Influence of ecological conditions on some secondary metabolites variations in Moroccan *Juniperus thurifera* L

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Abstract:

The aim of this work is to study the influence of ecological conditions on some secondary metabolites (phenols and flavonoïds) in 14 *Juniperus thurifera* L. stands collected from the High and the Middle Atlas Mountains of Morocco. Total phenol and flavonoïd contents were determined spectophotometrically using Folin-Ciocalteu and $AlCl_3$ reagents, respectively. The antioxidant activity of the methanolic extracts was examined by DPPH method. The total phenol contents ranged from 137 mg GAE/g DW (in Bouiblan) to 191 mg GAE/g DW (in Tizrag). The total flavonoïd contents ranged from 11 mg QE/g (in Bouiblan) to 28 mg QE/g (in Tizrag). The highest antioxidant activity was noticed in Tizrag and the lowest was noticed in Bouiblan. These results showed a positive correlation between phenols, flavonoïds and antioxidant activity. Comparison between the Middle and the High Atlas stands showed significant differences between stands. The Middle Atlas stands are statistically close but those of the High Atlas are statistically different.

Key Words: Antioxidant activity; Atlas mountains of Morocco; Flavonoïd contents; *Juniperus thurifera* L.; Phenol contents.

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Introduction

In recent years, several studies have been made on the secondary metabolites of plants and their biological effects, these studies showed different results even within the same species. In fact, specimens of the same plant species growing under different environmental conditions showed a significant differences in the production and accumulation of the primary and secondary metabolites (El hajjouji et al. 2019; Gutbrodt et al. 2012; Oh et al. 2009; Pavarini et al. 2012; Ramakrishna et al. 2011). Influenced by environmental factors, respective groups of secondary metabolites act as a chemical interface between the plant and its environment. The chemical interaction between plants and their environment is mediated mainly by the biosynthesis of secondary metabolites, which exert their biological roles as a plastic adaptive response to their environment. Such chemical interaction often includes variations in the production of plant metabolites (Gutbrodt et al. 2012; Miranda et al. 2015; Pavarini et al. 2012; Ramakrishna et al. 2011; Szakiel et al. 2010). Therefore, the study of these variations is very useful in the chemical characterization of plants of the same species which are collected from different regions. This is the stage when the different geographical origin of a plant material is taken into account (Celiktas et al. 2007; Jamshidi et al. 2009).

Juniperus thurifera L. is a typical component of vegetal landscapes of the semi-arid mountain regions within the western part of the Mediterranean region. It is a variable species, in part as a result of long-term isolation of populations dispersed in separate mountain massifs (Romo and Boratyński 2005). It's an uncompetitive species adapted to dry environments and occurs on inaccessible sites or only poorly accessible for other trees (Quézel and Médail 2003).

This work aims to study the influence of ecological conditions on some secondary metabolites (phenols and flavonoïds) in 14 *J. thurifera* L. stands collected from the High and the Middle Atlas Mountains of Morocco.

Materials and Methods

Plant material

Samples of *J. thurifera* L. were collected in October of 2015 at 14 locations in the High and the Middle Atlas Mountains of Morocco. 9 stands (Tinmel, Azzaden, Ghighaya, Tizrag, Ait Lakkak, Tizi n Tichka, Tabant, Tagleft and Tounfiyt) were sampled in the High Atlas and 5

stands (Col Zad, Aglmam Sidi Ali, Timahdit, Tizi Bouzaabel and Bouiblan) were sampled in the Middle Atlas. The 14 stands are chosen in order to cover the main geographical areas of the *J. thurifera* L. in Morocco.

Latitude, longitude and altitude of each habitat locality were determined by a GPS locator. Exposure is varied as well as the slope. The other parameters were determined by Badri (2003). The characteristics of collection sites are shown in table 1.

Aerial parts were identified in the Ecology and Environment Laboratory, Faculty of Sciences Ben M'Sik, University Hassan II of Casablanca, Morocco. Collected samples were air dried immediately after harvesting, at ambient temperature (25°C) and kept away from sunlight for 21 days until its weight became constant. They were packed in paper bags and kept in a dark, dry and cool place until analysis.

Preparation of methanolic extract

After drying the leaves at room temperature, 50 g of powdered leaves of the 14 samples were extracted with absolute methanol at room temperature (2×200 ml) for 24h. After the filtration, the methanolic extracts were evaporated to dryness.

Determination of total phenolic contents

The total phenolic contents were determined using the Folin-Ciocalteu reagent as described by Gulcin et al. (2004). The aliquot of the samples, dissolved in methanol, was mixed with Folin-Ciocalteu reagent (100 µL) and distilled water (3 mL). Sodium carbonate (300 µL, 15%) was added to the mix. The solution had its volume adjusted to 5 mL with distilled water. After 2 h, absorbance was measured at 760 nm. A standard curve was prepared using gallic acid with a concentration range from 0.5 to 25 µg/mL. Total phenolic contents were expressed as mg gallic acid equivalents (GAE)/g dry weight (DW) of plant extract.

Determination of total flavonoid contents

Total flavonoid content was determined by aluminium chloride colorimetric assay adapted from Chatatikun et al. (2013), with a slight modification. An aliquot of 1 mL of extract solution (25–200 µg/mL) or quercetin (25–200 µg/mL) were mixed with 75 µL of $AlCl_3$, 500 µL of NaOH (1N) and 2.5 mL of distilled water. The mixture was incubated for 30 min at room temperature followed with the measurement of absorbance at 510 nm against the blank. Total flavonoid contents were expressed as mg quercetin equivalents/g of dry weight (mg QE/gDW).

Antioxidant activity (DPPH radical scavenging assay)

The antioxidant activity of *J.thurifera* L. methanolic extracts were evaluated according to the slightly modified method of Pothitirat et al. (2009). 500 µl of the methanolic extract and standards BHT, vitamin C and α- tocopherol (concentrations 40 µg/ml) separately were added to 1 mL of methanolic solution of DPPH (100 µM). The reaction mixture was incubated in the dark at room temperature for 20 minutes. The optical density was monitored at 517 nm against blank containing methanol. The antioxidant activity was expressed as the inhibition percentage (%IP) according to the following equation: $\% IP = \{(A_0 - A)/(A_0)\} \times 100$

Where A_0 = absorbance value of the blank sample, and A = absorbance value of the analyzed sample.

Statistical analysis

Concerning the statistical processing of our data, we have set up, as a first step, a variance analysis by GraphPad. Prism7 software both for phenolic and flavonoid contents. The ANOVA shows that there is a significant difference between the different stands (P value lower than 0.001) and consequently we have conducted, as a second step, a multiple comparison analysis (Tukey test) which allowed us both to differentiate between the different stands and to group those that are identical. All the assays were carried out in triplicate. Data were expressed as mean Standard Deviation (SD).

Results and Discussion

Total phenol contents

Total phenol contents have received considerable attention due to their antioxidant activities and free radical scavenging abilities which potentially have beneficial implications in human health (Amezouar et al. 2012; Rahhal et al. 2019).

Total phenol contents of the 14 *J.thurifera* L. stands are presented in table 2. All the methanolic extracts were characterized by the presence of considerable amount of phenols. The highest amount was noticed in Tizrag (191.31 mg GAE/g DW) and the lowest was noticed in Bouiblan (137.03 mg GAE/g DW). These results are in agreement with those of El Jemli et al. (2016) for the same species collected in Oukaimeden (193.79 mg GAE/g DW).

Comparison between the Middle and the High Atlas stands showed significant differences between stands (Table 2). The statistical analysis showed 4 different groups (a, b, c and d). The Middle Atlas stands (Col Zad, Aglmam Sidi Ali, Timahdit, Tizi Bouzaabel and Bouiblan)

are connected by the same letter d, they are statistically close because of similar ecological conditions (same type of soil and same bioclimatic stage (Table 1)). The High Atlas stands are statistically different, a fact which can be explained by the difference in ecological conditions between stands. *Juniperus thurifera* L. grows most frequently in the Middle Atlas and receives more precipitations than that in the High Atlas. It forms loose forests above the vegetation zone of forests of *Cedrus atlantica* and/or *Quercus ilex*, even covering extended areas, especially in the central part of the Middle Atlas, while the distribution in the High Atlas is more dispersed (Romo and Boratyński 2005). The content of polyphenolic compounds in vegetables is contingent on a number of factors among which climate, agronomy, maturation phase, harvest time, storage conditions, temperature, tissue damage, genetic factors and varietal diversity (Çirak et al. 2007; Djouahri et al. 2014; Ebrahimi et al. 2008).

Total flavonoïd contents

The total flavonoid contents in the methanolic extracts ranged from 11.94 to 28.03 mg QE/g DW (Table 3). A high concentration of flavonoïds was determined in Tizrag accounting for 28.03 mg QE/g edw. The lowest concentration was noticed in Bouiblan (11.94 mg QE/g DW). Jemli et al. (2016) reported 14.93 mg QE/g DW in *J.thurifera* L. collected from wild populations located in Oukaimeden.

Statistics showed significant variations between Middle and High Atlas stands. They confirmed that some biological active compounds in analyzed plants varied between populations. According to Dunkić et al. (2012), the contents of total phenolic and flavonoïds in aerial parts of the investigated populations of *Satureja montana* L. and *S. subspicata* Vis. (Lamiaceae) revealed a statistically significant within-species difference, depending on the locality and plant organ used for determination. Variations between locations could be ascribed to biotic (vermin, alleopathy, diseases) and abiotic factors (climate, soil, fertilization) (Young et al. 2005). The content of biologically active compounds also depends on plant age and harvesting time (Kolodziej and Sugier 2013). Szakiel et al. (2010) reported that divergences may result from the environmental and phenological factors at play at the time of plant collection. Climate, seasons of the year, phenological stage, genetic load, temperature, altitude humidity, among other factors, can significantly affect the quality and/or the quantity of bioactive compounds.

Antioxidant activity

The DPPH free radical scavenging activity of *J.thurifera* L. are presented in table 4. The Inhibition Percentage (%IP) values of methanolic extracts are compared with the standards: BHT (Butyl Hydroxyl Toluen), Vitamine C and α-tocopherol.

All samples methanolic extracts were able to reduce the stable purple colored radical DPPH into yellow-colored DPPH-H. They were also more effective than the synthetic antioxidant (BHT, Vitamine C and α-tocopherol) (except for Bouiblan extract). Tizrag extract had the strongest free radical-scavenging activity with IP value of 98.23%. The lowest capacity was observed in Bouiblan extract with IP=82.08%. These results are in agreement with several works (Emami et al. 2007; Jemli et al. 2016) which showed an important antioxidant effects in several species of the *Juniperus* genus.

Comparison between the Middle and the High Atlas stands showed significant differences between these stands (Table 4). The Middle Atlas stands (Col Zad, Aglmam Sidi Ali, Timahdit, Tizi Bouzaabel and Bouiblan) are connected by the same letter d, they are statistically close (same soil and same bioclimatic stage). The High Atlas stands are statistically different which can be explained by the difference in ecological conditions between stands. Indeed, several climate factors such as water availability, temperature and solar radiation are well described as being able to influence the production of metabolites (Arbona et al. 2013; Jakobsen and Olsen 1994; Ramakrishna and Ravishankar 2011; Shulaev et al. 2008). Thus, plants under conditions of stress induced by climate factors (drought, high temperatures, freezing, wide thermal amplitude, high levels of solar radiation) may show changes in the production of different metabolite classes.

The Inhibition percentage varied from 82.08% to 98.23% in *J. thurifera* L. stands. The minimum percentage was noticed in Bouiblan stand (82.08 %), which has also the minimum contents in phenols and flavonoïds. Maximum percentage of inhibition was noticed in Tizrag stand (98.23%) that has also the highest values in phenols and flavonoïds. These results showed a strong correlation between phenols, flavonoïds and antioxidant activity. Several studies have shown that there is a positive correlation between total phenol contents and antioxidant activity of the plants material (Afshar et al. 2012; El Jemli et al. 2016). Flavonoïds, including flavonols, flavones and condensed tannins, are a class of plant phenolics, which contain hydroxyl groups, are responsible for the radical scavenging and chelating properties (Juan and Chou 2010; Sharififar et al. 2009). The strong correlations between the total antioxidant and the phenolic content indicate that the phenolic compounds

largely contribute to the antioxidant activities of these *Cupressaceae* species and therefore could play an important role in the beneficial effects of these important medicinal plants (El Jemli et al. 2016).

Conclusion

Based on the above results, it is possible to confirm that the chemical features of *J.thurifera* L. change in terms of environmental factors by taking into account the variations in metabolic profile perceived through the occurrence of major metabolites in various plant parts. The secondary metabolism of plants and the expressed metabolite levels may change considerably due to the influence of several abiotic stress signals.

Table 1. Characteristics of collection sites of the 14 *J.thurifera* L. stands.

	Stands	Latitude Longitude	Altitude (m)	Soil*	Precipitations* (mm)	Bioclimatic stage*
HIGH ATLAS	Tinmel	30°58'N 08°13'W	2250	Red sandstones	500-600	Subhumid
	Azzaden	31°11'N 07°97'W	2400	Schists	500-800	Semi-arid to subhumid
	Ghighaya	31°09'N 07°56'W	2100	Red sandstones	700-800	Subhumid
	Tizrag	31°13'N 07°53'W	2500	Red sandstones	500-600	Subhumid
	Ait Lakkak	31°14'N 07°51'W	2050	Red sandstones	500-600	Subhumid
	Tizi n Tichka	31° 15'N 07°23'W	2050	Schists	500-600	Upper semi-arid
	Tabant	31° 65'N 6°41'W	2200	Compact limestone	400-500	Semi-arid
	Tagleft	32°20'N 06°13'W	1980	Limestone	500-600	Subhumid
	Tounfiyt	32°45'N 05°22'W	1950	Frost shattering	400-500	Semi-arid
MIDDLE ATLAS	Col Zad	33°03'N 05°09'W	2100	Limestone	600-700	Subhumid
	Aglmam Sid Ali	33°09'N 04°99'W	2150	Limestone	600-700	Subhumid
	Timahdit	33°13'N 05°04'W	2200	Limestone	700-800	Subhumid
	Tizi Bouzaabel	33°65'N 04°05'W	2200	Limestone	700-800	Sudhumid
	Bouiblan	33°38'N 04°04'W	2250	Limestone	700-800	Subhumid

*(Badri et al. 2003)

Table 2. Total phenol contents of the 14 *J.thurifera* L. stands. Letters in superscript denote statistical differences between stands.

Stands	**Total phenol contents** (mg GAE/g DW)
Tinmel	181.50±6.08^{ab}
Azzaden	172.56±14.94^{abc}
Ghighaya	170.25±9.91 ^{abc}
Tizrag	191.31±5.89^{a}
Ait Lakkak	157.23±6.15^{cd}
Tizi n Tichka	157.25±5.82^{cd}
Tabant	143.79±4.05^{d}
Tagleft	142.29±6.39^{d}
Tounfiyt	157.83±9.11^{bcd}
Col Zad	151.74±9.95^{cd}
Aglmam Sidi Ali	145.18±5.49^{d}
Timahdit	144.93±5.81^{d}
Tizi Bouzaabel	144.32±5.76^{d}
Bouiblan	137.03±9.48^{d}

Table 3. Total flavonoïd contents of the 14 *J.thurifera* L. stands. Letters in superscript denote statistical difference between stands.

Stands	**Total flavonoid contents** (mg QE/g edw)
Tinmel	19.26±0.24^{c}
Azzaden	22.20±1.46^{b}
Ghighaya	22.30±0.98^{b}
Tizrag	28.03±0.67^{a}
Ait Lakkak	19.93±0.84^{c}
Tizi n Tichka	16.61±0.82^{de}
Tabant	16.78±0.11^{d}
Tagleft	15.44±0.37^{def}
Tounfiyt	16.32±0.67^{def}
Col Zad	15.62±0.47^{def}
Aglmam Sidi Ali	12.85±0.25^{gh}
Timahdit	14.72±0.17^{efg}
Tizi Bouzaabel	14.38±0.59^{fg}
Bouiblan	11.94±0.09^{h}

Table 4. DPPH scavenging activity of the 14 *J.thurifera* L. stands. Letters in superscript denote statistical difference between stands.

Stands	%IP
Tinmel	$97.45 \pm 0,7^{ab}$
Azzaden	$97.81 \pm 0,3^{abc}$
Ghighaya	$96.04 \pm 0,4^{abc}$
Tizrag	$98.23 \pm 0,6^{a}$
Ait Lakkak	$97.44 \pm 0,4^{c}$
Tizi n Tichka	$95.12 \pm 0,5^{c}$
Tabant	$96.68 \pm 0,3^{c}$
Tagleft	$91.90 \pm 0,6^{c}$
Tounfiyt	$89.78 \pm 0,4^{cd}$
Col Zad	$86.73 \pm 0,8^{cd}$
Aglmam Sidi Ali	$87.82 \pm 0,5^{d}$
Timahdit	$88.32 \pm 0,7^{d}$
Tizi Bouzaabel	$86.18 \pm 0,3^{d}$
Bouiblan	$82.08 \pm 0,7^{d}$
BHT	$86.02 \pm 0,9^{cd}$
Vitamine C	$85.82 \pm 0,3^{cd}$
α-tocopherol	$82.23 \pm 0,2^{d}$

IP: Inhibition Percentage

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