Solvent-free Microwave and Hydro-distillation Extraction of Essential Oils from the Sawdust of Pines: Correlation with Heat-map

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The chemical compositions were investigated for pine essential oils obtained through a solvent-free microwave assistance extraction system (ME) and a conventional hydrodistillation system (HD). The essential oils of *P. pinea*, *P. nigra*, *P. brutia*, and *P. sylvestris* sawdust were analyzed by gas chromatography and mass spectroscopy (GC-MS). The main components of the pine essential oils were D-limonene (52.8% for ME and 76.6% for HD), β-caryophyllene (12.4% for ME), β-myrcene (2.89% for ME and 1.48% for HD), sesquiterpene hydrocarbons (25% for ME and 9.79% for HD), and total sesquiterpene (25.25% for ME and 9.79% for HD) for *P. pinea*; D-limonene (28.1% for ME and 79.2% for HD) for *P. nigra*; α-pinene (76.6% for ME and 77.3% for HD), diterpene hydrocarbons (94.17% for ME and 95.62% for HD), and total diterpenes (94.94% for ME and 96.3% for HD) for *P. brutia*; β-pinene (36.7% for ME and 42.4% for HD), terpineol, (13.8% for ME and 6.06% for HD) diterpene alcohol (26% for ME and 12.57% for HD), and total oxygenated terpenes (26% for ME and 12.57% for HD)for *P. sylvestris.* Moreover, the ME was able to produce more diterpene alcohols and sesquiterpene hydrocarbons while the HD showed higher potential for the diterpene hydrocarbons. According to the heat-map correlation, *P. pinea* showed high similarity with *P. nigra*, while *P. sylvestris* was related to *P. brutia.*

INTRODUCTION

Scientific and ethno-botanic research on plants are of growing interest because of their use and identification. Secondary metabolisms, such as essential oils, of the plants in food flavoring, fragrance, folk medicine, and pharmaceutical industries are significantly valuable (Alma *et al.* 2012; Ilcim *et al.* 2014; Karaogul *et al.* 2016a, 2017; Karaoğul and Alma 2018). The essential oils, also known as the secondary metabolites, consist of terpenes or terpenoids (Marriott *et al.* 2001). They are important antioxidants that assist in food processing and storage due to their shelf life and properties (*e.g.*, flavor, fragrance, texture, and color of food) (Zeng *et al.* 2012), as well as their biological activities such as antimicrobial, antiviral, antiparasitic, and insecticidal effects (Bakkali *et al.* 2008). Some techniques can be used to obtain essential oils from various parts of the plant, including water or steam distillation through solvent extraction, microwave assistance extraction, expression under pressure, supercritical fluid, and subcritical water extractions (Tumen *et al.* 2010; Meullemiestre *et al.* 2014a). However, some technologies generally have several disadvantages including long processing times and high temperatures, as well as low essential oil content amounts (Rodríguez-Rojo *et al.* 2012; Meullemiestre *et al.* 2014b). In a few years, new, cleaner,

and green tendency techniques (*e.g.*, microwave assistance extraction system) could be preferred because of their shorter extraction times, lesser consumption abilities of both the organic solvent and energy, and their production of high-quality content of the essential oils (Gholivand *et al.* 2013; Meullemiestre *et al.* 2014b; Fernández-Agulló *et al.* 2015; Mellouk *et al.* 2016).

Four pine species are commonly known and commercially used in Turkey (*Pinus pinea, Pinus nigra, Pinus brutia*, and *Pinus sylvestris).* The essential oils of the pines are usually used as food and beverage additives for additional flavor, and as cosmetics and perfumery ingredients for fragrances (Ustun *et al.* 2006; Tumen *et al.* 2010). The objective of this study is to investigate and to compare the essential oils of the pine species (*e.g.*, *P. pinea, P. nigra, P. brutia, P. sylvestris)* through a solvent-free microwave assistance extraction system (ME) and a conventional hydro distillation system (HD).

EXPERIMENTAL

Materials

Four different fresh pine sawdust were used in this study. The pine species *P. pinea*, *P. nigra*, and *P. brutia* were obtained from Kahramanmaras province located in the Mediterranean region of Turkey. *Pinus sylvestris* was provided from Kars province located in the East Anatolian region of Turkey. The form of sawdust was approximately (30 x 2.5 x 0.25 mm) as well as air dried of their moisture content. The pine species were identified at the Dept. of Forest Engineering at Kahramanmaras Sutcu Imam University. The sawdust's were air dried before use.

Isolation of the Essential Oil

The essential oils of the pines were extracted by a conventional hydro-distillation system (HD) and a microwave assistance extraction system (ME), (NEOS Milestone laboratory oven, Sorisole, Italy) (Fig. 1.). A Clevenger apparatus was also used for the hydro-distillation (SAHA laboratory, Ankara, Turkey) unlike ME. Using 150 g of the pine sawdust, the essential oils were collected at 4 h by HD, 45 min by the ME, and then dried over anhydrous sodium sulfate in a vial until further analysis. The HD process was distilled 400 mL volume of water. At 300 W microwave irradiation power of the ME system, the samples were heated using a fixed power. Each extraction process was repeated three times to find a correct standard deviation.

Pine Essential Oil Analysis

Gas chromatography-mass spectrometry (GC-MS) was performed using a Clarus 680 instrument (Perkin-Elmer Technologies, Waltham, MA, USA) equipped with an HP-5MS capillary column (30 m/320 μm i.d., 0.25 μm film thickness). The carrier gas was helium and was delivered at a 1.0 mL/min flow rate. The temperature of the column oven was set starting from 40 °C (hold time 4 min) to 180 °C at a 3 °C/min heating rate. The temperature program after the 5 min of hold time at 180 °C was continued at 20 °C/min to 230 °C for 20 min. The injection and the MS-transfer line temperature were held at 250 °C, which ran in the electron impact ionization mode at a 70-eV electron energy level. Compounds were identified by comparison of the mass spectra with the NIST 09, replib, mainlib and WILEY11 mass spectral database (Salem *et al.* 2015) and the measured retention index (RI) values of the components with a homologous series of C7-C30 n-alkane standards (about 99% purity). The quantitative area-percent measurements were based on peak areas from the GC-MS data.
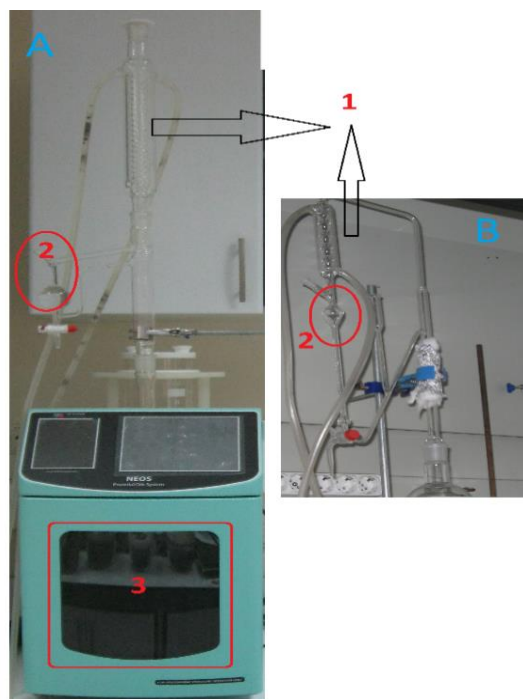
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Fig. 1. Isolation apparatus of the essential oils; (A) microwave assistance extraction system, (B) conventional hydro-distillation system, (1) refrigerant system, (2) collected oil fraction, (3) microwave oven

Statistical Analysis

The statistical analysis was calculated by Excel 2016 program. The results were subjected to an analysis of variance at the 95% confidence level, and significant differences between mean values of the results were investigated using ANOVA Single Factor.

RESULTS AND DISCUSSION

The essential oils of the pine species were obtained through a solvent-free microwave assistance extraction system (ME) and a conventional hydro-distillation system (HD). Terpene groups in the essential oils of the *Pinus* species were investigated and compared. The groups were categorized into diterpene hydrocarbons, diterpene alcohols, sesquiterpene hydrocarbons, and sesquiterpene alcohols. These aromatic diterpenes can be used for anti-inflammation, anti-carcinogenesis, and neuroprotection (Kiyama 2017). The terpene categories and their amounts in various pine sawdust are shown in Table 2. Additionally, the essential oil compositions, retention time, and retention index of the various pine species sawdust are showcased in Table 1. A total of 34 various essential oil components were determined for all pine species. As can be seen in Table 1, total numbers of determined essential oil compounds were in the range of 13 to 21 chemical compounds according to each pine species. It was clear that the essential oil compounds number by the ME were a higher amount of than the HD. This phenomenon could be related back to the microwave energy effect (Orio *et al.* 2012; Mellouk *et al.* 2016)

The percent amount of the main component was affected by the extraction procedures and pine species. Percent compositions of the main component of the essential oils in the sawdust of the pine species are shown in Figs. 2 and 3. The main compositions of the essential oil in *P. pinea* were D-limonene at 52% to 76%, β-

caryophyllene at 12%, α-pinene at 4.5% to 7%, β-pinene at 1.8% to 6.8%, longifolene at 5.6%, β-myrcene at 1.4% to 2.9%, and terpineol at 1.2% to 1.9%. The main components of *P. nigra* were D-limonene at 28% to 79%, α-pinene at 4.75% to 37%, eremophilene at 7.45%, δ-cadinene at 3.5%, β-caryophyllene at 3.31%, and terpineol at 1.6% to 4.2%. The main components of *P. brutia* essential oil were α-pinene at 76% to 77%, β-pinene at 12%, and D-limonene and camphene at 1.2% to 1.3%. The main composition of *P. sylvestris* was β-pinene at 36% to 42%, α-pinene at 24% to 32%, terpineol at 6% to 13.8%, 4-terpineol at 6% to 11.6%, and D-limonene at 2.7% to 2.8%.

The percent compositions of α-pinene, β-pinene, and D-limonene of the essential oils in the pine species are illustrated in Fig. 2. The α-pinene was at highest proportion in the *P. brutia*, and the lease proportion in the *P. pinea*. However, the α-pinene in the *P. nigra* and *P. sylvestris* were not significantly affected, unlike with the HD in the *P. nigra*. Moreover, the α-pinene was increased by the ME in the *P. pinea* and *P. nigra*, unlike in the *P. brutia* and *P. sylvestris*. The results showed that the highest to lowest amount of the β-pinene in each species was *P. sylvestris, P. brutia, P. pinea*, and *P. nigra* (Megawati *et al.* 2019). The highest amount of D-limonene was in the *P. nigra*, followed closely by the *P. pinea*.
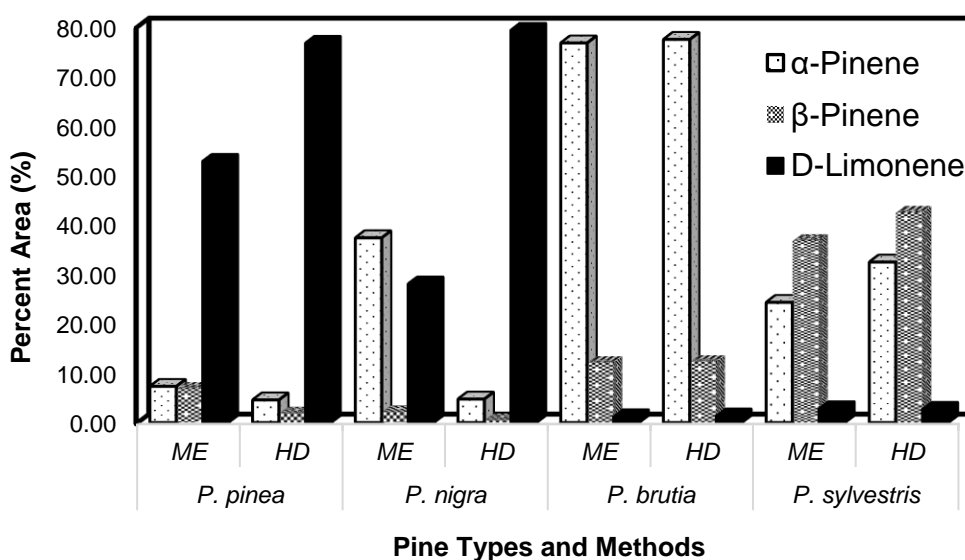
Fig. 2. Percent compositions of α-pinene, β-pinene, and D-limonene as the main component in the sawdust of the pine species

β-myrcene, terpineol, and β-caryophyllene of the essential oils in the pine species were shown as the percent compositions in Fig. 3. β-myrcene was the highest in *P. pinea*, followed by *P. brutia*, *P. pinea,* and *P. sylvestris*. Moreover, β-myrcene could be obtained by HD because of its polarity. Terpineol composition was the highest in *P. sylvestris*, while *P. brutia* had the lowest amount. It was confirmed that the highest amount of β-caryophyllene was in *P. pinea*, followed by *P. nigra*. Moreover, according to statistical analysis, there were significant differences among the HD and ME treatments (p < 0.05) for α-pinene, β-pinene, D-limonene, terpineol, and β-caryophyllene in *P. pinea* and *P. nigra*. Besides, the terpineol (in *P. brutia*), α-pinene, D-limonene, β-caryophyllene (in *P. sylvestris*) were p < 0.05. However, there were no significant differences among the HD and ME treatments (p > 0.05) for α-pinene, β-pinene, D-limonene, and β-caryophyllene in *P. brutia* and β-pinene and terpineol in *P. sylvestris*.

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Table 1. Percent Compositions, Retention Time, and Retention Index of the Essential Oils Among the Pine Species

No	RT[1]	RI[2]	CN[3]	*P. pinea*		*P. nigra*		*P. brutia*		*P. sylvestris*	
				ME[4]	HD[5]	ME	HD	ME	HD	ME	HD
1	16.7	1135	α-Pinene	7.3±0.32	4.58±0.38	37.4±0.4	4.75±0.45	76.6±0.67	77.3±0.38	24.3±0.37	32.5±1.5
2	17.4	1160	camphene	0.18±0.02	0.08±0.01	0.52±0.08	0.09±0.01	1.23±0.07	1.33±0.07	0.80±0.1	1.05±0.05
3	18.8	1185	β-Myrcene	2.89±0.11	1.48±0.02	ND	1.64±0.24	1.67±0.2	1.79±0.21	1.20±0.2	1.41±0.09
4	19.8	1198	β-Pinene	6.79±0.21	1.80±0.2	2.09±0.09	0.43±0.07	12.0±0.96	12.2±0.24	36.7±0.73	42.4±0.44
5	21.1	1213	D-Limonene	52.8±0.86	76.6±0.67	28.1±1.14	79.2±0.73	1.21±0.08	1.37±0.13	2.85±0.15	2.73±0.23
6	22.4	1236	α-Tricyclene	ND	ND	ND	ND	0.07±0.009	0.07±0.01	0.05±0.009	ND
7	23.1	1246	carene	0.18±0.02	0.36±0.05	0.10±0.02	ND	0.10±0.02	0.08±0.01	0.20±0.02	ND
8	26.5	1296	Sabinene	ND	ND	ND	ND	ND	ND	0.18±0.02	0.20±0.02
9	27.4	1310	terpineol	1.93±0.07	1.24±0.14	4.28±0.28	1.62±0.12	0.39±0.03	0.41±0.03	13.8±0.19	6.06±0.06
10	29.2	1336	α-Ylangene	0.15±0.01	ND	0.47±0.03	ND	ND	ND	0.03±0.009	ND
11	30.7	1365	o-Cymene	ND	ND	ND	ND	0.12±0.009	0.03±0.009	ND	0.13±0.02
12	31.1	1365	β-Phellandrene	ND	ND	ND	ND	1.03±0.03	1.16±0.16	1.44±0.06	1.44±0.16
13	32.1	1380	α-Longipinene	2.64±0.36	0.94±0.06	0.45±0.05	0.25±0.05	0.02±0.009	0.02±0.009	ND	ND
14	32.5	1390	α-Copaene	0.26±0.03	ND	0.37±0.03	ND	ND	ND	0.16±0.02	0.08±0.01
15	33.2	1395	γ-Terpinene	ND	ND	ND	ND	0.13±0.02	0.15±0.01	0.53±0.03	0.49±0.01
16	33.4	1401	Sativen	0.17±0.02	0.05±0.01	0.24±0.01	ND	ND	ND	ND	ND
17	34	1405	Eremophilene	ND	2.29±0.21	7.45±0.45	ND	0.02±0.009	ND	ND	ND
18	34.1	1410	Longifolene	5.65±0.35	ND	ND	ND	ND	ND	ND	ND
19	34.2	1413	β-Caryophyllene	12.4±0.46	ND	1.55±0.05	3.31±0.31	0.22±0.02	0.24±0.04	ND	ND
20	35.1	1426	α-Caryophyllene	ND	0.87±0.03	1.29±0.01	0.52±0.02	0.14±0.009	0.33±0.02	ND	ND
21	35.5	1437	γ-Muurolene	0.53±0.03	ND	ND	ND	ND	ND	ND	ND
22	35.8	1424	β-Cubebene	1.31±0.11	ND	1.81±0.19	ND	ND	ND	0.88±0.02	0.33±0.05
23	35.9	1449	caryophyllene	0.16±0.02	5.65±0.35	ND	ND	ND	ND	ND	ND

24	36.1	1442	α-Muurolene	1.02±0.02	ND	ND	ND	ND	ND	0.99±0.01	0.43±0.07
25	36.5	1454	δ-Cadinene	0.76±0.06	ND	3.57±0.43	0.23±0.03	0.24±0.01	ND	0.29±0.01	0.10±0.01
26	38.4	1472	Fenchol, exo-	ND	ND	ND	ND	0.18±0.009	0.11±0.01	0.34±0.04	0.20±0.02
27	39.8	1508	α-Cadinol	0.15±0.01	ND	0.50±0.06	0.40±0.04	0.01±0.009	ND	ND	ND
28	41.7	1529	Borneol	ND	ND	ND	0.07±0.01	0.16±0.01	0.08±0.01	0.30±0.05	0.15±0.01
29	42.1	1540	4-Terpineol	ND	ND	ND	0.05±0.009	0.04±0.009	0.08±0.009	11.6±0.38	6.16±0.16
30	49.7	1679	Cycloisosativene	ND	ND	0.28±0.05	0.05±0.009	ND	ND	0.05±0.01	0.02±0.009
31	50.7	1695	Cryptopinone	1.60±0.2	ND	ND	ND	ND	ND	ND	ND
32	50.9	1701	Eremophilene	ND	ND	ND	1.03±0.03	ND	ND	ND	ND
33	51.4	1704	D-Germacrene	ND	ND	0.32±0.03	0.12±0.02	0.01±0.009	ND	ND	ND
34	52.6	1730	Isoledene	ND	ND	2.27±0.27	0.18±0.02	ND	ND	0.20±0.03	0.08±0.009
Total Number of Determined Compounds				**20**	**13**	**19**	**17**	**21**	**18**	**21**	**19**

[1]RT: Retention Time**, [2]RI:** Retention Index**, [3]CN:** Component name, **[4]ME:** solvent-free microwave assistance extraction system, **[5]HD:** conventional hydro distillation system, ND: no detectable

Table 2. Diterpen and Sesquiterpene Percent Compositions of the Essential Oils in the Pine Species

Terpene Groups	*P. pinea*		*P. nigra*		*P. brutia*		*P. sylvestris*	
	ME[3]	HD[4]	ME	HD	ME	HD	ME	HD
Diterpene Hydrocarbons	70.21±0.25	84.96±0.22	68.15±0.35	86.31±0.30	94.17±0.21	95.62±0.12	68.34±0.17	82.38±0.28
Diterpene Alcohol	1.93±0.07	1.24±0.14	4.28±0.28	1.74±0.04	0.78±0.01	0.68±0.01	26.07±0.16	12.57±0.16
Sesquiterpene Hydrocarbons	25.10±0.12	9.79±0.11	20.07±0.13	5.76±0.08	0.74±0.02	0.59±0.03	2.59±0.03	1.04±0.04
Sesquiterpene Alcohol	0.15±0.01	ND	0.50±0.06	0.40±0.04	0.01±0.009	ND	ND	ND
Total Diterpenes	72.14±0.16	86.21±0.18	72.43±0.31	88.04±0.17	94.94±0.11	96.30±0.07	94.41±0.17	94.96±0.17
Total Sesquiterpenes	25.25±0.07	9.79±0.11	20.57±0.10	6.16±0.06	0.76±0.01	0.59±0.03	2.59±0.03	1.04±0.04
Total Oxygenated Terpenes	2.08±0.04	1.24±0.14	4.78±0.17	2.13±0.04	0.79±0.01	0.68±0.01	26.07±0.16	12.57±0.06

ND: no detectable, **[3]ME:** solvent-free microwave assistance extraction system, **[4]HD:** conventional hydro distillation system, ND: no detectable
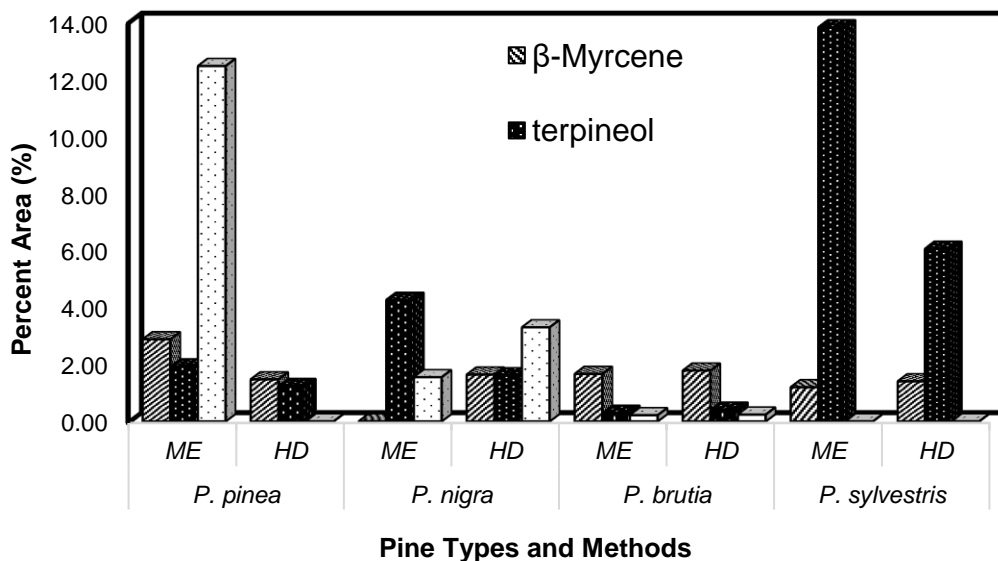
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Fig. 3. Percent compositions of the β-myrcene, terpineol, and β-caryophyllene as the main component in the sawdust of the pine species

The diterpene hydrocarbons, diterpene alcohols, sesquiterpene hydrocarbons, sesquiterpene alcohols, total diterpenes, total sesquiterpenes, and total oxygenated terpenes of the essential oils in the pine species are shown in Table 2, Fig. 4, Fig. 5, and Fig. 6.

As can be seen from Fig. 4, the diterpene alcohols were significantly higher in the *P. sylvestris.* Besides, the diterpene alcohols of the *P. nigra, P. pinea,* and P. *brutia* were listed from increasing to low, respectively. The sesquiterpene hydrocarbons were at the highest amount in *P. pinea*, followed by in the *P. nigra.* Nevertheless, there was a small amount of sesquiterpene hydrocarbons in the *P. sylvestris* and P. *brutia.*
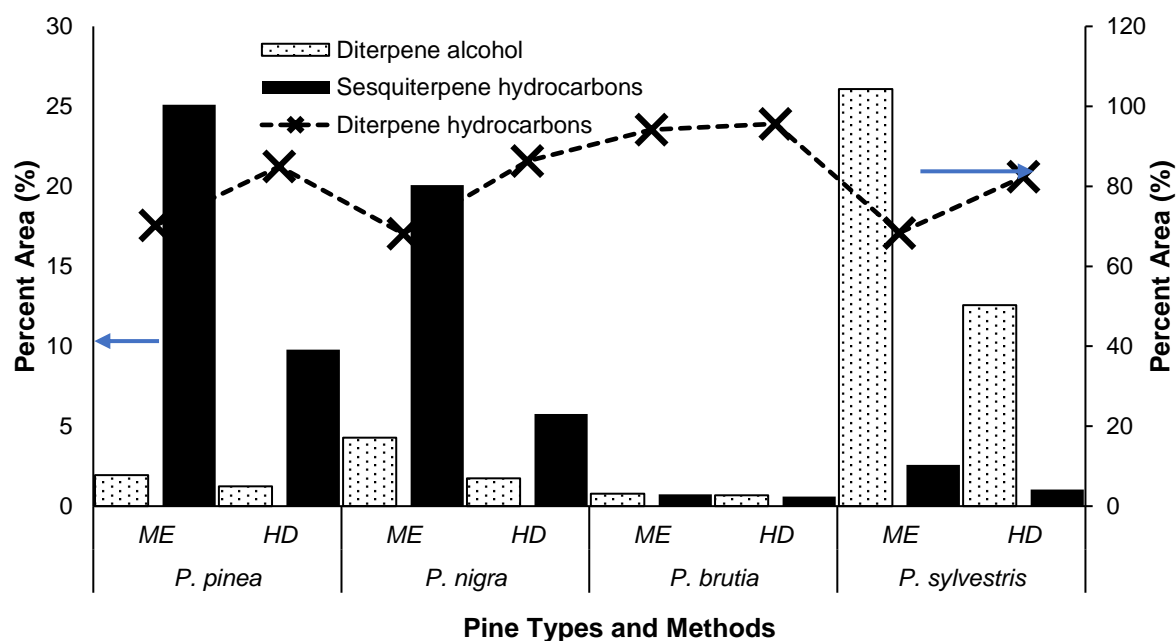

Fig. 4. Percent amount of the diterpene hydrocarbons, diterpene alcohols, and sesquiterpene hydrocarbons in the pine species

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More diterpene alcohol and sesquiterpene hydrocarbons were obtained by the free solvent microwave assistance extraction system. This could be attributed to the higher power of the microwave, as well as the use of less solvent, heat, and the mass transfer phenomena.

The rapid extraction efficiency of the ME accelerates the extraction under microwaves (Farhat *et al.* 2011; Desai and Parikh 2015). This phenomenon could also be explained with energy transfer correlation among biological compounds (13.6 eV), microwave energy on the extraction (1.24×10^{-6} - 1.24×10^{-3} eV), covalent bond energies (5 eV), and hydrogen bond energies (2 eV) (Meullemiestre *et al.* 2014a,b). Moreover, the diterpene hydrocarbons were high for almost all species (Tumen *et al.* 2010). But the highest amount was in the P. *brutia*, followed by the *P. nigra*, *P. sylvestris*, and *P. pinea.* The conventional hydro distillation system has great potential for the diterpene hydrocarbons due to its relationship with water.
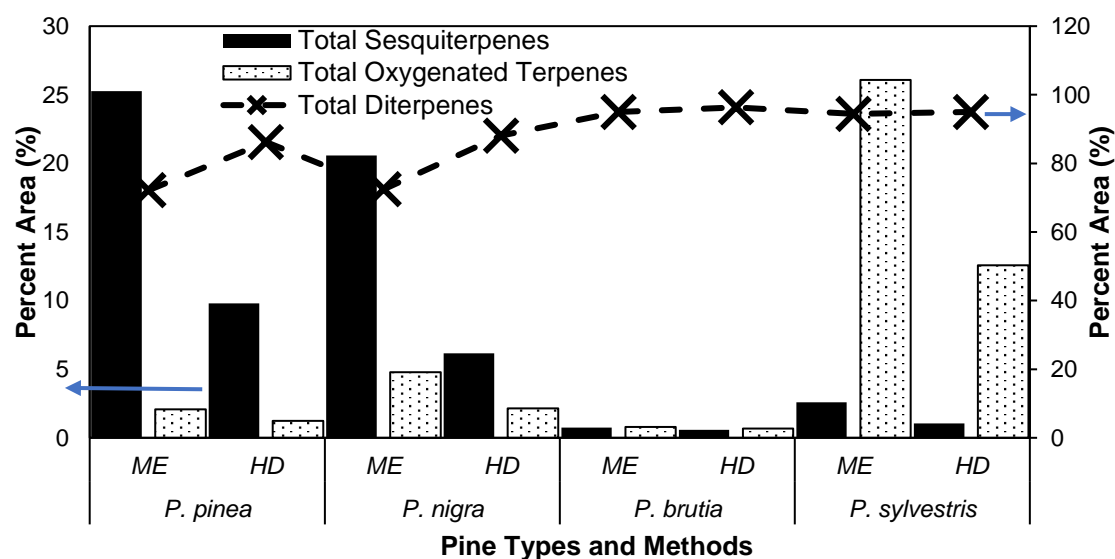

Fig. 5. Percent amount of the total diterpenes, total oxygenated terpenes, and total sesquiterpene in the pine species for each of the extraction methods

It is clearly evident from Fig. 5 that the total sesquiterpenes had higher potential in *P. pinea*, followed by *P. nigra.* However, there was also a lower total sesquiterpene in *P. sylvestris* and *P. brutia.* Moreover, the total oxygenated terpenes were considerably higher in *P. sylvestris.* Besides, the total oxygenated terpenes, in order from a high to low amount, were first in *P. nigra,* then *P. pinea,* and finally P. *brutia.* The results showed that the total sesquiterpenes and total oxygenated sesquiterpenes reached greater potentials through the ME. This phenomenon could be related to the higher power of the microwave and the less solvent used (Elyemni *et al.* 2019). Besides, the total diterpenes were high for almost all of the species, especially *P. brutia* and *P. sylvestris*, and was slightly lower for *P. nigra* and *P. pinea.* The HD significantly influenced the amount of the total diterpenes because of its hydrophilicity.

Figure 6 demonstrates the heat-map correlation of the pine species according to the main components, total diterpenes, total oxygenated terpenes, and total sesquiterpene in each. According to the correlation relationship of the heat-map, the higher or lower amounts are displayed in a bold red color to bold green color. *P. pinea* and *P. nigra* were very similar according to the heat-map correlation analysis and in regard to their chemical compositions. Moreover, the *P. brutia* and *P. sylvestris* illustrated much similarity. The results of the chemical composition of the pine essential oil showed that the *P. pinea* and *P. nigra* were different from the *P. brutia* and *P. sylvestris.*
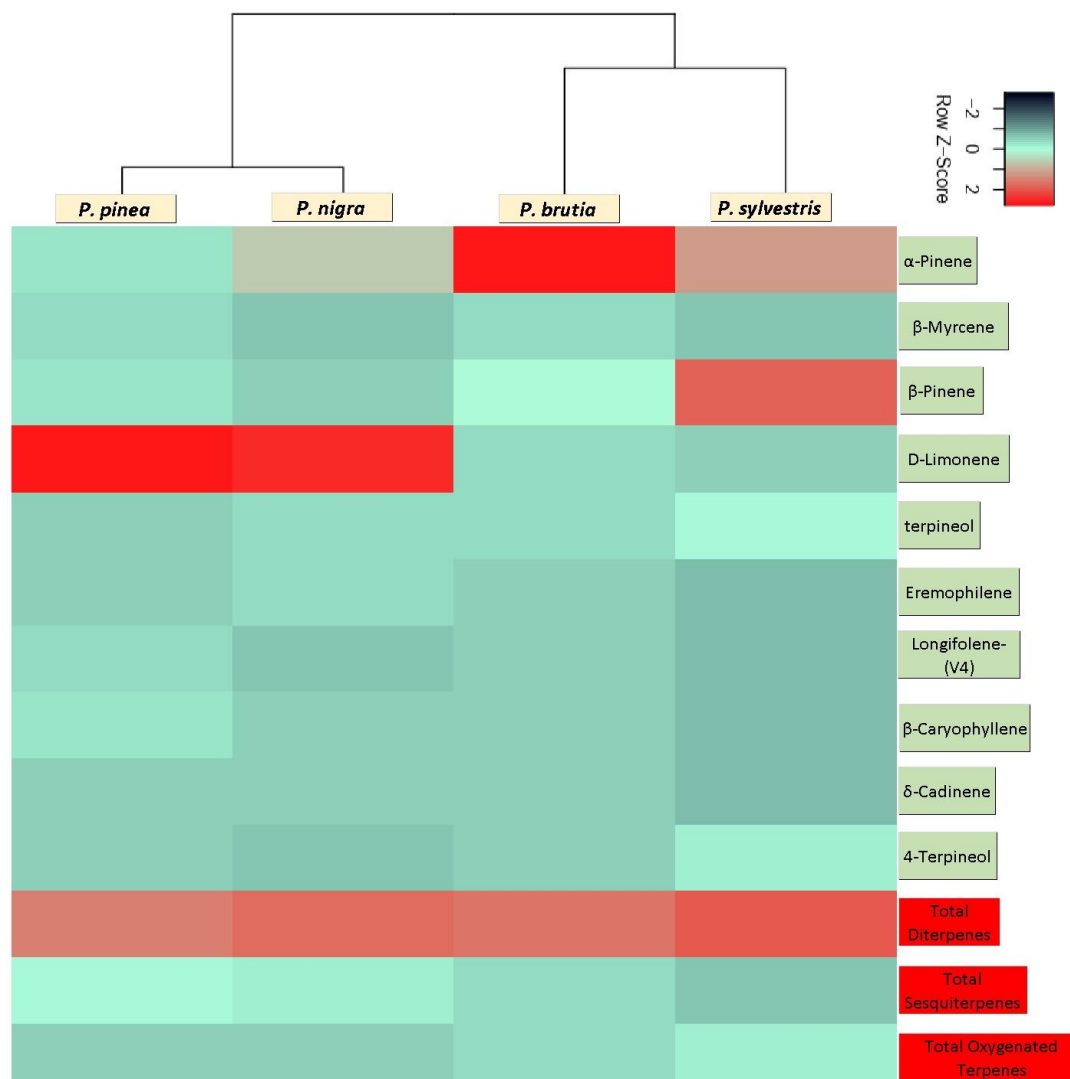
Fig. 6. Heat-map correlation of the pine species according to the main components, total diterpenes, total oxygenated terpenes, and total sesquiterpenes

CONCLUSIONS

1. The essential oils of *P. pinea*, *P. nigra*, *P. brutia*, and *P. sylvestris* produced through a solvent-free microwave assistance extraction system (ME) and conventional hydro distillation system (HD) were investigated by GC-MS. The extraction methods and pine species affected the chemical compositions of the essential oils. According to the chemical compositions of the essential oils, the terpene groups were calculated as diterpene hydrocarbons, diterpene alcohols, sesquiterpene hydrocarbons, and sesquiterpene alcohols.

2. A total of 34 various essential oil compositions were determined for all the pine species. The main compositions of the essential oils were α-pinene, β-pinene, D-limonene, β-caryophyllene, longifolene, β-myrcene, terpineol, eremophilene, δ-cadinene camphene, and 4-terpineol by ME system.

3. The composition of the essential oil in *P. pinea* had higher amounts of D-limonene, β-caryophyllene, β-myrcene, sesquiterpene hydrocarbons, and total sesquiterpenes. The *P. nigra* had a greater potential of D-limonene. Additionally, the *P. brutia*

illustrated higher amounts of α-pinene, diterpene hydrocarbons, and total diterpenes. Finally, the *P. sylvestris* had the highest amount of β-pinene, terpineol, diterpene alcohol, and total oxygenated terpenes.

4. More diterpene alcohol and sesquiterpene hydrocarbons were produced through the ME process. This could be related to the higher power of the microwave, the free solvent in the process, and their hydrophobicity. However, the HD showed higher potential for the diterpene hydrocarbons because of its hydrophilicity.

5. When the results of the essential oils from the pines were investigated according to the heat-map correlation, the *P. pinea* showed similarity with *P. nigra.* And also, *P. sylvestris* was similar to the *P. brutia.*

6. In summation, this examination demonstrates the potential importance of the diterpene alcohol, sesquiterpene hydrocarbon, and total oxygenated terpene unlike total diterpene hydrocarbon by ME system. Also, the pine wastes known as sawdust have possible value.

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