillation timeframes (DT) fractions

As mentioned above, *P. heldreichii* is a Tertiary relict and Balkan subendemic. Associated communities of the species are relict, monodominant (Valchev and Rusakova, 2015). Due to the limited distribution of the species, mainly in the western part of the Balkan Peninsula, there are not many phytochemical studies. This study evaluated the EO composition in the

293 different DT of samples from Bulgaria for the first time. The effect of DT was presented in
294 Table 4 which shows multiple mean comparison results for the response variables with
295 significant DT effect. A total of seven EO components were identified by the GC-MS-FID
296 analysis, representing 90.3 - 98.2% of EO (Table 4). Limonene and α-pinene were the main
297 EO components, with limonene prevailing up to 70.5 - 77.0% in EO (Supll. Table 2). Their
298 amount was the highest at the beginning of the distillation (0-15; 15-30), with limonene
299 reaching 76.9 -77.0% and α-pinene 12.47 -15.54% of the total oil (Table 5). Comparing our
300 results for the EO composition of *P. heldreichii* with data from other authors, it is clear that
301 limonene was present in all samples from Bulgaria (Naydenov et al., 2005), Serbia (Bojović´
302 et al., 2011; Nikolić et al., 2007, 2015; Simić et al. 1996), Kosovo (Basholli-Salihu et al.,
303 2017) and Greece (Graikou et al., 2012) but in much smaller quantities and the distillations
304 took a longer period. Also, some of the studies cited above indicated a qualitatively different
305 EO composition of *P. heldreichii*. For example, in samples from Greece, Graikou et al. (2012)
306 indicated the contents of longifolene (6.89%), cembrene (23.82%) and kaurene (5.88%) while
307 in samples from Serbia, the contents were: germacrene D (42.64-42%), and β-caryophyllene
308 (10.58–13.32%) (Simić et al., 1996). Obviously, there are phytochemical differences in the
309 species as a result of many factors such as genetic features, ecological and geographical
310 features, the studied part and the distillation method. For example, Naydenov et al., (2005)
311 investigated the genetic structure and terpene analysis of the species from four Bulgarian
312 locations and distinguished two groups of EOs that corresponded to the geographical
313 locations (Naydenov et al., 2005).

314 In this study, shorter distillation (0-30 min) in *P. heldreichii* resulted in high-limonene
315 EO. The statistical analysis indicated the effect of DT was significant (or marginally
316 significant) on all response variables except germacrene D (Suppl. Table 1).

317

318 **Table 4.** Mean concentration (%) of *α*-pinene, *β*-pinene, *β*-myrcene, limonene, bornyl acetate,
319 *β*-caryophyllene, total monoterpenes (MT), and sesquiterpenes (ST) of *P. heldreichii* obtained
320 from the 4 distillation times (DT) and control (straight 120 min).

DT (min)	*α*-pinene	*β*-pinene	*β*-myrcene	limonene	bornyl acetate	*β*-caryophyllene	Total	MT	ST
0-15	15.54 a[1]	1.21 b	2.225 a	77.0 a	0.445ab	1.47 c	98.2a	96.4a	1.82b
15-30	12.47bc	1.40 b	0.945 b	76.9 a	0.396ab	2.98 ab	97.5a	94.1ab	3.35ab
30-60	11.32 c	1.53 ab	1.375 b	72.6 ab	0.001 b	3.61 a	90.6b	87.0b	3.61a
60-120	13.50 b	1.88 a	1.375 b	70.5 b	1.045 a	2.04 bc	90.3b	88.3b	2.04ab
control	13.68 b	1.42 b	1.945 a	73.4 ab	0.630ab	2.63 abc	94.4ab	91.1 ab	3.25 ab

321 [1]Within each column, means sharing the same letter are not significantly different.

322

323 *3.2.2. The composition of essential oil (EO) of P. peuce in different distillation timeframes*
324 *(DT) fractions*

325 *Pinus peuce* is a Balkan endemic, Tertiary relict and it forms monodominant or mixed
326 phytocenoses in the upper border of threes in the mountains in Bulgaria (Rusakova, 2015b).
327 In general, classes of monoterpenes (MT) (75.6 -89.9%) and sesquiterpenes (ST) (8.36 -
328 17.07%) predominated in EO of *P. peuce* samples, representing 92.4 -98.3% of EO (Table 5).
329 The amount of monoterpenes was highest in the first distillation intervals (0-15min) (MT
330 89.9%), then it decreased (Table 5). *α*-Pinene (34.26 - 43.75%), limonene (19.2 -22.2%) and
331 *β*-pinene (9.24 -11.48%) are the main compounds in MT and in DT 0-15 min the largest
332 amount is released (Table 5; Suppl. Table 2). Compared to the published results, a similar

qualitative EO composition of *P. peuce* (*α*-pinene, limonene, *β*-pinene) was found in samples from Macedonia (twigs with needles) (Karapandzova et al., 2014) and Greece (twigs) (Papadopoulou and Koukos, 1996) which were obtained by distillation with different duration (2, 3 or 4 hours). For samples of *P. peuce* from Serbia, Montenegro and Scardo-Pindic mountain it was also stated that *α*-pinene, germacrene D and *β*-pinene prevailed but limonene was not found (Nikolić et al., 2008, 2011, 2014) (Table 1). In addition, samples from the studied species from Macedonia (steam distillation, for 4 hours) and Greece contained phellandrene, citronellol (Koukos et al., 2000; Karapandzova et al., 2014) which are components that were not found in the present study. Our study analyzed eight EO compounds found at all times of the experiment. This relatively persistent EO composition contradicts our working hypothesis. In particular, we identified quantitative differences in the identified components of EOs. The effect of DT was significant (or marginally significant) in 9 of the response variables but it was not significant on camphene (overall mean = 7.90%), *β*-myrcene (overall mean = 1.80%), and limonene (overall mean = 20.71%). Table 5 shows multiple mean comparison results for the 9 response variables with significant DT effect. Overall, our study shows that the amounts of *α*-pinene (34.26 - 43.75%), limonene (19.2 - 22.2%), *β*-pinene (9.24 -11.48%) and camphene (7.99 -8.42%)) obtained in the first min of distillation (0-15 min) are close to the quantities obtained for 3, 4 or 5 hours of distillation (Hajdari et al., 2016; Nikolić et al., 2008; Papadopoulou and Koukos, 1996).

The sesquiterpenes (ST) was the second class of compounds of EO from which germacrene D is the prevalent one, especially in the interval of 15-30 min (Table 5).

Generally, we can conclude that 2-3 hours of distillation is not required to obtain EO from *P. peuce* with a high content of *α*-pinene, limonene, camphene, and *β*-pinene. If one wants to saves time and energy to obtain EO with the desired composition (*α*-pinene, limonene, camphene, *β*-pinene), a DT of 15 min should be sufficient.

Table 5. The mean concentration (%) of *α*-pinene, *β*-pinene, bornyl acetate, *β*-caryophyllene, germacrene D, total, monoterpenes (MT), and sesquiterpenes (ST) obtained from the 3 distillation times (DT) and control (straight 360 min) of *Pinus peuce*.

DT (min)	*α*-pinene	*β*-pinene	bornyl acetate	*β*-caryophyllene	germacrene D	Total	MT	ST
0-15	43.75a	11.48a	2.07 c	2.220 d	6.13 b	98.3a	89.9a	8.36b
15-30	34.26 b	9.32 c	4.00 a	4.335 a	12.73 a	92.7b	75.6c	17.07a
30-60	36.08 b	9.24 c	3.85 a	3.475 b	10.20 ab	92.4b	78.7c	13.68ab
Control	39.71ab	10.51 b	3.02 b	2.905 c	8.14 ab	95.5ab	84.5 b	11.04 ab

[1]Within each column, means sharing the same letter are not significantly different.

3.2.3. The composition of essential oil (EO) of P. mugo at different distillation timeframes (DT) fractions

Pinus mugo is an Alpine species and in Bulgaria it is an edificator and dominant of the phytocenoses it forms in Rila and Pirin mountains (Rusakova 2015). Populations of the species are a resource for obtaining of aromatic EO (Hajdari et al., 2015). As mentioned in the introduction section, samples from Bulgarian populations of *P. mugo* have not been studied. Furthermore, an assessment of DT on the qualitative composition of EO has not been researched. In our study, we conducted two experiments with the species, namely (1) Grinding of raw material and (2) Non-grinded. Statistical analysis showed that grinding of raw material has an impact mainly on the EO yield (Table 3). The ANOVA results and the multiple means comparison results for the other response variables are shown in Tables 6 – 8.

375 The data reveal that at least one of the effects was significant in all response variables except
376 terpinolene and its overall mean = 2.165%. According to the GC-MS-FID analysis, 13
377 components of EO were identified in the tested samples of *P. mugo* which represent about
378 69.0 - 95.3% of the analyzed EO (Suppl. Table 3; Table 9 and Table 10). The studied samples
379 in our EO study are dominated by monoterpenes class which coincides with previous studies
380 on the type of samples from Macedonia (Karapandzova et al. 2019), Kosovo (Hajdari et al.
381 2015), Poland (Lis et al., 2019) and Italy (Garzoli et al., 2021).

382 **Table 6.** ANOVA *p*-values that show the significance of the main and interaction effects of
383 distillation time (DT) and grinding (Gr) of *P. mugo* on the concentration (%) of *α*-pinene,
384 camphene, *β*-pinene, *β*-myrcene, *α*-phellandrene, *β*-phellandrene, terpinolene, and bornyl
385 acetate. $\sqrt{MSE}$ = square root of the Mean Square Error (MSE) estimates the common
386 standard deviation (σ).

Source of Var.	*α*-pinene	camphene	*β*-pinene	*β*-myrcene	*α*-phellandrene	*β*-phellandrene	terpinolene	bornyl acetate
DT	0.023	**0.060**	**0.001**	0.001	0.001	0.001	0.412	**0.003**
Gr	0.010	**0.006**	**0.008**	0.001	0.667	0.983	0.239	0.564
DT*Gr	**0.025**	0.173	0.117	**0.007**	**0.045**	**0.010**	0.109	0.542
$\sqrt{MSE}$	0.915	0.178	0.225	0.130	0.812	1.003	0.332	0.353

387 [1]Significant effects that require multiple means comparison are shown in bold.

388

389 In general, *α*-pinene, *α*-phellandrene and *β*-phellandrene are the main EO components of *P.*
390 *mugo*. Their amount is the highest in the first 15 min of distillation (Table 9). Overall, as the
391 duration of DT increases, the amounts of *α*-phellandrene and *β*-phellandrene decrease.

Previous studies on the species have reported EO composition with a high content of δ-3-carene, myrcene, (E)-β-caryophyllene, α-pinene and limonene (Garzoli et al., 2021; Lis et al., 2019; Mitić et al., 2018) while β-phellandrene was not found and α-phellandrene was in insignificant amount. Probably the studied population is a new chemical type because in *Pinus* species, there is a high genotypic variability which corresponds to the phytochemical composition of the EOs (Petrakis et al., 2001).

Table 7. Mean concentration (%) of camphene, β-pinene, and δ-cadinene of *P. mugo* obtained from the two grindings (plant materials).

Grinding	camphene	β-pinene	δ-cadinene
Grinded	1.56 b	5.56 b	2.30 a
Non-grinded	1.83 a	5.89 a	1.65 b

[1]Within each column, means sharing the same letter are not significantly different

Table 8. ANOVA *p*-values that show the significance of the main and interaction effects of distillation time (DT) and grinding (Gr) of on β-caryophyllene, germacrene D, bicyclogermacrene, δ-cadinene, spathulenol, total monoterpenes (MT), and sesquiterpenes (ST) of *P. mugo*. $\sqrt{MSE}$ = square root of the Mean Square Error (MSE) estimates the common standard deviation (σ).

Source of Var.	β-caryophyllene	germacrene D	bicyclogermacrene	δ-cadinene	spathulenol	Total	MT	ST
DT	0.001	**0.001** [1]	**0.001**	**0.001**	0.001	0.001	0.001	0.001
Gr	0.011	0.355	0.247	**0.005**	0.001	0.296	0.028	0.003
DT*Gr	**0.015**	0.224	0.616	0.143	**0.001**	**0.062**	**0.012**	**0.039**

$\sqrt{MSE}$	0.906	0.512	0.355	0.400	0.194	3.680	1.958	2.341

[1]Significant effects that require multiple means comparison are shown in bold.

Table 9. Mean concentration (%) of *α*-pinene, *β*-myrcene, *α*-phellandrene, *β*-phellandrene, *β*-caryophyllene, spathulenol, total monoterpenes (MT), and sesquiterpenes (ST) of *P. mugo* obtained from the 10 combinations of grinding and distillation times (DT, with Control being straight 360 min).

Grinding (GR)	DT (min)	*α*-pinene	*β*-myrcene	*α*-phellandrene	*β*-phellandrene	*β*-caryophyllene	spathulenol	MT	ST	Total
GR	0-15	17.84a[1]	4.28 a	23.07ab	28.12 a	4.54 b	1.43 cd[1]	86.6 a	8.53 c	95.2 a
GR	15-30	15.32ab	3.28b	19.97bcd	21.55 b	10.23 a	2.92 a	72.3bc	20.93ab	93.2 ab
GR	30-60	12.68 b	2.75 c	16.23 e	16.58 c	10.50 a	2.99 a	58.5ef	24.02a	82.5abcd
GR	60-120	15.71ab	2.64 c	17.13de	12.13 d	4.53 b	2.35 ab	56.4 f	12.61 bc	69.0 d
GR	Control	15.97ab	3.62b	22.02 abc	21.93 b	5.52 b	2.12 bc	74.4 b	12.24 bc	86.7abc
NGr	0-15	16.48 a	4.28 a	23.76 a	28.79 a	4.83 b	0.30 ef	85.6 a	6.66 c	92.3abc
NGr	15-30	17.68 a	3.58b	19.15cde	18.32bc	7.24 ab	0.001 f	70.8bcd	13.26 bc	84.1abc
NGr	30-60	16.45 a	3.40 b	17.52de	16.32 c	6.63 b	0.01 f	65.5cde	14.13 bc	79.6bcd
NGr	60-120	16.27ab	3.35 b	17.41de	15.71cd	5.43 b	1.25 d	64.1def	13.79 bc	77.9 cd
NGr	Control	17.12 a	3.64 b	19.78cd	21.24 b	4.89 b	0.85 de	73.4 b	10.15 c	83.6abc

[1]Within each column, means sharing the same letter is not significantly different; Grinding – GR; Non grinded – NGr.

Table 10. Mean the concentration (%) of camphene, *β*-pinene, bornyl acetate, germacrene D, bicyclogermacrene, and *δ*-cadinene of *P. mugo* obtained from the 4 distillation times (DT) and control..

DT (min)	camphene	*β*-pinene	bornyl acetate	germacrene D	bicyclogermacrene	*δ*-cadinene
0-15	1.49 b[1]	7.46 a	1.22 c	1.19 b	0.52 c	0.33 c
15-30	1.83 ab	5.71 b	2.43 a	3.20 a	1.95 a	1.76 b
30-60	1.73 ab	4.84 c	2.23 ab	3.64 a	2.44 a	2.94 a
60-120	1.85 a	4.63 c	1.70 abc	1.92 b	1.63 ab	3.24 a
Control	1.57 ab	5.99 b	1.48 bc	1.83 b	1.08 bc	1.59 b

[1]Within each column, means sharing the same letter are not significantly different.

3.3. Antimicrobial Activity

The antimicrobial activity of the EOs of *Pinus peuce*, *P. heldreichii* and *P. mugo* was evaluated in this study. The antimicrobial activity of *P. heldreichii* ranged between 3.00 and 8.00 mm. The best antimicrobial activities of *P. heldreichii* were found against Gram-negative bacteria *S. enterica* subsp. *enterica* and *E. coli* (8 mm) and the worst against *C. glabrata* (3.00 mm). The effect of DT&Population for *P. heldreichii* was marginally significant only on *Candida tropicalis* (p = 0.068). The means are shown in Table 11. Since the effect of DT&Population was not significant on the other 8 antimicrobial activities, their overall means are shown in Table 11. Mitić et al. (2019) tested antimicrobial activity against *Klebsiella pneumoniae, Eschcerichia coli, Morganella morgani,* and *Staphylococcus aureus* subsp. *aureus*. They found similar results as in our study, that the best antimicrobial potency was found against *E. coli*. Mitić et al. (2018) found the best antimicrobial activity of two *Pinus*

species, similar with our results against Gram-negative bacteria panel. The EO of *P. heldreichii* wood was found to have inhibitory effect against *S. aureus*, *K. pneumoniae* and *E. coli* (Graikou et al, 2012).

Table 11. Overall mean of *S. aureus* subsp. *aureus*, *L. monocytogenes, B. cereus, S. enterica* subsp. *enterica, P. aeruginosa, E. coli, C. albicans*, and *C. glabrata* where there was no significant difference among the 3 DT & Population levels; and the mean *C. tropicalis* values where the effect of DT & Population is marginally significant (*Pinus heldreichii*).

Antimicrobial activity	Overall mean in mm	DT&Population	*C. tropicalis*
S. aureus subsp. *aureus*	5.56	0-360minPirin	3.67 a[1]
L. monocytogenes	7.89	0-15minSlavianka	2.67 ab
B. cereus	5.67	0-360minSlavianka	2.33 b
S. enterica subsp. *enterica*	8.00		
P. aeruginosa	5.33		
E. coli	8.00		
C. albicans	5.33		
C. glabrata	3.00		

[1]Within *C. tropicalis* column, means sharing the same letter are not significantly different.

The ANVOA results for *P. mugo* are shown in Table 12. Accordingly, at least one effect was significant on *S. enterica* subsp. *enterica* and *P. aeruginosa* and the means are shown in Table 13. The overall means of the other 7 antimicrobial activities that were not significantly different are also shown in Table 13.

Table 12. ANOVA p-values that show the significance of the main and interaction effects of distillation time (DT) and Grinding (Gr) on *S. aureus* subsp. *aureus* (SA), *L. monocytogenes* (LM), *B. cereus* (BC), *S. enterica* subsp. *enterica* (SE), *P. aeruginosa* (PA), *E. coli* (EC), *C. albicans* (CA), *C. glabrata* (CG), and *C. tropicalis* (CT) of *P. mugo*. $\sqrt{MSE}$ = square root of the Mean Square Error (MSE) estimates the common standard deviation (σ).

Source of var.	SA	LM	BC	SE	PA	EC	CA	CG	CT
DT	0.631	0.631	0.172	**0.011**	**0.004**	0.999	0.594	0.172	0.580
Gr	0.631	0.631	0.172	**0.011**	**0.004**	0.999	0.594	0.172	0.580
DT*Gr	0.631	0.631	0.172	0.438	0.347	0.169	0.594	0.172	0.122
$\sqrt{MSE}$	0.577	0.577	0.577	0.707	0.577	0.764	1.041	0.577	1.000

Significant effects that require multiple means comparison are shown in bold.

Antimicrobial activity of *P. mugo* ranged between 2.17 to 8.00 mm. The highest antimicrobial activity was against *E. coli* and the lowest against *C. tropicalis*. The results were very similar as antimicrobial activity of *P. heldreichii*. In a previous study, Kačániová et al. (2017) tested

458 *P. mugo* EO against ten *Pseudomonas* species. The best antimicrobial activities in this study
459 were found against *P. agglomerans, P. brassicacearus, P. koreensis, P. ludensis, P. mandelii*
460 and *P. veronii.* The only significant effects were the main effects of GR and DT on *S. enterica*
461 *subsp. enterica (p = 0.011)* and *P. aeruginosa* (p = 0.004).

462 **Table 13.** Overall mean of *S. aureus* subsp. *aureus* (SA)*, L. monocytogenes* (LM)*, B. cereus*
463 (BC)*, E. coli* (EC)*, C. albicans* (CA)*, C. glabrata* (CG), and *C. tropicalis* (CT) where none of
464 the effects was significant; and the mean *S enterica* susp. *enterica* (SE), and *P. aeruginosa*
465 (PA) values where the main effects of Grinding and DT were significant (*Pinus mugo*).

Antimicrobial activity	Overall mean in mm	Grinding	SE	PA	DT	SE	PA
S. aureus subsp. *aureus*	5.58	Grinded	9.17 a[1]	6.50 a	0-15 min	9.17 a	6.50 a
L. monocytogenes	5.58	NotGrinded	7.83 b	5.17 b	0-360 min	7.83 b	5.17 b
B. cereus	3.08						
E. coli	8.00						
C. albicans	2.17						
C. glabrata	3.58						
C. tropicalis	2.17						

466 [1]Within SE and PA column, means sharing the same letter are not significantly different.

467

Since the effect of DT for *P. peuce* was not significant on all 9 antimicrobial activities, their overall means are shown in Table 14. This non-significant effect of DT shows that there is no specific DT that can give desired or undesired antimicrobial activity.

Table 14. Overall mean of *S. aureus* subsp. *aureus, L. monocytogenes, B. cereus, S. enterica subsp. enterica, P. aeruginosa, E. coli, C. albicans, C. glabrata,* and *C. tropicalis* where there was no significant difference between the 2 DTs (*Pinus peuce*).

Antimicrobial activity	Overall mean in mm
S. aureus subsp. *aureus*	5.67
L. monocytogenes	8.00
B. cereus	5.50
S. enterica subsp. *enterica*	8.17
P. aeruginosa	5.33
E. coli	8.00
C. albicans	5.83
C. glabrata	3.17
C. tropicalis	3.17

Karapandzova et al. (2014) in their study used the disk diffusion and the broth dilution methods for antimicorbial screening of the *P. peuce* essential oils against one strain of

Candida albicans and bacterial isolates of Gram-negative bacteria and Gram-positive bacteria. They found out the highest antimicrobial activity of *P. peuce* EOs mainly against *Streptococcus agalactiae, S. pyogenes, Enterococcus* and *Candida albicans*, followed by *Haemophilus influenzae, Acinetobacter* spp., *Escherichia coli, Salmonella enteritidis, Staphylococcus aureus* and *S. epidermidis*. We also found out in our study the best antimicrobial activity of *P. peuce* but against *L. monocytogenes* and *E. coli*.

4. Conclusion

In the present study, the EO from *P. heldreichii, P. peuce* and *P. mugo* was extracted at different time intervals. The results illustrated that most of the EO was extracted during the first 15 mins of distillation (0-15); up to 70% of the total EO in *P. heldreichii,* 53.85% in *P. peuce* and 54.63% in *P. mugo*. This result is a prerequisite for reducing the distillation duration as well as saving energy and time. The EO in the three species in the different time intervals of the experiment had a relatively constant qualitative composition, which refutes the working hypothesis of this study. The concentration of compounds in the EO extracted during the 15 min was as follows: limonene (76.9-77.0%), α-pinene (12.47-15.54%) for *P. heldreichii*; α-pinene (34.26 - 43.75%), limonene (19.2-22.2%), β-pinene (9.24-11.48%) for *P. peuce;* and α-pinene (16.48-17.84%), α-phellandrene (23.07-23.76%), β-phellandrene (28.12-28.79%) for *P. mugo*;. The concentration of most individual EO compounds in the 0-15 min fraction of this study were comparable to that in previous studies where distillation times of 2, 3, 4, 6, 24 hours or even 20 days was used. This study demonstrated that *P. heldreichii, P. peuce* and *P. mugo* EOs desirable composition can be obtained in relative short time and therefore, both energy and time can be saved.

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CRediT authorship contribution statement

Author Contributions: "Conceptualization, **I.S.** and **V.D.Zh**.; methodology, **I.S**., **V.D.Zh**., **Ch.C., M.C.**; software, **T.A**.; validation, **I.S., C.R**., **V.D.Zh**., **Ch.C**. and **T.A**.; formal analysis, **T.A**.; investigation, **I.S., C.R., V.D.Zh**., **Ch.C., T.A., M.C., D.B**; resources, **I.S., C.R**., **V.D.Zh**., **Ch.C., T.A., M.C., D.B**.; data curation, **I.S. and V.D,Zh**.; writing-original draft preparation, **I.S., C.R**.; writing-review and editing, **V.D.Zh**., **Ch.C., I.S**., **T.A., C.R**., **M.C., D.B**.; visualization, **Ch.C**., and **T.A**.; project administration**, I.S**.; funding acquisition, **I.S**.

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