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Antioxidants and chlorophyll in *Pinus pinaster* Arn. needles

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Abstract
The aim of this study is to determine antioxidant substances, chlorophyll (A and B), carotenoids and the antioxidant activity of ethanolic extracts of needles from two *Pinus pinaster* varieties (var. Maghrebiana (Morocco) and var. Renoui (Tunisia)) growing in the Northwest of Tunisia. The total phenolic content was determined using Folin–Ciocalteu reagent. The total flavonoid was assessed using 2% aluminum chloride and condensed Tanins was determined with the vanillin. The antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. Significant differences were found between the two varieties ($p<0.0001$).Var. Maghrebiana presented the highest polyphenol content (28.87 mg GAE/g), tannin (28 mg CE/g), Chlorophyll A (10.88 mg/L), chlorophyll B (1.06 mg/L) and carotenoids (1.22 mg/L).The most important antioxidant potential was reached by Maghrebiana variety (% inhibition DPPH= 88.53%; IC_{50} = 11.25 µg/mL). Renoui variety presented a lowest antioxidant activity (% inhibition DPPH = 84.97%; IC_{50} = 16.50 µg/mL).

Keywords: *Pinus pinaster*, polyphenols, flavonoids, tannin, chlorophyll, antioxidant activity

1. Introduction
Polyphenols are phytochemicals, playing an important role in maintaining health and wellness. For this purpose, the addition of polyphenols to foods has great interest due to their well-known abilities to scavenge free radicals. The generation of free radicals plays an important role in the progression of a great number of pathological disturbances, such as atherosclerosis [1], brain dysfunction [2] and cancer [3, 4] and also has great effects on inflammatory diseases. Plants are considered as the most important source of natural antioxidants. Polyphenols are found in many woods plants such as Pine, especially *Pinus sylvestris* L. and *Pinus pinaster* [5]. *Pinus pinaster* (maritime pine) is a pine native in the Mediterranean region. It is found in some countries such as France, Portugal, Spain, Italy, and in some North Western African countries such as Algeria, Morocco and Tunisia [6, 7]. Many studies have showed that these species is characterized by their Pharmaceutical and nutraceutical properties [8, 9].
The purpose of this study is to evaluate, for the first time, the total polyphenols, tannins, flavonoids, chlorophyll A, chlorophyll B, carotenoids and antioxidant activity of two North African varieties of *P. pinaster* growing in Tunisia.

2. Materials and Methods
2.1 Plant Materiel
Needles of two varieties of *Pinus pinaster* (Var. Renoui from Tunisia and Var. Maghrebiana from Morocco) were collected in April 2015 from Souiniet arboretum in West-Northern Tunisia (8 ° 48 'E, 35 ° 54' N, 492 m). It is characterized by a cold and humid Mediterranean bioclimate. The average annual precipitation is approximately 1140 mm/yr. The mean annual temperature is 15.6°C.

2.2 Extract preparation and extraction yield
20 g of dried powder of *Pinus pinaster* needles was weighted and soaked in 200 ml of solvent ethanol for 24 hours. The plant extracts were filtered and the filtrates were dried until a constant dry weight of each extract was obtained. The residues were suspended in ethanol and used for the experiments.
The results of extraction yield were expressed as the percentage (%) of grams of extract per gram of dry needles.

2.3 Determination of total phenolic compounds
The total phenols content was determined by Folin Ciocalteu method [10]. 2.5 ml of Folin-Ciocalteu reagent (1:10 diluted with distilled water) was added to 2 ml of 7.5% sodium carbonate and 0.5 ml of needles extracts. The mixtures were allowed to stand for 30 min and the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 0.03, 0.06, 0.12, 0.25 and 0.5 g/L solutions of gallic acid. The content of phenolics was expressed in mg of gallic acid per g of dry weight of needles (mg GAE/g).

2.4 Determination of flavonoids content
The flavonoids content in extracts was determined according to Lamaison and Carnat [11], using a method based on the maximum absorbtivity at 430 of a complex flavonoid–aluminium. 1 ml of diluted sample was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm. Rutin was used to make the calibration curve. The flavonoids content was expressed in mg per g of rutin per g of dry weight of needles (mg RE/g Ext).

2.5 Determination of condensed tannins
Condensed tannin content was determined with the method described by [12]. 3 ml of vanillin (4% in methanol) was added to 0.5 ml of the aqueous extract and 1.5 ml of HCl. The mixture was then kept in the dark for 15 min at 20°C. The

absorbance was read at 500 nm. A calibration curve was prepared with a solution of catechin. The results were obtained in mg of catechin equivalent per g of dry weight of needles (mg CE/g Ext).

2.6 Determination of chlorophyll and carotenoids

Total pigments were extracted from needles of *P. pinaster* by grinding in 80% acetone. Then absorbance reading was taken using a spectrophotometer at wavelengths: 750, 664, 646 and 453 nm and the results were expressed in mg/L, calculated by the formula [13, 14]:

Chlorophyll a (mg/L) = 11.78*(A664-A750)-2.29*(A646-A750)

Chlorophyll b (mg/L) = 20.05*(A646-A750)-4.77*(A664-A750)

Carotenoid (mg/L) = 4.76*(A653-A750)-0.226*(chl a-chl b)

2.7 Free radical scavenging activity

5 ml of DPPH solution (0.004%, in ethanol) was incubated with varying concentrations of the extracts (3–85 g/L). The reaction mixture was mixed and incubated for 30 min at room temperature and the absorbance of the resulting solution was read at 517 nm against a blank [15]. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$Scavenging\ effect\ (\%) = \left[\frac{1 - A_{Sample\ (517\ nm)}}{A_{Control\ (517\ nm)}}\right] x\ 100$$

IC_{50} values were determined.

2.8 Statistical analysis

The statistical significance was evaluated with the SAS (9.0) program (General Linear Models procedure). P values less than 0.05 were considered statistically significant. All values are the mean of three replications. Correlations were performed by SPSS.20 program.

3. Results

Highest yield was reached by needles from Maghrebiana variety (8.45%). Renoui variety exhibited a lowest yield of 5.77%.

The total polyphenols contents of *P. pinaster* needles measured according the Folin-Ciocalteu method varied significantly between the two studied varieties. The highest amount was found in ethanolic extract of Var. Maghrebiana (28.87 mg GAE/g) while the lowest was in ethanolic extract of Var. Renoui (12.82 mg GAE/g of extract).

Var. Maghrebiana showed also the most important amount in tannin contents (28 mg CAT/g Ext) (Table1), Chlorophyll A (10.88 mg/L), chlorophyll B (1.06 mg/L) and carotenoids (1.22 mg/L) (Table 2).

No significant difference was registered for the flavonoids content between the two studied varieties.

The best antioxidant potential was shown by Maghrebiana variety (88.53% DPPH inhibition) with the lowest IC_{50} value (11.25µg/mL), while the lowest antioxidant power (84.97% DPPH inhibition) corresponding to the highest IC_{50} value (16.50 µg/mL), was reached by Renoui variety. These IC_{50} values are close to the IC_{50} value of BHT (15.50 µg/mL) which was used as a positive control (Table 3).

A positive correlation was observed between the antioxidant activity and the total polyphenols, tannin, flavonoids, chlorophyll (A and B) and carotenoids content ($r > 0.9$).

4. Discussion

Pine is known as one of the richest plant species in phenols. *Pinus pinaster* needle extract showed an important amount of phenols, tannin, chlorophyll and carotenoids. *P. pinaster* needles exhibited high phenols content when compared with other pine species. Kähkönen [16] demonstrated that needles of *Pinus sylvestris* contained 17.50 mg GAE/g of total phenols. Furthermore, Pasqualini *et al*. [17] showed that *Pinus halepensis* needles contain 23 mg GAE/g of total phenols.

In literature, it is well known that secondary metabolite such as polyphenols and flavonoids play an important role in free radical resistance [18, 19, 20]. Moreover, phenols have been reported to play a preventive role in the case of heart disease and cancer and to improve the antioxidant level in humans' plasma [21, 22, 23].

Pigments such as chlorophylls and carotenoids have been designated to prevent oxidative damage of genetic material and lipid peroxidation [24].

Antioxidant activity, determined using the DPPH test, showed an important antioxidant power of *P. pinaster* needles extracts. These values are comparable to those determined for *Pinus sylvestris* needles (85%) [16].

A positive correlation was registered between the total phenols content and the antioxidant activity of the studied needles extracts.

This positive correlation was confirmed by several other studies [25, 26, 27].

It is also known that phenolic compounds such as flavonoids, phenolic acids and tannins contribute to the antioxidant capacity of several plants [28].

Considering the two tested extracts, significant differences were registered in the rates of secondary metabolite and pigments. These differences could be explained by different geographical origins of the studied varieties. Several studies have explained the interspecific and intraspecific variability's of the phenolic compounds, which may be related to the different geographical sources and/or to the genotype [29, 30]. Aloui [31] proved that the Moroccan variety Maghrebiana growing in North west Tunisia, which was found the richest in phenolic compounds and pigments in this study, is well adapted to soil and climatic conditions when compared to the Tunisian variety Renoui. This result suggests the presence of a genetic diversity between the two varieties.

5. Tables and Figures

Table 1: Yield, polyphenols, flavonoids and tannins content in extracts from *Pinus pinaster* needles

	Unit	Maghrebiana	Renoui
Yield	%	8.45^{a}± 0.5	5.77^{b}±0.8
Polyphenols	mg GAE/g	28.87^{a}±0.6	12.82^{b}±2.5
Flavonoids	mg RE/g	4.86^{a} ±0.5	4.27^{a}±0.9
Tanins	mg CE/g	28^{a}±5.9	16.02^{b}±0.7

Values with the same letter are not statistically different ($p<0.5$)

Table 2: Average levels of chlorophyll and carotenoids of extracts from *Pinus pinaster* needles

	Unit	Maghrebiana	Renoui
Chlorophyll A	mg/L	10.88^{a}±0.04	7.99^{b}±0.02
Chlorophyll B	mg/L	1.06^{a}±0.1	0.15^{b}±0.1
Carotenoids	mg/L	1.22^{a}±0.01	1.07^{b}±0.04

Values with the same letter are not statistically different ($p<0.5$)

Table 3: Antioxidant activity of extracts from *Pinus pinaster* needles

	Unit	Maghrebiana	Renoui	BHT
DPPH inhibition	%	88.53^{a}±1.05	84.97^{b}±1.04	
IC_{50}	µg/mL	11.25^{a}±2.4	16.5^{b}±0.5	15.5

Values with the same letter are not statistically different ($p<0.5$)

6. Conclusions

In conclusion, Maghrebiana Var. showed the highest total polyphenol and pigments content. This high amount of total phenolic compounds in its needles needs to be supported by studying their biological properties and their nutritional value. More studies are recommended to identify the phenolic profiles and to characterize the chemical composition of needles. Such studies could contribute to the valorization of this plant as a source of natural antioxidants.

7. References

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