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Variability of phenolic compounds and antioxidant efficacy in needles extracts of *Pinus nigra* Arn

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Abstract – The phenolic compounds and antioxidant capacity of ethanol extracts, obtained from nineteen *Pinus nigra* provenances growing in Northwestern Tunisia, were invetigated. Phenols (Folin–Ciocalteu method), flavonoids (method based on the formation of a complex flavonoid–aluminium) and tannin (vanillin method) content were evaluated. The antioxidant capacity was determined using both phosphomolybdenum method and free radical scavenging activity (DPPH test). Total phenols ranged from 15.67 to 47.53 mg GAE/g of dry matter. The amount of flavonoids varies from 1.69 to 3.97 mg RE /g. Extracts showed an important antioxidant capacity ranging from 242.50 to 1206.87 GAE en mg/g for the total activity and from 36.08 to 99.05% for the free radical scavenging activity.

Keywords: *Pinus nigra* Arnold, variability, subspecies, phenolic compounds, antioxidant capacity

1. Introduction

Plants are a source of numerous free radical scavenging molecules and have high concentrations of various natural antioxidants, such as polyphenols, carotenoids and tocopherols which have a high antioxidant potential (Kahkonen et al. 1999; Zheng and Wang 2001; Kasinulainen and Holopainen 2002). It is knowing that natural antioxidants have been associated with the health benefits (Block et al. 1992; Parr and Bolwell 2000). Moreover, these compounds have been reported to have an essential role in plants defense, against herbivory and pathogens (Bravo 1998; Winks and Schimmer 1999; Blodgett et al. 2007).

Within conifers, Pine species are considered as a natural source of antioxidant compounds (Zulaica-Villagomez et al. 2005; Guri et al. 2006; Cretu et al. 2013). Almost pines were reported to have a high antioxidant capacity (*Pinus halepensis*, *Pinus pinea*, *Pinus sylvestris*, *Pinus nigra*…) (Laracine-Pittet and Lebreton 1988; Robles et al 2003; Yesil-Celiktas et al. 2009).

Pinus nigra Arnold is one of the most known medicinal plants in the Mediterranean countries (Tuzlaci and Erol 1999). This plant is known for its use in traditional medicine for respiratory diseases and for protection against both endoparasites and ectoparasites (Yeilada et al. 1995; Arı 2014; Grieve 1984; Fakir 2009; Gülçin 2003).

This species is discontinuously distributed around the northern Mediterranean from Southwest Europe to Asia Minor, extending to the Crimea and is also found in North Africa (Morocco and Algeria). The species is divided into six subspecies: *P. nigra* subsp. nigra (Host); *P. nigra* subsp. salzmannii (Dun.) *P. nigra* subsp. dalmatica (Vis.); *P. nigra* subsp. pallasiana (Lamb.); *P. nigra* subsp. laricio (Poir.) and *P.nigra* ssp mauretanica (Quézel and Médail 2003). This collective species is characterized by a high genetic, biochemical, phenotypic and morphological diversity (Arbez et al. 1974).

The aim of this study was to compare, for the first time, the total phenols, flavonoids, tannin content and the antioxidant capacity of needles collected from nineteen samples corresponding to different provenances from different regions which have been planted and grown in North West of Tunisia.

2. Materials and methods

2.1. Plant materiel

Needles of nineteen samples of *Pinus nigra* were collected in April 2015 from Souiniet arboretum in West-Northern Tunisia (8°48'E, 35°54'N, 492m). The nineteen samples correspond to different

provenances from different regions which have been planted and grown in North West of Tunisia (Table 1) according to a comparative plantation assay since 1966.

Table 1. Characterization of the nineteen samples of *Pinus nigra*

Sub-species	Provenances	Country	Code
Pinus nigra salzmanni	Brougatles Alès	France	Broug
Pinus nigra laricio var. calabrica	Trenta	Italie	Tent
Pinus nigra laricio var calabrica	Les Barres	France	Barr1
Pinus nigra laricio var. calabrica	Cosenza	Italie	Cos
Pinus nigra laricio var. corsicana	Bois frerot (Ardennes)	France	Bfr
Pinus nigra nigra var. austriaca	Puget-Théniers	France	Pug
Pinus nigra nigra var. nigricans	Kustendil	Bulgarie	Kus
Pinus nigra pallasiana	Alaçam	Turquie	Ala
Pinus nigra laricio var. calabrica	Cantanzaro	Italie	Cant
Pinus nigra laricio var. corsicana	les Barres (leint)	France	Barrleint
Pinus nigra salzmanni	St Guilhem (Herault)	France	Guil
Pinus nigra pallasiana	Crimée	Russie	Crim
Pinus nigra laricio var. calabrica	Grancia	Italie	Gran
Pinus nigra laricio var. calabrica	Aspromonto	Italie	Aspr
Pinus nigra salzmanni	Cazorla	Espagne	Caz
Pinus nigra laricio var. calabrica	Tavola	Italie	Tav
Pinus nigra salzmanni	Olette (Pyr-Orient)	France	Olet
Pinus nigra laricio var. calabrica	les Barres	France	Barr2
Pinus nigra laricio var. corsicana	Marghese	Corse	Marg

2.2. Extract preparation

Needles of *Pinus nigra* were dried and then ground into a powder. 20 g of needle powder were mixed with 200 ml of solvent ethanol for 24 hours with continuous shaking. The filtrated plant extracts were dried to evaporation of all solvent. The residues were suspended in ethanol and used for the experiments. Needles extracts were conserved in obscurity at 4°C.

2.3. Extraction yield

Extraction yield was expressed as the percentage (%) of grams of extract per gram of dry needles.

2.4. Determination of total phenolic compounds

The total phenolic content of the extract was determined by the Folin–Ciocalteu method (Singleton and Rossi, 1965). 0.5 ml of needle extracts were mixed with 2.5 mL of Folin–Ciocalteu reagent (1:10) for 3 min, followed by the addition of 2 mL of sodium carbonate (7.5%). The mixture was allowed to stand for a further 30 min in the dark, and absorbance was measured at 765 nm. The total phenolic content was calculated from the calibration curve using 0, 0.03, 0.06, 0.12, 0.25 and 0.5 g/L solutions of gallic acid in water, and the results were expressed as mg of gallic acid equivalent per g dry weight (mg GAE/g).

2.5. Determination of flavonoids content

The total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method (Quettier Deleu et al. 2000). 1 ml of diluted sample was mixed with 1 ml of 2% aluminum chloride methanolic solution. The mixture was allowed to stand for 15 min, and absorbance was measured at 430 nm.

The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per g dry weight (mg RE/g Ext).

2.6. Determination of condensed tannins

The method described by Broadhurst and Jones (1978) was used to determine the total condensed tannin content in needle extracts. 0.5 ml of the extract was mixed with 3 ml of vanillin (4% in methanol) and 1.5 ml of Hydrochloric acid. After incubation for 15 min at 20◦C in the dark, the absorbance was read at 500 nm. The condensed tannin content was calculated from a calibration curve prepared with a solution of catechin (30 ppm). The results were expressed in mg of catechin equivalent per g of dry weight of needles (mg CE/g).

2.7. Total antioxidant activity

The total antioxidant activity was evaluated according to the method described by Prieto et al. (1999). 0.5 mL of extracts was combined with 4.5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). 0.5 mL of ethanol was used in case of blank. The tubes were placed in a boiling water bath at 95°C for 90 min. The samples were than cooled, and the absorbance was measured at 695 nm against blank. The antioxidant activity was expressed as equivalents of Gallic Acid (mg/g of dry matter).

2.8. Free radical scavenging activity

5 µl of the extracts was mixed with 5 ml of DPPH solution (0.004%, in ethanol). The reaction mixture was incubated for 30 min at room temperature and the absorbance was read at 517 nm against a blank (Brand-Williams et al. 1995).
The radical scavenging activity was calculated using the following formula:

$$\text{scavenging effect (\%)} = \left[1 - \frac{\text{DO sample}}{\text{DO control}}\right] \times 100$$

2.9. Statistical analysis

The statistical analysis were done with the GLM procedure (General Linear Models) of the SAS (9.0) program. Correlations were performed by SPSS.20 program. Principal component analysis was evaluated with R (version 3.1.1) program.

3. Results

3.1. Extraction yield

Yields of extracts from needles of *P. nigra* are shown in table 2. Highest yield was reached by needles of Broug samples (31.29%). Bfr, Ala and Olet samples exhibited the lowest yield with 5.3%, 5.66% and 5.58%, respectively.

Table 2. Yeilds extracts and total phenol, tannins and flavonoids content in needles of P. Nigra (Values with the same letter are not statistically different (p<0.5))

Sample	Yield (%)	Phenols (mg GAE/g)	Tanins (mg CE/g)	Flavonoids (mg RE /g)
Broug	31.29^{a}±0.1	26.79^{gde}±1.81	22.72^{cb}±1.06	3.26^{bdac}±0.32
Tent	6.62^{l}±0.05	17.08^{h}±1.53	14.30^{ed}±0.44	2.19^{edgcf}±0.00
Barr1	14.88^{g}±0.09	22.88^{fghe}±1.31	24.19^{b} ±1.57	2.10^{egf}±0.30
Cos	9.33^{j}±0.04	20.70^{fgh}±1.13	27.73^{a} ±1.30	2.10^{egf}±0.38
Bfr	5.3^{n}±0.01	27.53^{fde}±3.26	36.81^{a} ±0.67	1.69^{g}±0.32
Pug	22.97^{c}±0.2	20.70^{h}±0.31	27.73^{a} ±0.34	2.10^{edgcf}±0.02
Kus	14.67^{g}±0.4	30.38^{cd}±1.46	14.33^{ed}±0.96	3.08^{ebdacf}±0.20
Ala	5.66^{n}±0.1	33.97^{cb}±0.33	17.59^{cd} ±1.13	2.70^{ebdgcf}±0.11
Cant	11.86^{i}±0.02	19.72^{gh}±1.36	24.78^{b} ±3.82	2.10^{egf}±0.10
Barrleint	17.30^{f}±0.5	22.88^{fghe}±2.65	26.36^{b} ±2.29	2.45^{edgcf}±0.11
Guil	13.20^{h}±0.3	22.60^{fghe}±3.66	32.61^{a} ±3.09	3.97^{a}±0.17
Crim	6.28^{m}±0.6	18.84^{h}±1.29	18.52^{cd} ±0.59	3.21^{bdac}±0.03
Gran	8.55^{k}±0.5	23.17^{fghe}±3.57	27.17^{b}±1.12	1.99^{gf}±0.22
Aspr	9.67^{j}±0.1	37.50^{b}±0.92	22.24^{cb} ±0.78	3.76^{a}±0.43
Caz	21.31^{d}±0.09	15.67^{h}±1.95	10.44^{e}±1.64	2.45^{edgcf}±0.23
Tav	25.41^{b}±0.6	21.79^{fghe}±2.93	16.65^{d}±2.07	2.52^{edgcf}±0.28
Olet	5.58^{n}±0.8	28.69^{cde}±2.98	18.39^{cd} ±0.29	3.71^{ba}±0.81
Barr2	13.15^{h}±0.15	38.02^{b}±1.06	15.17^{ed}±0.61	3.34^{bac}±0.36
Marg	18.01^{e}±0.15	47.53^{a}±1.32	24.09^{b} ±0.75	3.55^{ba}±0.07

Statistical analysis showed that samples from *nigra* subspecies presented the most important yields while samples from *pallasiana* subsp. showed the lowest values.

3.2. Phenols, flavonoids and tannins contents

Results showed significant variations in the total phenols, flavonoids and tannin contents in *P. nigra* needles (p<0.0001).

The amount of total phenols ranged from 15.67 to 47.53 mg GAE/g of dry matter (Table 2).
The highest values was reached by needles of Marg sample and the lowest by Pug, Crim, Tent and Caz samples with respective values of 20.7,18.84, 17.08 and 15.67 mg GAE/g of dry matter. It has been demonstrated that total phenol content was higher in *laricio* subsp. than the other subspecies.
The amount of flavonoids varies from 1.69 to 3.97 mg RE/g. The highest values were found in needles of Guil and Aspr samples (3.97 and 3.76 mg RE/g respectively). Needles of Bfr sample showed the lowest flavonoids content with 1.69 mg RE/g. Samples of *salzmanni* subsp. exhibited the most important flavonoids content.
Needles of Bfr, Purg and Guil samples were the richest in tannins. Its total contents were respectively 36.81; 27.73 and 32.61 mg CE/g. Samples belonging to *nigra* and *laricio* subspecies were the richest in tannins.

3.3. Antioxidant activity

Total antioxidant activity of needles extracts ranged from 242.5 to 1206.87 mg GAE/g (Table 3). The highest value was reached by Bfr, Ala and Crim samples with respective values of 1287.5, 1206.87 and 1179.37 mg GAE/g. Caz sample showed the lowest total antioxidant activity.

Table 3. Total antioxidant activity (gae en mg/g) and free radical scavenging activity (% DPPH inhibition) of P. Nigra needles extracts (Values with the same letter are not statistically different ($p<0.5$))

Sample	Total antioxidant activity (GAE en mg/g)	% DPPH inhibition
Broug	741,87 ^{ab}±1,875	91,67^{ab}±2,35
Tent	1116,25 ^{ab}±31,25	82,97^{ab}±3,58
Barr1	743,75 ^{ab}±6,25	88,22^{ab}± 0,97
Cos	1123,75 ^{ab}±1,25	77,12 ^{ab}± 3,63
Bfr	1287,5 ^{a}±40	80,16 ^{ab}±1,08
Pug	459,68 ^{ab}±25,31	73,01 ^{ab}±2,27
Kus	903,75 ^{ab}±15	99,05^{a}±0,03
Ala	1206,87^{a}±15	66,39^{b}±0,96
Cant	631,87 ^{ab}±73,12	79,79 ^{ab}±0,14
Barrleint	838,12 ^{ab}±86,87	91,00 ^{ab}±0,38
Guil	996,25 ^{ab}±0,62	89,53 ^{ab}±0,33
Crim	1179,37^{a}±81	91,12 ^{ab}±2,90
Gran	1028,12 ^{ab}±32,62	85,61 ^{ab}±1,75
Aspr	751,87 ^{ab}±78,125	87,83 ^{ab}±1,02
Caz	242,50 ^{b}±14,375	36,08^{c}±4,93
Tav	733,12 ^{ab}±46,25	78,25 ^{ab}±1,52
Olet	1037,59 ^{ab}±6,25	84,95 ^{ab}±0,22
Barr2	895,62 ^{ab}±15,625	89,86 ^{ab}±0,32
Marg	900,62 ^{ab}±75,125	93,26^{a}±0,11

Using the free radical scavenging method, the highest percentage of inhibition was reached by needles of Kus and Marg samples (99.05 and 93.26%, respectively). Such as total antioxidant activity, Caz sample showed the less important free scavenging activity (36.08%).
Samples belonging to *pallasiana* subspecies showed the highest total antioxidant activity.

3.4. Correlation between antioxidant activity and total phenol, total flavonoids and tannin contents

The relationships between total phenols, flavonoids and tannins content and the antioxidant activity measured in *P. nigra* needles was determined using correlation analysis (Table 4).
There was a significant linear correlation between the antioxidant activity and total phenols, flavonoids and tannins contents in the case of *salazmani*, *pallasiana* and *nigra* subspecies. However, no significant relationship between antioxidant activity and total phenol, flavonoids and tannins was recorded for *laricio* subspecies.

Table 4. Linear correlation of antioxidant activity versus the total phenol, tannin and flavonoids contents in needles of *Pinus nigra.*

subspecies		Correlation coefficients		
		phenols	**tannins**	**flavonoids**
salazmani	Total Antioxidant activity	Y=0.013x+13.61 R² = 0.680	Y=29.10x+142.1 R² = 0.541	Y=537.8x-1045 R² = 0.953
	DPPH inhibition	Y=0,1896x+9,1 R² = 0,756	Y=2,2739x+27,71 R² = 0,6299	Y=34,75x-40,79 R² = 0,7592
pallasiana	Total Antioxidant activity	Y=0,027x- 5,95 R² = 0,9816	Y=0,0025x+14,44 R² = 0,7475	Y=0,0008x+2,07 R² = 0,984
	DPPH inhibition	Y=-1,198x+107,18 R² = 0,9386	Y=0,1188x+8,802 R² = 0,888	Y=0,023x+1,23 R² = 0,9843
laricio	Total Antioxidant activity	Y=1.0x–58.35 R² = 0.381	Y=12.59x+16.5 R² = 0.162	Y=5.306x+1.74 R² = 0.432
	DPPH inhibition	Y=1,0001x-58,353 R² = 0,3812	Y=-0,1476x+88,9 R² = 0,0262	Y=5,306x+71,74 R² = 0,4324
nirga	Total Antioxidant activity	Y=34.659x-145.59 R² = 0.836	Y=-23.51x+12.35 R² = 0.86	Y=0.011x+1.92 R² = 0.851
	DPPH inhibition	Y=1,8922x+39,51 R² = 0,6961	Y=-0,4832x+65,8 R² = 0,6781	Y=0,0209x+0,96 R² = 0,6063

The strongest correlation was registered by *pallasiana* subspecies between antioxidant capacity (total antioxidant activity and DPPH inhibition) and total flavonoids ($R^2 = 0.984$).

3.5. Principal component analysis

The results of principal component analysis showed that total phenol content, total antioxidant activity and radical scavenging activity were the most significant variables for classification of the *P. nigra* extracts. These parameters were considerably loaded into the two major principal components (Dim1 and Dim 2) explaining more than 66% of the variance.

According to the analysis, three different groups were revealed (Figure 1). The first group contained Marg, Aspr and Barr2 samples which had the main concentrations of phenols and the strongest antioxidant activity. The second group contained only Caz sample which showed the lowest amount of phenols and the lowest antioxidant activity. The third group regrouped all the other samples studied.

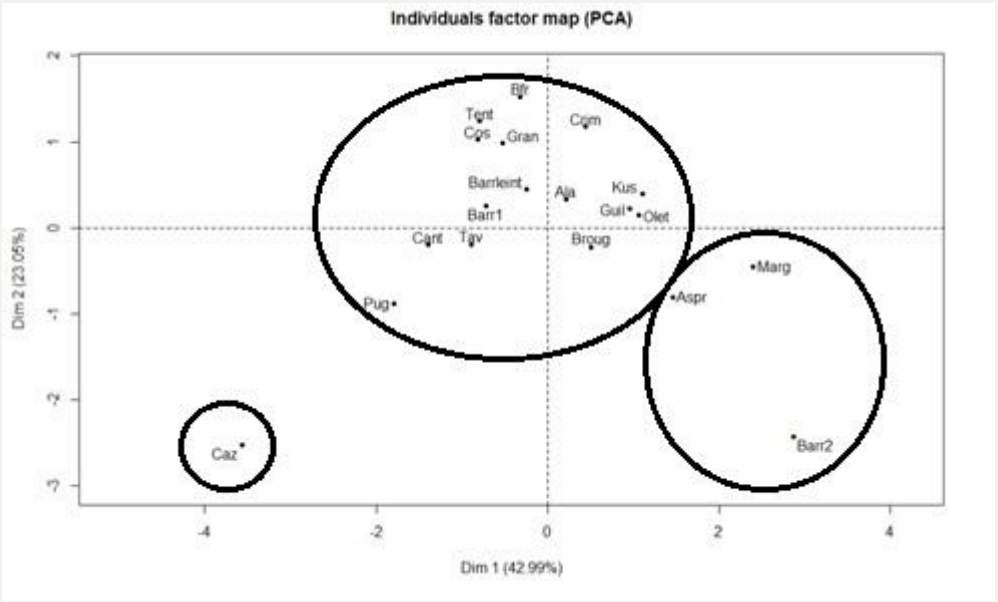

Figure 1. Indivudials factor map obtained from the PCA of data about total phenol, tannins and flavonoids content and antioxidant capacity of *P. Nigra* needles extracts

4. Discussion

Extracts yields of black pine (*P. nigra*) needles of several provenances are more significant than those found in maritime pine (*P. pinaster*) varieties growing in the Souiniet site of Ain Drahem province (Maghrebiana variety (8.45%) and Renoui variety (5.77%)) (Fkiri al. 2018).

The amounts of total phenols in the *Pinus nigra* needles obtained in this study are more important than those found in other species of the same genus such as *Pinus halepensis* (25 mg GAE/g) (Laracine-Pittet and Lebreton, 1988), *Pinus pinaster* (17 mg GAE/g) (Alonso et al. 2002), *Pinus roxburghii* (10 mg GAE/g) and *Pinus wallichiana* (5 mg GAE/g) (Maimoona et al. 2011). Results showed that total phenol content was higher in *laricio* subsp. which is in accordance with levels reported in the literature (Cannac et al. 2007). Levels of total flavonoids were of the same importance as those found in other species, such as *Pinus halepensis* (Laracine-Pittet and Lebreton 1988) and *Pinus sylvestris* Robles et al (2003). In addition, needles of *P. nigra* showed a high amount of tannins when compared with *P. pinaster* needles (Alonso et al. 2002).

The analyses of variance showed that rates of total phenols, flavonoid and tannin contents varied considerably between the nineteen samples studied. In the literature, several factors were reported to explain the variability of these compounds. Numerous studies demonstrated that this diversity is related to the bioclimatic conditions especially rainfall, soil and temperature (Bhavana et al., 2013; Bencsik et al., 2011). Other authors explain it by a genetic variability.

In our case, significant difference found among provenances seem to support the hypothesis of high genetic variability of black pine. The divergence between the nineteen *P. nigra* samples, from different origins and growing under same bioclimatic and substrates conditions cannot result from local environmental factors. Arbez et al. (1974) highlighted a high degree of genotypic variability observed in *P. nigra* subspecies that was reflected in the biochemical variability of this plant (Fratianni et al. 2007).

Moreover, Boscaiu et al. (Boscaiu et al. 2010) showed that there is a positive correlation between the stress caused by environmental factors and the rate of phenolic compounds accumulated in the plants, signifying that these compounds appear to be good markers of stress in a lot of species. According to these results, we can suggest that samples of *P. nigra* which showed the highest amount of phenols and flavonoids were the less adapted to the bioclimatic conditions of the growing site and vice versa.

P. nigra needles exhibited an important antioxidant activity expressed as total antioxidant activity and DPPH free radical scavenging activity. It showed significant antioxidant activity when compared to alpha-tocopherol (Vijayakumar 2012).

The antioxidant capacity of *Pinus nigra* needles is more important than that found in other species such as *P. roxburghii*, *P. wallchiana* and *P. gerardiana* (Sharma et al. 2016). Many studies have revealed that there is an important relationship between antioxidant activity and phenolic and flavonoids compounds in plants (Cai et al. 2004; Shyi-Neng et al. 2014; Sharma et al. 2016). Needles of *P. nigra* showed a high amount of phenols and flavonoids. This can explain its important antioxidant capacity. On the other hand, antioxidant activity exhibited a high variability among the samples studied. This may be explained by the variability observed in antioxidant compounds, especially that a considerable correlation was revealed between the total phenols and the antioxidant capacity in the samples. Needles of *Pinus nigra* could be used as a source of natural antioxidants which is known by their positive effects on human health, due to their antioxidant properties, in particular protection from coronary heart disease and cancer (Catherine et al. 1997; Serafini et al. 1998; Knekt et al. 1996; Thomas 2000). It could be also used as a possible food supplement (Zupko et al. 2001) or in cosmetical and pharmaceutical industry (Lupo 2001).

5. Conclusion

This study showed the great presence of antioxidant compounds and confirmed high level of antioxidant activities in *Pinus nigra* needles. Significant differences were revealed between subspecies and provenances. Based on the results of our study, *P. nigra* needles are a potent source of natural antioxidants applied in pharmaceutical and food industries. To highlight the properties of this plant, additional investigations concerning more biological activities require to be conducted.

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