Phytochemical studies on essential oils of *Pinus pinaster* Aiton and evaluation of their biological activities

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Abstract:

This study focus on the analysis of the chemical composition of the aerial parts essential oils (Eos) (needles, cones and branches) of Tunisian *P. pinaster* Aiton, and to evaluate their antioxidant activities, their inhibition toward germination and seedling growth of weeds and to assess their antifungal activities against phytopathogenic fungi. Eos were obtained by hydrodistillation and analyzed by GC and GC/MS analysis. A total of 27, 25, and 15 compounds were identified respectively in needles, cones and branches. All analyzed oils were rich in hydrocarbonated monoterpenes (32.57-90.48%). α-pinene (25.11-80.95%), β-pinene (1.86-33.12%) and β-caryophyllene (0.28-21.34%) were the dominant compounds in the volatile oils. All tested samples exhibited interesting antioxidant activities. The phytotoxicity of Eos was evaluated against tow weeds: *Sinapis arvensis* L. and *Phalaris canariensis* L. and one cultivated specie *Triticum tirgidum* L. The antifungal activity was investigated *in vitro* using five targeted fungal strains. Tested samples were differently effective toward tested plants and target fungi depending on the variability of their chemical compositions. All sample oils showed a significant phytotoxic effects and needles oils exhibited the highest herbicidal effects against all tested species. The highest fungitoxic properties were obtained with needles and cones Eos against all fungi.

Keywords:*Pinus pinaster* Aiton, essential oils, antioxidant activity, herbicidal activity, antifungal activity.

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Introduction

Maritime pine *(Pinus pinaster.Aiton*) is an evergreen conifer species appertaining to *Pinaceae* family and *Pinus* genus. Its area of distribution ranges from Portugal to Greece and from Morocco to Tunisia, whether as continuous ancient or recent areas (Caudullo et al. 2017). In Tunisia, pine forests occupy 400.000 Ha, which represent more than 65% of the forest area. Maritime pine is one of the most important forest species in Tunisia, next to *Pinus halepensis* Mill. and *Pinus pinea* L. used especially for the stabilization of dunes and the production of plant nursery substrates (Mutke et al. 2011) .

Its feature and stand structures allow a wide range of uses. In fact their softwood has a low quality essentially due to knots frequency and resin secretion, but generally used in pulpwood and locally used for construction and furniture (Polge 1992). The most major non woody products is resin, which is a natural product that has multiple applications and it is very demanded within several industries of coatings, adhesives, printing inks, insecticides and disinfectants (Salim et al. 2019). Generally, resin and decoction of all pine trees are known to be antiseptic, diuretic, rubefacient vermifuge and antidiabtic (Manganelli et al. 2001). Recently, the assessment of biological activities of resin extracted from Tunisian pine forests showed that Maritime pine was the major resin production, followed by Aleppo pine and Stone pine with 50, 30 and 20% resin yield, respectively (Aloui et al. 2022; Amri et al. 2022). Maritime pine as a fast-growing conifers species ensure a particular value, in terms of provisioning, regulating, and supporting ecosystem services, make it a specie of primordial importance in several countries. In fact, *Pinus* genus is an allelopathic tree, known for their richness in Eos. That have been studied in dozen research for their promising in industrial and pharmaceutical applications, which recommended as antimicrobial agents as well as potential insecticidal. Needles Eos from Tunisian *Pinus halepensis* Mill. has been investigated against *Ectomyelois ceratoniae* Zeller the date moth, which is a serious pest to stored products in all countries producing date palm (*Phoenix dactylifera* L.) (Amri et al. 2014). Actually, allelopathy in natural and agricultural ecosystems is receiving increasing attention because allelochemicals significantly reduce the plant growth and the yields of crop plants (Wang et al. 2014). Herbicidal activity from Tunisian *Pinus radiata* needles Eos has been studied on seed germination and seedling growth being significantly more effective on dicots *Sinapis arvensis* L. and *Trifolium campestre* Schreb. than monocots *Phalaris canariensis* L. (Ismail et

al. 2021). Furthermore, the herbicidal activity of needles Eos from Tunisian Aleppo pine investigated for three common weeds in Tunisian cereal crops was very strong and seed germination was inhibited at a low concentration (Hamrouni et al. 2015). Accordingly, the aims of this study were to determine the chemical composition of the essential oils of cones, branches and needles of *P. pinaster*, to assess their antioxidant activity and to evaluate their herbicidal activity against *Phalaris canariensis* L., *Sinapis arvensis* L., and *Triticum durum* L., and lastly, the evaluation of the antifungal activity has been provided against five plant pathogenic . As far as we know, there are no previous studies on the variability of p. pinaster Eos.

Material and Methods

Plant Material

Cones, needles and branches of *Pinus pinaster* Aiton have been collected during January from the Souinet arboretum of the National Institute of Researches on Rural Engineering, Water and Forests. Samples collected from different trees were harvested and mixed for homogenization. The experimental site is located in Ain Draham, in the north of Tunisia at an altitude of 492 m, where humid climate prevails. Dr Lamia HAMROUNI, National Institute of Researches on Rural Engineering, Water, and Forests, TUNISIA, identified the plant and voucher specimens (PPR-1401, PPR-1402 and PPR-1403) were submitted to the herbarium division of the Institute.

Isolation of the essential oils

Fresh and finely grounded materials were prepared for hydro distillation using a Clevenger type apparatus, a weight of 200 g versed in 500 ml of distilled water for 4 h, this experiment were repeated four times for each sample. The total amount of the Eos obtained was collected and dried over using anhydrous sodium sulfate and stored in sealed glass brown vials in a refrigerator at 4 °C until further analysis and bioassay studies. Yield based on dry weight of the sample was calculated (w/w %).

Gaz chromatography and mass spectrometry analysis

Gaz chromatography analysis with FID detection

The essential oils were analyzed using a Hewlett Packard 5890 II GC equipped with Flame Ionization Detector (FID) and HP-5 MS capillary column (5 % phenyl/95 % dimethylpolysiloxane: 30 m×0.25 mm id, film thickness 0.25 µm). Injector and detector temperature were set at 250 and 280 °C, respectively. Oven temperature was kept at 50 °C for

1 min then gradually raised to 250 °C at 5 °C/min and subsequently, held isothermal for 4 min. Nitrogen was the carrier gas at a flow rate of 1.2 ml/min. Diluted samples (1/100 in hexane, v/v) of 1.0 µL were injected manually in the splitless mode. Quantitative data were obtained electronically from FID area percent data without using correction factors.

Gas chromatography analysis with MS detection

Analysis of the oils was performed using a Hewlett Packard 5890 II GC, equipped with a HP 5972 mass selective detector and a HP-5 MS capillary column (30 m×0.25 mm id, film thickness 0.25 µm). For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, a scan time of 1.5 s and mass range 40-300 amu, was used. Helium was the carrier gas at a flow rate of 1.2 ml/min. Injector and transfer line temperatures were set at 250 and 280 °C, respectively. The oven temperature program was the same with GC/FID analysis. Diluted samples (1/10 in hexane, v/v) of 1.0 µL were injected manually in the splitless mode. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library) or with authentic compounds and confirmed by comparison of their retention indices either with those of authentic compounds or with data published in the literature(Adams 2005). Further confirmation was done from retention index data generated from a series of *n*-alkanes retention indices (relative to C9-C28 on the HP-5 MS capillary column).

Total phenolic content

The total phenolic compounds content of *P. pinaster* Eos was determined by using the Folin–Ciocalteu reagent, according to the procedure described by Tzanakaki et al (2011). 125 µL of Eos were mixed with 125 µL of the Folin–Ciocalteu reagent. The mixture was shaken before the addition of 1.25 mL of 7% Na_2CO_3, adjusted with distilled water to a final volume of 3 mL, and mixed thoroughly. After incubation in the dark for 90 min, the absorbance was measured at 755 nm in comparison to the prepared control. The obtained results was expressed as mg of gallic acid equivalent per gram of dry weight (mg GAE/g DW) through a calibration curve prepared with gallic acid (Tzanakaki et al. 2011). All samples were analyzed in triplicate.

Antioxidant activity

Total antioxidant capacity

In order to quantify the total antioxidant capacity of *Pinus pinaster* Eos, a spectrophotometric method was adopted (Prieto et al. 1999) which is based on the reduction of molybdenum (VI)

ions to molybdenum ions (V) by the plant extract to form the phosphate-complex-Mo^{5+} with green color and acidic pH. The total antioxidant activity solution was prepared as follows: In one volumetric flask, 0.394 g of sodium phosphate ($Na\ H_2PO_4$, 28mM) and 0.494 g ammonium heptamolybdate [$(NH_4)_6MO_7O_{24}$, $4H_2O$, 4 mM] are mixed with 3.195 mL of sulfuric acid (0.6 N) and finally, the volume is adjusted with distilled water to 100 mL. 100 µL of the diluted extract were mixed with 1 mL of the total antioxidant activity solution, previously prepared. The mixture was then placed in a water bath at 95 °C for 90 min. After that, the reading was made at 695 nm. The total antioxidant activity is expressed in mg gallic acid equivalent per gram of dry weight (mg GAE/g DW).

DPPH Assay

The scavenging activity of 2.2-diphenyl-1-picrylhydrazyl (DPPH) free radical of *P. pinaster* Eos was monitored according to method described by Tohidi et al (2017) with some modifications.

Briefly, 1 mL of various concentrations of Eos in methanolic solution ranging from 5-100 µL/mL was added to 0.25 mL of a 0.2 mmol/L DPPH methanolic solution. The reaction mixture was vigorously shaken and left standing at room temperature and in the dark for 30 min., the absorbance was read spectrophotometrically against a control at 517 nm.

The antiradical activity (three replicates per treatment) was expressed as IC50 (µL/mL), the concentration required to cause a 50% DPPH inhibition. The percentage of inhibition activity of DPPH radical was calculated by using the following equation:

$$\textbf{DPPH scavenging effect (\%)} = [(A_C - A_S) / A_C] \times 100$$

A_S: the absorbance of DPPH with Eos

Ac: the absorbance of DPPH without sample (control).

Seed germination and seedling growth experiments

Seeds of weeds (*Sinapis arvensis* L., *Phalaris canariensis* L.) and cereal crop (*Triticum turgidum* L.) were collected from parent plants growing in crop fields (Tunisia_July 2019), then sterilized with 15% sodium hypochlorite during 20 min and rinsed with abundant distilled water.

On petri dishes, seeds were putted on double-layered Whatman N°1filter paper contained different concentrations (0, 1, 2 and 3 µL/ mL) of Eos dissolved in 1% tween 20 solution (Tworkoski 2009).Then every cultures was incubated under controlled conditions (25° C, 70% of relative humidity and a 16/8 photoperiod of 1500 lux light). In addition, every Pétri

dishes were closed and sealed with adhesive tape. The assays were treated in triplicates, the number of germinated seeds was daily counted, and seedling lengths were measured.

Antifungal activity

Fusarium culmorum, Fusarium graminaruim, Fusarium avenaceum, Bipolaris sorokiniana and Botrytis cinerea were the tested species of pathogenic fungi from the culture collection of the Tunisian National Institute of Agronomic Research.

For each culture of the fungi species, a potato dextrose agar PDA was used and stored at 4 °C and in 1 ml of glycerol 25% at -20 °C. Each Eos samples was at first step dissolved in 1 ml of Tween 20 (0.1% v/v) and in second step added into 20 ml PDA at 50 °C. A 5 mm in diameter of mycelial disk, extracted from the periphery of a 6-day-old culture, was inoculated of each PDA plate (in the center) and finally incubated in the dark at 24°C for 7 days. Each tests were treated in triplicates, the PDA plates treated with tween 20 (0.1%) were considered as a negative control. A formula was used to calculate the growth inhibition considered as the percentage of inhibition of radials growth to the control.

The formula: % growth inhibition= [C-T]/C*100

C: the average of three replicates of hyphal extension (mm) of controls

T: the average of three replicates of hyphal extension (mm) of plates treated with Eos (Cakir et al. 2005).

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using the SPSS 23 software package. Differences between means were tested through Student– Newman–Keuls and values of $P \leq 0.05$ were considered significantly different.

Results and discussion

Essential oils composition

Chemical composition of needles, branches and cones Eos are shown in Table 1. GC–MS analysis of *P. pinaster* Eos showed 27, 15 and 25 different components in needles, branches and cones, respectively, which correspond to 93.73, 94.62, and 95.99% of the total identified oil. The Eos were mixtures of five chemical classes including monoterpene hydrocarbons (32.57-90.48%), oxygenated monoterpene (0-5.01%), sesquiterpene hydrocarbons (4.04-37.49%) oxygenated sesquiterpenes (0-4.79%) and diterpenes hydrocarbons (0-16.13%).

The major components in needles oils were α-pinene (25.11%), β-caryophyllene (21.34%), abietadiene (5.93%) and germacrene D (5.9%).

The oil composition obtained from the different aerials part showed a clear diversity. Quantitively, pinene isomers was overwhelmingly presented in branches Eos (84.85%). On the other hand, needles and cones Eos were qualitatively richer composition as compared to branches samples. These results agree with the literature, in particular with the studies of Macchioni et al (2003) who reported α-pinene as the main compound in the different parts of *P. pinaster* essential oils growing in central Italy (Macchioni et al. 2003).

As for needles Eos, similar result was reported by Amri et al (2013) showing that α-pinene, germacrene D, and α-caryophylene were the major oil components (Amri et al. 2013).

However, Hmamouchi et al (2001) reported germacrene D (62.5%) as the main compound of *P. pinaster* needles grown in Morocco, which is slightly different to our finding. this difference can be attributed to numerous factors including the soil conditions and ecological climatic conditions, age of plant and the season of harvest. On the other hand, physiological variations (organ development, type of plant material and Type of secretory structure) can explained particularly the observed difference of obtained from diverse plant part.

Nevertheless, the comparison of the Eos composition described in different studies is sometimes difficult, because not only different methodologies of isolation and analysis may have been used, but also when different plant parts and various developmental stages are involved (Figueiredo et al. 2008; Saoud et al. 2013; Aljaiyash et al., 2022; Mohand et al., 2022).

Table 1. Chemical composition of needles, branches and cones essential oils from *Pinus pinaster*

N°	Compounds	I.R.	*P. pinaster*		
			Needles	Branches	Cones
1	Tricyclene	925	-	0.19	0.1
2	α-Pinene	941	25.11	80.95	29.1
3	Camphene	955	1.02	1.58	1.2
4	Sabinene	978	1.84	-	-
5	β-Pinene	981	1.86	3.9	33.12
6	β-Myrcene	993	0.67	3.17	4.9

7	α-Phellandrene	1006	-	0.26	-
8	Δ-3-Carene	1013	0.37	-	-
9	Limonene	1032	1.33	0.43	3.08
10	β-Phellandrene	1033	0.37	-	0.85
11	Terpinolene	1089	0.4	-	0.51
12	α-Campholenal	1128	-	-	0.31
13	(*Z*)-Pinocarveol	1141	0.37	-	1.35
14	(*Z*)-Verbenol	1143	0.38	-	-
15	Pinocarvone	1164	-	-	0.38
16	α-Terpineol	1190	-	-	1.63
17	Verbenone	1206	-	-	0.43
18	(*Z*)-Carveol	1219	-	-	0.1
19	Carvone	1244	-	-	0.1
20	isobornyl acetate	1286	1.6	-	0.2
21	Longipinene	1352	2.8	-	2.5
22	Longifolene	1404	1.56	2.26	13.9
23	β-Caryophyllene	1420	21.34	0.63	0.28
24	β-Farnasene	1453	-	0.18	0.49
25	α-Humulene	1459	3.11	0.34	-
26	Germacrene-D	1484	5.9	0.2	-
27	α-Muurolene	1499	1.32	0.16	-
28	trans-γ-Cadinene	1514	-	0.18	-
29	δ-Cadinene	1524	1.46	0.19	-

30	Longicamphenylone	1561	-	-	0.47
31	Caryophyllene oxide	1583	2.46	-	-
32	Longiborneol	1586	-	-	0.37
33	Guaiol	1597	1.6	-	0.3
34	Humulene oxide	1608	0.38	-	-
35	α-Eudesmol	1654	0.35	-	-
36	Pimaradiene	1892	-	-	0.16
37	Abietatriene	2055	5.37	-	-
38	Patulane	2069	2.73	-	-
39	Phenanthrene	2074	2.1	-	-
40	Abietadiene	2082	5.93	-	0.16
Total identification %			93.73	94.62	95.99
Hydrocarbonated monoterpenes %			32.57	90.48	72.35
Oxygenated monoterpenes %			2.75	-	5.01
Hydrocarbonated sesquiterpenes%			37.49	4.14	17.17
Oxygenated sesquiterpenes%			4.79	-	1.14
Hydrocarbonated diterpenes%			16.13	-	0.32

-: not detected

Total polyphenol content

The total polyphenol contents, varied significantly ($P \leq 0.05$) between the studied parts. Branches Eos was the richest on polyphenol (67.22 mg GAE/g DW) followed by cones with 42.39 mg GAE/g DW and the lowest polyphenol content was detected in needles 20.77 mg GAE/g DW.

Total antioxidant activity

The total antioxidant assay based on the reduction of molybdenum, it just a quantitative estimation method of total antioxidant capacity of the *P. pinaster* Eos. Total antioxidant capacity was determined as acid Gallic equivalents in milligrams per gram of dry weight (mg GAE/g DW). The phenolic compounds are well known by their antioxidant activity, indeed results showed that branches and cones have the highest total polyphenol,and the highest total antioxidant capacity (283.166 and 274.83 mg GAE/g DW, respectively). However, needles showed the lowest activity 89.64 mg GAE/g DW.

DPPH scavenging activity

Essential oils extracted from branches, needles and cones of *P. pinaster* were tested for their radical scavenging ability, due to the importance of this activity in the prevention of diseases caused by antioxidants and free radicals. The DPPH test is widely used. As noted from table 2, branches showed the highest antioxidant activity with IC50 = 30.33 µL/mL, followed by cones (IC50 = 75.25 µL/mL). The lowest activity was detected in needles (IC50 = 95.40 µL/mL). However, Tümen et al. (2018) studied cones Eos of Turkish origin revealed more strength in DPPH, ABTS reducing capacity and OH-radical inhibition assays than branches and needles. Far in the literature, a number of researchers reported strong antioxidant activity of essentials oils from pine. Bouyahya et al. (2019) reported that *P. halepensis* volatile oils extracted from leaves contain bioactive compounds that could have potential application against oxidative stress related diseases.

In addition, Ulukanli et al. (2014) revealed notable antioxidant potential of Eos extracted from resins of *Pinus brutia* and *Pinus pinea* growing in Turkey.

Considering our findings, we have observed that there is a link between total phenol amount and antioxidant effect, which is in agreement with the study of Meullemiestre et al. (2014) on essential oil of *P. pinaster* indicating a clear correlation between TPC and DPPH assays confirming that the total phenols highly contributes to the antioxidant activity. Antioxidant activities of Eos from medicinal plants are mainly attributed to the active compounds. It is very difficult to attribute the antioxidant effect of these Eos to one or a few active principles, because Eos always contains a mixture of different chemical compounds. In addition to the major compounds, also minor compounds may make a significant contribution to the oil activity (Bouajaj et al., 2014; Bentoura et al., 2021; Khammassi et al., 2022).

Herbicidal activity

Essentials oils extracted from different parts of *P. pinaster* were assessed on the germination and the seedling growth of *T. durum* and two weeds *S. arvensis, P. caraniensis* that reduce crops.

Germination and seedling growth were strongly inhibited under essential oil treatment in a rate-dependent way, and all weeds tested displayed different degree of sensitivity toward Eos. At the dose of 2µL/mL, Eos extracted from cones and needles ensured a completely inhibition of the germination and seedling growth of *S. arvensis* (Table 2).

Table 2. Inhibitory effect of increasing doses of pine species branches, needles, cones essential oils on percentage of seed germination of tested plants.

Herbs species	Doses (µL /mL)	Germination%		
		branches	Needles	Cones
Sinapis arvensis	Control	100±0.00a	100±0.00 c	100±0.00 d
	1	100±0.00a	43.33±5.77 b	53.33±5.77 b
	2	100±0.00a	3.33±5.77 a	23.33±5.77 c
	3	100±0.00a	0±0 a	0±0 a
Phalaris canariensis	Control	86.66±5.77a	86.66±5.77 b	86.66±5.77d
	1	80.00±10.00a	76.66±5.77b	53.33±15.27c
	2	80.00±0.00 a	63.33±11.54a	26.66±11.54b
	3	76.66±11.54a	46.66±15.27a	6.66±5.77 a
Triticum durum	Control	100±0.00a	100±0.00 c	100±0.00 d
	1	100±0.00a	83.33±15.27c	63.33±5.77c
	2	100±0.00a	70.00±10.00b	50.00±0.00 b
	3	100±0.00a	50.00±10.00a	30.00±1.00a

Means in the same column by the same letter are not significantly different for the Student–Newman–Keuls test ($p \leq 0.05$).

While *P. canariensis* appears to be slightly resistant at the same dose to the three tested essential oil. However, a low phytotoxic effect was obtained with branches oil when compared to needles and cones Eos. Needles, cones, and branches oils of *P. pinaster* (concentration tested ranged from 1–3 µL/mL) exhibited significant inhibitory seedling growth against all tested species (Tables 3 and 4).

Table 3. Inhibitory effect of increasing doses of pine species branches, needles, cones essential oils on shoot growth of tested plants.

Herbs species	Doses (µL /mL)	Shoot growth (cm)		
		branches	needles	cones
Sinapis arvensis	Control	12.30±1.47d	12.30±1.47d	12.30±1.47d
	1	8.43±0.66 c	9.23±0.68 c	7.40±0.65 c
	2	5.16±1.04 b	6.10±1.25 b	4.43±0.51 b
	3	2.50±0.55 a	0.00±0.00a	0.00±0.00 a
Phalaris canariensis	Control	13.03±0.89d	13.03±0.89c	13.03±0.89d
	1	9.33±1.04 c	11.83±0.76c	6.86±1.62 c
	2	6.00±1.00 b	6.50±0.50 b	4.30±1.08 b
	3	2.83±0.76 a	1.46±1.28 a	2.13±0.32 a
Triticum durum	Control	15.36±0.72d	15.36±0.72d	15.36±0.72d
	1	8.80±1.47 c	10.16±1.25c	12.53±1.36c
	2	6.40±0.65 b	7.26±1.41 b	7.66±2.08 b
	3	3.50±0.50 a	4.83±0.76 a	3.96±0.55 a

Means in the same column by the same letter are not significantly different for the Student–Newman–Keuls test ($p \leq 0.05$).

Table 4. Effect of increasing doses of pine species branches, needles, cones essential oils on the root growth of tested plants

Herbs species	Doses(µL /mL)	Radical elongation(cm)		
		branches	needles	cones
Sinapis arvensis	Control	13.10±1.34d	13.10±1.34d	13.10±1.34d
	1	8.63±0.77 c	8.20±0.91 c	7.56±1.35 c
	2	4.16±0.90 b	6.33±0.70 b	3.90±00.00 b
	3	1.60±0.45 a	0 ±0 a	0 ±0 a
Phalaris canariensis	Control	13.73±1,11c	13.73±1.11c	13.73±1.11c
	1	8.20±0.65 b	6,83±0.76 b	6.60±0.87 b
	2	6.33±0.57 a	5.16±0.76 b	2.76±0.58 a
	3	4.83±0.76 a	1.501±0.32a	2.03±0.35 a
Triticum durum	Control	12.16±1.60d	12.16±1.60d	12.16±1.60c
	1	7.23±0.68 c	8.16±1.04c	11.16±0.76c
	2	4.50±0.50 b	5.66±0.76 b	5.93±2.44 b
	3	2.63±0.40 a	2.46±0.50 a	2.44±1.76 a

Means in the same column by the same letter are not significantly different for the Student–Newman–Keuls test ($p \leq 0.05$).

Regarding *S. arvensis*, volatile oil extracted from cones is the most potent oil, suppressing shoot elongation by 40% and 64% at 1 and 2 µL/mL respectively. When the concentration of oil was increased to 3 µL/mL, the inhibitory effect was enhanced significantly, reaching 100% compared to the control. Similarly, for root elongation, the suppressive effect was enhanced to 42% by 1 µL/mL oil, and 70% at the dose of 2 µL/mL. However, at 3 µL/mL cones oil almost completely killed the seedlings (100%). On the other hand, according to statistical analysis, phytotoxic effect was greater on weeds than *T. durum* wheat cultivated crop. In fact, germination of *T. durum* is slightly affected at 3 µL/mL dose, recording an inhibition, which is far from total about 50% after cone's Eos application and 70% for the needles.

Obviously, herbicidal activity varied toward dose, tested oils and tested herbs. If we compare the effect of such Eos, it may be concluded that the Eos from cones and needles possessed more effective herbicidal effect than branches Eos.

In addition, *S. arvensis* (dicot weeds) was more sensitive to all tested oils than *P. canariensis* (monocot weeds), while *T. durum* (cultivated crops) was the most resistant.

To the best of our knowledge, the inhibitory effect of cones and branches Eos from *P. pinaster* on seed germination and seedling growth of cultivated plants and weeds was not previously reported. Nevertheless, in agreement with this study, previous studies reported that some of pine species were found to possess herbicidal effects on seed germination of weeds and crops species. Our study is in perfect concordance with previous reports that showed interesting phytotoxic potential of Pine species essential oils.

Tunisians needles Eos extracted from *P. pinea*, *P. nigra*, and *P. halepensis* confirmed ours finding and reported an inhibitory effects against the germination and seedling growth of weeds and cultivated crop (Amri et al. 2012; 2013 a; 2017; 2022).

Whether the observed phytotoxic effects of *P. pinaster* oils are due to sesquiterpenes, monoterpenes, or combined synergistic effects of all the components, the exact essential oil mechanisms on germination and seedling growth inhibition remains unclear. However, the interaction between allelochemicals and the physiological, biochemical processes in target species can explain such inhibitory effects (Ben Ghnaya et al., 2016).

In reference to Table 1, the observed germination and growth inhibition effects could be attributed to the presence of various chemical compounds. In fact, in tested oils, α-pinene was found to be the major monoterpene (25.11-80.95%), which is described as an allopathic compound of a number of plant species, it disturbs energy metabolism by acting on the decoupling of oxidative phosphorylation and by inhibiting the electron transport chain (Abrahim et al. 2003). However, it is widely known that monoterpenes in Eos, through their inhibitory potential, produce the inhibition of cell proliferation in the roots of apical meristems (Singh et al. 2006).

In addition, other major and minor components in the Eos of needles, cones and branches can lead to the herbicidal effects, it has demonstrated that β-pinene (1.86-33.12%) in tested oils (Chowhan et al. 2011), limonene (0.43-3.08%) (Fagodia et al. 2017), and caryophyllene (Araniti et al. 2018) exhibited an important phytotoxic activity. Similarly, β-caryophyllene (0.28-21.34% in pine oils) have been reported to have herbicidal effects against *Arabidopsis thaliana* and produced alterations to plant water status, oxidative stress and physical

damages to the photosynthetic machinery, indeed, the rosettes of plants treated were characterized by a corkscrew shape indicating micro-tubular alterations (Araniti et al. 2018) .

In fact, De Feo et al (2002), have tested the phytotoxic effects of 10 compounds from *Ruta graveolens* Eos; they demonstrated that α-pinene significantly inhibits the germination and root growth of radish and they indicated that α-pinene was the most potent inhibitor of germination and seedling growth (De Feo et al. 2002) .

In another study on the herbicidal effects of 27 monoterpenes such as α-pinene and its isomer β-pinene against germination and seedling growth of *Lepidium sativum* and *Raphanus sativus*, results obtained from this study revealed significant phytotoxic properties of these two compounds (Martino et al. 2010).

According to Singh et al (2006), α-pinene not only inhibited the germination and seedling growth of *Cassia occidentalis* but also the exposure of seedlings to α-pinene provoked solute leakage, and increased levels of malondialdehyde, proline and hydrogen peroxide, indicating lipid peroxidation and induction of oxidative stress (Meullemiestre et al. 2014). In addition, in the same report, activities of the antioxidant enzymes like superoxide dismutase, catalase, guaiacol peroxidase, ascorbate reductase and glutathione reductase were significantly elevated,there by indicating the enhanced generation of reactive oxygen species upon α-pinene exposure(Singh et al. 2006) . Pinenes were also reported to reduce chlorophyll content in Oryza sativa seedlings, cell respiration, enzymatic activity of proteases, α and β-amylases, peroxidases and polyphenol oxidases activities increased in a dose-dependent way as a defense mechanism (Chowhan et al. 2011) .

Actually, there are many successful examples for the use of Eos or their constituents as commercial herbicides. For example, clove oil is used as the major component in the commercial herbicide Burnout II, the herbicide cinmethylin is the derivative of 1,4-cineole, which is a oxygenated monoterpene that can be found in the volatile oils of many plant species (Qaderi et al. 2003). The potent inhibitory activity of Eos obtained from different aerial parts of *P. pinaster*, indicated their potential value of being further explored as environment friendly herbicide.

Antifungal activity

Essential oils isolated from different parts of *P. pinaster* have been tested for their antifungal activity against five phytopathogenic fungi, which mainly attack cereals and cause dramatic

effects. In fact, volatile oils obtained from needles, cones and branches significantly reduced the growth of all the tested fungi (Table 5).

Based on the statistical analysis, a clear difference was observed in the growth inhibition among the studied fungal strains. Based on the statistical analysis, *Fusarium* species especially *F graminarium* strains appears more sensible to Eos rather than *B. sorokiniana* and *B. cinerea*. Indeed, Eos from cones ensure an inhibition up to 40% of the tested fungal species. While, the lowest growth inhibition percent of all the studied fungal species was obtained with branches Eos. Particularly, there is no effect enregestrited for *B. sorokiniana.*

In fact, Pine genus are reported for their antifungal potential, EOs isolated from Tunisian needles of *P. pinea, P. halepensis, P. pinaster, and P. nigra* have been reported to possess interesting antifungal effect which confirm our finding (Amri et al., 2012;2013a;2013b; 2017) . Other researchers have reported a great antifungal potential of ethanolic extract obtained from the *P. pinaster* bark *against Trametes versicolor* (Özgenç et al. 2017).

Actually, the observed differences in the susceptibility of the studied fungal strains to Eos could be related to the chemical Composition mentioned above, the relative high content of α-pinene, β-pinene and β-caryophylene could be responsible for the observed antifungal activity (Fikri et al., 2020).

In this respect, Ghanmi et al (2007) revealed a powerful antifungal activity of the turpentine characterized by a high percentage of α-pinene as main compound versus *Penicillium parasiticus* and *Aspergillus niger* (Ghanmi et al. 2007). Additionally, previous studies indicated that the presence of hydrocarbons monoterpenes in agar medium generated a growth inhibition of *F. oxysporum* (Singh et al. 2002; Tzanakaki et al. 2011).

Several authors have demonstrated the antifungal properties of these compounds; Chang et al (2008) reported the fungicidal activity of β-caryophyllene, α-pinene and β-pinene against *Colletotrichum gloeosporioides* and *Fusarium solani*, the highest fungiotoxic effect was correlated to *Z*-caryophyllene than α- and β-pinene (Chang et al. 2008),Which is in great concordance with our finding.

Furthermore, Piper et al (2001) reported that these compounds are capable to rise fungal cell permeability and membrane fluidity causing leakage (Piper et al. 2001). Different research reported these modifications in permeability and increases in membrane fluidity after treatment with terpenes (Bard et al. 1988; Hammer et al. 2004; Metoui et al., 2015). Moreover, they insert between fatty acyl chains that make up membrane lipid bilayers, disrupting lipid packaging and causing changes in membrane properties and functions. These

changes include interaction with membrane enzymes and proteins, such as H+/ATPase membrane pumping which induces a flow of protons out of the cell causing changes in the cells (Cristani et al. 2007 ; Tatsadjieu et al. 2009).

Table 5: Inhibitory effect of pine species cones branches and needles essential oils on the growth of five phytopathogenic fungi

Fungi strains	*Pinus pinaster*						
	Control	cones		branches		needles	
	Growth (cm)	Growth (cm)	inhibition %	Growth (cm)	inhibition %	Growth (cm)	inhibition %
Bipolaris sorokiniana	8.50±0.0	4.90±0.14	42.35±1.66a	8.50±0.00	0.00±0.00a	6.10±0.14	28.23±1.66a
Botrytis cinerea	8.50±0.0	5.10±0.14	40.00±1.66a	7.70±0.42	9.41±4.99ab	6.60±0.14	22.35±1.66a
Fusarium. graminarium	5.10±0.28	2.85±0.21	44.14±1.06a	2.35±0.21	53.73±6.72b	1.25±0.35	75.25±8.30b
Fusarium avenaceum	3.60±0.56	2.10±0.14	40.62±1.25a	2.80±0.28	20.62±0.32ab	1.10±0.14	68.75±8.83b
Fusarium culmorum	5.85±0.63	3.25±0.07	44.17±4.86a	5.25±0.35	9.39±1.90ab	4.15±0.21	28.83±4.11a

Means in the same column by the same letter are not significantly different for the Student–Newman–Keuls test ($P \leq 0.05$)

Conclusion

Essential oils obtained by hydrodistillation of *P. pinaster* showed a diversity in chemical composition between the three different aerial part (needles, cones and branches). According to our results, all analyzed oils were rich in hydrocarbonated monoterpenes: α-pinene, β-pinene and β-caryophyllene were the dominant compounds in the volatile oils. However, each Eos distinguished relatively by the variability in minor component which can be related to the observed difference in biological activities. Antioxidant, herbicidal and antifungal activities were depending on the origin of the extracted oils and the tested species. Although differing in efficacy, demonstrated their capability to be useful and valuable leads for sustainable agriculture programs

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