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Evaluation of essential oil composition and antioxidant capacity of hydromethanolic extracts of *Tetraclinis articulata*, depending on location and seasonal variations

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Abstract

This work describes the seasonal and geographical variation in essential oils composition, the in vitro lipid peroxidation inhibitions, the anti-radical activities and total phenolic contents of two aqueous methanol (methanol: water, 80:20 and 50:50 v/v) and essential oils fractions of aromatic wild plant, *Tetraclinis articulata* (Vahl) Masters, grown in two arboreta in the North East Tunisia. Chemical composition of the essential oils was evaluated by GC/MS and GC/FID. The inhibition of lipid peroxidation and DPPH radical scavenging ability were studied to evaluate the antioxidant activity for six extracts of *Tetraclinis articulata*. The results show that essential oil content was not affected by geographical origin and was significantly affected by season ($p \leq 0.05$). The essential oil yields ranged from 0.10% to 0.26%, the maximum amount was observed during winter (January) and the minimum was obtained in spring (May). GC/MS analysis revealed 29 constituents in this plant. For both geographical origins (Korbous and Jbel abderrahmen) and four season periods (January; April; May and November 2010), highly significant differences were observed for oil compositions ($p \leq 0.05$). The main compounds identified in the leaves of this plant were α-Pinene (26.3 -36.6%), bornyl acetate (19.0 - 25.4%), camphor (5.5 - 15.3%), and limonene (5.3 - 8.9%). Others representative components were detected such as β-myrcene (3.9 - 6.6%), borneol(3.0 - 6.8%) and caryophyllene (0.2 - 5.1%). For the antioxidant data, the methanol: water (50:50 v/v) fraction of the January harvest exhibited the highest DPPH radical scavenging activity, and exerted the highest percentage inhibition of the induced autoxidation of methyl linoleate. The total phenolic content of various extracts was in the range of 17.7±0.7 to 30.1±1.0 eq. gallic acid/g. This study demonstrated that inhibition of lipid peroxidation and the DPPH radical scavenging ability of *Tetraclinis articulata* leaf are correlated with the amount of phenolic constituents.

Keywords: *Tetraclinis articulate*, essential oil, chemical composition, hydromethanolic extracts, antioxidant activity, total phenolic content.

1. Introduction

Extensive studies carried out by many authors on the medicinal plants documented the influence of the harvest season and geographical origin of variety on chemical composition and biological activities of essentials oils and various organic extracts [1-4]. Regarding *Tetraclinis articulata* (Vahl) Masters, some recent studies have focused on chemical and biological properties of this plant [5-10].

Tetraclinis articulata (Atlas cypress or Barbary thuja) is a small conifer of the Cupressaceae family. The monospecific genus has a predominantly North African distribution (Morocco, Algeria and Tunisia) as well as a number of other stations namely the Sierra di Cartagena (south-east of Spain), Malta, Cyprus and Libya [11]. In Tunisia, it is distributed in the North East but has never been found in the South [12]. The tree flowers in April, cones mature in summer and seeds are shed in early autumn [1]. The branches of *T. articulata* emit a pleasant smell; they are used in compresses for children to treat fever and in drinks to treat diarrhoea [13]. In North Africa, different parts of the tree are used in traditional and veterinary medicine principally against intestinal and respiratory ailments as well as skin condition [1]. Various parts of the plant are traditionally used in Maroccan popular medicine as antipyretic, anti-diarrheal and diuretic [14]. Obtaining bioactive compounds of

essentials oils from this plant is performed by hydro-distillation (HD) [1, 5, 8, 16], supercritical carbon dioxide (SFE) [9] and microwave-assisted hydro-distillation (MAHD) [5]. The essential oil composition of some organs of *T. articulata* has been the subject of some previous studies and the principal compounds identified in this plant are α-pinene, camphene, limonene, ρ-cymene, camphor, bornyl acetate, linalool acetate, α-camphenol, trans-pinocarveol , cis-verbenol, verbone, cedrol, 1,7-di-epi-cedrol, 4,6-dimethyl-octane-3.5-dione, Z-muurolene, and germacrene D [1, 5-9,13, 15,16]. Also, biological studies have demonstrated considerable antioxidant, antibacterial, and anti-inflammatory effects for *T. articulata* [5-9, 17].
Therefore, the aims of this study were to characterize and compare the chemical composition of the essential oils of *T. articulata* growing in two different arboreta in North East Tunisia and collected in four periods (November, January, April and May), and to evaluate the in *vitro* lipid peroxidation inhibition, antiradical activity profiles and total phenol content of essential oil and hydromethanolic extracts of this plant.

2. Materials and methods

2.1. Chemicals
Methyl linoleate (LH), a gift of Novance, was distilled in a glass oven (Büchi). 2,2_-Azobis-isobutyronitrile (AIBN) (Fluka, >98%), butanol-1 (Sigma, 99.8%), oxygen (Air Liquide, 99.995%), (-)–catechin hydrate (Fluka, >96%),(±)-α-tocopherol (Fluka, ≥97%) methanol (Merck, 99.8%), Folin–Ciocalteu reagent (Sigma–Aldrich), ethanol (Merk 99.8%), and distilled water were used without further purification.

2.2. Plant material
Tetraclinis articulata Leaves were collected from the arboretum of Korbous during four periods (Collect 1: 5 January 2010, Collect 2: 23 April 2010, and Collect 3: 25 May 2010; Collect 4: November 2010) and from Jbel abderahman arboretum during one period (Collect 1: 25 May 2010). Leaves of similar age were taken from three trees in order to get homogeneous samples. Both arboreta are located in the North East Tunisia with sub-humid bioclimatic conditions.
The arboretum of Korbous (latitude 36°48'N; longitude 10°33′E; altitude 370 m) has an area of 16 ha. Temperature recorded during the hottest month is about 37°C whereas it decreases to 6.8°C for the coldest month. The site is characterized by an annual average rainfall of 540 mm and a soil rich in sand, sandstone and clay [2].
The arboretum of Jbel abderrahmman (latitude 36° 42′; longitude 10°40′; altitude 200 m) had an average annual rainfall of 550 mm. The average maximum temperature of the warmest month is 32.4°C whereas the average temperature of the coldest is around 7.9°C. Lithological composition of the slope is based on sandstone and clay.
The Taxonomic identification was performed by botanists of the National Institute of Research in Rural Engineering, Water and Forestry (INGREF), Tunisia. A voucher specimen (IRT 61151) for each plant has been deposited in the herbarium of this institute. The trees were 47 years old in 2010.

2.3. Extraction of essential oil
The harvested material was air-dried at room temperature (20-25°C) for two week and then stored in cloth bags. Moisture content was assessed by constant weight at 103°C and by Karl Fischer volumetric titration: 9.2% w/w. The dried leaves *T. articulata* were subjected to hydrodistillation using a Clevenger apparatus for 180min..The yields were averaged over three experiments and calculated according to dry weight of the plant material. The essential oils were dried over anhydrous sodium sulphate until the disappearance of the last traces of water and then stored at 4°C.

2.4. Extraction with methanol-water mixtures
A total of six different extracts was prepared from the leaves of *T. articulata* collected in three periods (January, April and November) from Korbous. The dried plant materials from leaves (5 g) were separately powdered and dissolved in methanol: water (80:20 or 50:50 (v/v)), with a solid/liquid ratio 1/10(w/v) for 48h at room temperature. Following this, they were filtered through a Whatman n^{o} 1 filter paper. The extracts were concentrated under vacuum at 45°C using a rotary evaporator (Stuart) until constant weight and finally kept in the dark at -20°C until analysis. The extract yields were determined as the increase of the flask weight.

2.5. Chemical analysis of essential oils
2.5.1. GC/FID analysis
The essential oil was analysed using a Trace GC 2000 chromatograph (Thermoquest) equipped with a FID detector and a 5% diphenyl 95% dimethyl polysiloxane fused-silica capillary column (EC-5, 30 m x 0.25mm x 0.25 µm, Alltech). Injector and detector temperature was set at 250°C. Oven temperature was kept at 60°C for 1 min, then gradually raised to 300°C at 15°C/min. Hydrogen was the carrier gas at a flow rate of 1mL/min at 60°C. 1µL of diluted oil sample (1/100 in ethyl acetate (v/v)) was injected manually in the split mode. Quantitative data were obtained (ChromCard program, Thermoquest) from FID area percent data without the use of correction factors.

2.5.2. GC/MS analysis

Qualitative analyses of essential oils were carried out by gas chromatography-mass spectrometry (GC/MS). One mg of different essential oils was diluted in 1 mL of anhydrous ethyl acetate. GC/MS analysis was performed on a Clarus 500 gas coupled to a Clarus 500 MS quadrupole mass spectrometer (Perkin Elmer Inc., USA). Gas chromatography was carried out on a 5% diphenyl /95% dimethyl polysiloxane fused-silica capillary column (DB-5ms, 30m×0.25mm, 0.25µm film thickness, J&W Scientific, USA) with helium as carrier gas at a constant flow of 1mL/min. The gas chromatograph was equipped with an electronically controlled split/splitless injection port. The injection (1µL) was performed at 250°C in the split mode. The oven temperature program was as follows: 60°C for 1 min, 60°C to 300°C at a rate of 15°C/min. Ionisation was achieved under the electron impact mode (70 eV). The source and transfer line temperatures were 250°C and 330°C, respectively. Detection was carried out in scan mode: m/z =35 to m/z=700. The detector was switched off during the initial 2 min (solvent delay). Compounds were identified by comparison of their Kovats indices relative to C8–C16 *n*-alkanes obtained on a non-polar DB-5MS column with those provided in the literature, by comparison of their mass spectra with those recorded in the NIST (US National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral library and reported in published articles and by co-injection of available reference compounds. All samples were analyzed in triplicate.

2.6. Total phenol assay

Total phenolic constituents of the essential oil were analysed by the Folin and Ciocalteu method using gallic acid as standard [18, 19]. For the preparation of gallic acid stock solution, 0.5 g of dry gallic acid was dissolved in10 mL of ethanol and diluted to 100 mL with doubly distilled water (ddH_2O). Then 0, 1, 2, 3, 5, and 10 mL of the gallic acid stock solution were diluted to 100 mL with ddH_2O in volumetric flasks giving an effective concentration range of 0, 50, 100, 150, 250, and 500 mg/L gallic acid.

The extracts (3.5µL) of a 1 mg/mL methanolic solution or standard solutions of gallic acid (3.5 µL) or ddH_2O as blank were added to separate test tubes and mixed thoroughly with 276.5 µL ddH_2O and 17.5 µL of Folin- Ciocalteu reagent. After 8 min 52.5 µL of 7% Na_2CO_3 solution were added, and mixed thoroughly. The final concentration of the extracts in each well was 10µg/mL. The solutions were incubated at 20°C for 2h and the absorbance versus blank (0 mg/L gallic acid) was read at 765 nm using CE 7400 Double Beam UV/ VIS spectrophotometer. In addition, a sample containing only methanol was processed in the same way to account for any background due to methanol. The total phenol content of the sample was determined by comparing with a calibration curve of the gallic acid standard and represented as mg gallic acid equivalents (GAE)/g of dried samples. The standard curve equation is absorbance = 0.008 [gallic acid/µg.mL^{-1}] + 0.0056 (R^2= 0.9984). Each sample was assayed at least six times.

2.7. Antioxidant activity

2.7.1 DPPH assay

Free radical-scavenging activity of the sample extracts was evaluated with the modified DPPH (1,1-diphenyl-2-picrylhydrazil radical) assay [20, 21] which is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Briefly, a stock solution of DPPH (10^{-4} M) was prepared in aqueous methanol: water (50:50 and 80:20 (v/v)). One millilitre of sample was added to 3 mL of the DPPH solution. The mixture was then shaken vigorously and allowed to stand at room temperature in the dark. With our extracts, the half-reaction times were not more than a few minutes; after 30 min, the decrease in absorbance at 517 nm was measured against a blank (aqueous methanol solution) by using a CE 7400 Double Beam UV/ VIS spectrophotometer. A mixture of 1 mL of aqueous methanol and 3 mL of DPPH solution was used as the control. The radical stock solutions were freshly prepared every day, stored in a flask covered with aluminium foil and kept in the dark. The results were compared with catechin and α-tocopherol as standards. The radical scavenging activities of the samples, expressed as the percentage of DPPH reacted, were calculated according to the formula:

% DPPH reacted = $(A_B-A_A)/A_B$ x 100.

where A_B and A_A are the absorbance values of the control and of the test sample, respectively. The tests were carried out four times. The extract concentration for which 50% of DPPH disappears (efficient concentration EC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (100, 80, 50, 30, 20, 10, and 5mg/L).

2.7.2. Oxygen uptake method

Lipid peroxidation inhibitions by various aqueous methanol extracts were investigated by evaluating oxygen uptake inhibition during oxidation of methyl linoleate. The induced oxidation by dioxygen was performed in a gas-tight borosilicate glass apparatus [22, 23]. The solvent was butan-1-ol. Temperature was set to 60° C and initial conditions inside the vessel were as follows: methyl linoleate concentration: 0.4 M; 2, 2'-azobisisobutyronitrile (AINB) concentration: 9.10^{-3} M; extract concentration: 0.1 mg/mL; oxygen pressure 140 Torr. Oxygen uptake was monitored continuously by a pressure transducer (Viatron model 104); without any additive, oxygen uptake is roughly linear (see figures 4 and 5) and constitutes the control. In the presence of an antioxidant extract, oxygen consumption is slower, and we estimated the antioxidant capacity of the extract by its oxygen uptake inhibition (OUI), i.e. the ratio of oxygen uptake at a chosen time (3.5 h) in the presence and in the absence of the extract:

OUI (%) =[$P(O_2)_{3.5h}$ in presence of extract − $P(O_2)_{3.5\ h}$ in absence of extract] /[$P(O_2)_{0h}$ in absence of extract - $P(O_2)_{3.5h}$ in absence of extract] × 100.
This ratio defines antioxidative capacity as an oxygen uptake inhibition index (OUI); it should spread from 0 to 100%, for poor and strong antioxidants, respectively, and would be negative for prooxidants. All measurements were carried out in triplicate.

2.8. Statistical analysis
All the experiments were conducted at least in triplicate and statistical analysis of the data were performed by analysis of variance (ANOVA) using Tukey's range (HSD) tests. Differences were considered significant at $p \leq 0.05$. Data are presented as mean values ± standard deviation. Principal component analysis was performed using R software and the FactoMiner package [24].

3. Results and discussion

3.1. Yields and composition of essential oils
Table 1 shows the yields of essential oils, constituents identified in them, percentage composition and their Kovats index (KI) values listed in order of elution from the DB-5 capillary column as affected by seasonal changes and by geographical region.
Yield of leaf essential oils of the plants harvested in January is significantly higher than that of April and May. This result agrees with findings of other authors[3, 25, 26] : yield is negatively correlated with temperature. Some others studies revealed that plants or herbs exhibit remarkable fluctuation in volatile oil yields with progress of seasons [2,4].
No significant difference of yield was observed for volatile oil contents of both origins (last column of table 1). The population of Korbous and Jbel abderrahmen areas were grown in similar sub-humid climate, leading to similar oil contents, which is in agreement with Djouahri et al. [7] and Lei et al. [27]. This result may be compared to the previous studies which report oil yields ranging from 0.05 to 1.18% for leaves, branches (woody and not woody), cones, seeds and roots [1, 5-9, 10, 15, 16].
The yield of leaf volatile oils of several locations and periods has been measured by some authors: 0.61, 1.61, 19.2, 25.9 g/kg by HD, SFE at 90, SFE at 280, SFE at 1000 bar, respectively, for korbous arboretum during October 2010 reported by Herzi et al.,[9]; 0.22 to 0.78% by HD for four locations in Algeria during March 2012 reported by Djouahri et al. [6]; 0.54 and 0.56% by MAHD and HD, respectively, during March 2011 reported by Djouahri et al. [5]; 0.75, 0.78, 0.35% for three areas reported by Toumi et al. [10]; 0.22% during February 2005 reported by Bourkhis et al. [29]; 0.7% during April 1988 reported by Barrero et al. [16]. The essential oil contents (0.10 - 0.26%) in the present study agrees with these reports. However, one may observe significant differences in the quantity and quality of essential oil in *T. articulata*, which may be attributed to the method of extraction and to environmental factors, or different parts of the plant [5, 7, 9].
The GC/MS analysis of the four hydro-distillation essential oils from different seasonal periods revealed 28, 27, 22, 22 various compounds, representing 94.3, 95.1, 93.1, 93.8% of total oils in November, January, April and May, respectively (table1).
In fact, the most abundant components among all samples were α-pinene, (-)-bornyl acetate, camphor, and limonene. Other compounds were detected in appreciable quantities such as β-myrcene, borneol, and caryophyllene. The variations of the content of all these compounds (bold numbers in table 1), except α-pinene, are significant ($p \leq 0.05$), but the maximum or minimum values occur at very different periods.
The sesquiterpene caryophyllene is present in very high quantity in January. This compound is a biogenic volatile organic compound, emitted by numerous plants with a relatively high potential for secondary organic aerosol formation in the atmosphere. It is eager to be photooxidized by ozone in sunlight [29].
The regional variation of the chemical composition of the *T. articulata* essential oils can be seen in the last column of table 1. These leaves volatile oils were characterized by the presence of 22 components vs. 21 components corresponding to 93.8 % vs. 91.9% for Korbous vs. Jbel abderrahman.
When comparing amounts of the main components of *T. articulata* volatile oils for both origin during the May period, significant differences were observed ($p \leq 0.05$) for α-pinene, (-)-bornyl acetate, camphor, limonene, β-myrcene, and some other minor compounds, whereas no significant variations were detected for borneol.
Based upon recent studies done on the leaf essential oil composition of *T. articulata* we note considerable qualitative and quantitative differences between our region (Korbous arboretum) and others regions where different chemotypes have been documented. To the best of our knowledge, no comparative study have been reported on seasonal variation of essential oils of T. *articulata* and only one comprehensive research on the diversity of the volatile oils among this plant in various geographic areas of Algeria has been done [7, 10].

Seven main compounds of leaf essential oil were detected in the present study with an average percentage higher than 2% : α-pinene (26.3-36.6%), bornyl acetate (19.0-25.4%), camphor (5.5-15.3%), limonene (5.3 - 8.9%), β-myrene (3.9 - 6.6%), borneol (3.0 - 6.8%) and caryophyllene (0.2 - 5.1%). However, previous reports on the constituents of the leaf volatile oils of this plant from same area (Korbous) and year (2010) during October suggested the presence α-Pinene (24.9-31.3%), linalool acetate (21.4-18.2%), alloaromadendrene (1.1-7.6%), camphene hydrate (1.3-4.4%), and γ-caryophyllene (2.5-4.2%) by HD and SFE [8, 9]. These results show the absence of bornyl acetate and camphor, while our results showed the absence of linalool acetate and alloaromadendrene.

Table 1: Effect of seasonal changes and geographical regions on the yield and the chemical composition of the *Tetraclinis articulata(*Vahl) Masters essential oils. Values are means ± standard deviation of three separate experiments. Identification was obtained by MS, NIST and Wiley libraries spectra and the literature, and Kovats indices on a DB-5MS column. [a]: Kovats index relative to n- alkanes (C8-C16) on a DB-5MS column; [b]: identification also based on co-injection with authentic standard compounds; -: Not detected

			Korbous				Jbel Abderr.	Place evol.
N°	KI[a]	Compounds	Concentration (%)					
			November	January	April	May	May	
0	-	Yield (w/w %)	0.20±0.07^{AB}	0.26±0.03^{B}	0.11±0.01^{A}	0.10±0.03^{A}	0.14±0.02	no
1	929	Tricyclene	1.23±0.07^{A}	1.24±0.05^{A}	0.95±0.05^{B}	0.93±0.05^{B}	0.86±0.04	no
2	940	α-Pinene [b]	36.6±1.28^{A}	35.7±1.17^{A}	32.3±1.17^{A}	35.4±1.24^{A}	26.3±1.22	yes
3	956	Camphene	1.74±0.96^{A}	1.50±1.10^{A}	1.72±1.17^{A}	2.36±1.20^{A}	1.63±1.24	no
4	971	β-Thujene	0.39±0.01	-	-	-	-	no
5	985	β-Pinene	0.76±0.05^{B}	0.70±0.03^{AB}	0.57±0.02^{A}	0.85±0.03^{B}	0.70±0.07	no
6	992	β-Myrcene	5.10±0.41^{B}	3.85±0.33^{A}	5.67±0.12^{BC}	6.60±0.30^{C}	4.52±0.28	yes
7	1016	α-Phellandrene	0.14±0.02^{A}	0.07±0.01^{A}	0.40±0.01^{B}	0.07±0.03^{A}	-	no
8	1035	Limonene	8.91±0.40^{B}	7.51±0.32^{B}	5.34±0.42^{A}	8.41±0.30^{B}	7.52±0.42	no
9	1040	1,8-Cineole	0.3±0.010^{B}	0.11±0.04^{A}	0.56±0.05^{C}	0.09±0.03^{A}	0.59±0.03	yes
10	1064	γ-Terpinene	0.29±0.01^{A}	0.16±0.07^{A}	-	-	0.30±0.02	yes
11	1089	α-Terpinolene	0.48±0.04^{A}	0.34±0.04^{A}	-	-	0.29±0.05	yes
12	1095	α-Campholenal	0.33±0.19^{A}	-	0.76±0.20^{A}	0.42±0.12^{A}	1.40±0.22	yes
13	1152	Unknown	0.73±0.04	-	-	-	-	no
14	1158	Camphor	7.27±0.20^{B}	5.46±0.07^{A}	7.15±0.23^{B}	7.85±0.18^{B}	15.2±0.44	yes
15	1167	Cis-β-terpineol	0.85±0.02^{A}	0.46±0.01^{A}	-	0.33±0.02^{A}	-	no
16	1179	Borneol [b]	3.99±0.22^{A}	3.02±0.08^{A}	6.77±0.90^{B}	3.93±0.23^{A}	3.88±0.27	no
17	1193	4-Terpineol	0 .85±0.01^{B}	0.71±0.04^{A}	1.05±0.02^{C}	1.14±0.02^{C}	-	yes
18	1226	Unknown	0.46±0.03^{A}	0.78±0.05^{B}	-	-	-	no
19	1295	(-)-Bornyl	19.00±0.87^{A}	21.9±0.1^{B}	23.4±0.70^{B}	20.4±0.7^{AB}	25.3±0.50	yes
20	1357	α-Terpinyl	0.86±0.35^{A}	0.72±0.40^{A}	1.05±0.42^{A}	1.98±0.57^{A}	0.85±0.50	no
21	1382	α-Copaene	0.21±0.20^{A}	0.55±0.23^{AB}	-	1.10±0.22^{B}	-	yes
22	1389	α-Gurjunene	0.18±0.01^{A}	0.82±0.01^{B}	-	-	-	no
23	1434	Unknown	0.23±0.18^{A}	1.01±0.1^{B}	0.67±0.17^{AB}	0.61±0.18^{AB}	-	no
24	1444	Caryophyllene [b]	0.19±0.20^{A}	5.08±0.11^{B}	0.57±0.19^{A}	0.27±0.21^{A}	0.35±0.01	no
25	1521	δ-Cadinene	1.06±0.25^{A}	0.37±0.20^{A}	0.27±0.18^{A}	0.43±0.22^{A}	0.28±0.20	no
26	1565	Caryophyllene	0.38±0.07^{A}	1.78±0.12^{B}	2.19±0.07^{B}	0.37±0.10^{A}	1.00±0.17	yes
27	1614	Cubenol	0.30±0.24^{A}	0.26±0.34^{A}	0.38±0.40^{A}	0.22±0.44^{A}	0.44±0.28	no
28	1632	Tau-Cadinol	0.97±0.22^{A}	0.72±0.23^{A}	0.76±0.20^{A}	-	-	no
29	1646	Tau-Muurolol	0.49±0.02^{B}	0.22±0.01^{A}	0.57±0.01^{C}	-	0.37±0.01	yes
		Total	**94.27**	**95.08**	**93.11**	**93.82**	**91.88**	

Djouahri et al. [7] have studied essential oils of leaves and of wood of *T. articulata* harvested in four regions of Algeria and have observed significant differences in the chemical compositions according to the geographic origin of the population and to the parts of the plant. In the essential oils obtained from leaves, the main components were α-Pinene (16.3 - 25.3%), camphor (17.2 - 21.4%) and bornyl acetate (17.6 - 20.6%) while volatile oils from the wood were richer in camphene (15.5 - 18.8%), cedrol (10.5 - 12.5%), and α-acorenol (8.2 - 10.8%). Other studies of Djouahri et al., [5, 6] upon the chemical composition of essential oil from cone obtained by HD and leaves by HD and MAHD of *T. articulata* have shown that the leaves were rich in bornyl acetate (24.4 - 32.4%), α-pinene (12.5 - 23.6%), camphor (18.3 - 21.4%), limonene (4.3 – 7.0%), myrcene (0 - 5.5%), and α-terpineol acetate (0 - 6.8%) and that volatile oil from the cone was rich in α-campholenol (16.3%), trans-pinocarveol (15.5%), cis-verbenol (12.4%) and verbenone (13.4%). These results on chemical composition showed that qualitative and quantitative differences of individual compounds exist.

The results reported by Toumi et al. [10] on leaves essential oil of *T. articulata* from areas in west Algeria have shown that essential oil samples were rich in camphor (23.4 -31.6%), bornyl acetate (17.1 - 25.8%), borneol (6.6 - 14.1%), limonene (2.3 - 10.1%), and α-pinene (3.6 - 11.3%). The plant's vegetative cycle (secondary metabolism) and edaphic factors (humidity, rainfall, latitude, temperature, and soil conditions) are key reasons for chemical variability in secondary metabolites between populations. These factors influence the plant biosynthetic pathways and consequently the existence of different chemotypes which distinguish *T. articulata* essential oils of different origins. This diversity in yields and chemical compositions in bioactive compounds can be increased by using different extraction techniques (HD, SFE, and MAHD), solvents, pressures, temperatures and times [5, 9].

To search for a possible effect of the season on the yield and composition of the essential oil, we have carried out a principal component analysis (PCA) with numerical data centered and reduced by using values relative to annual averages. We so obtained the loadings of the four months on four principal components. As table 2 shows, the first three principal components account for 100% of the variance. As one can see on figure 1, April is definitely a particular period regarding the global composition of the essential oil.

Table 2 – Loading of the four months on the principal components

	PC1	PC2	PC3	PC4
November	0,79	0,10	-0,61	0,00
January	-0,90	0,43	-0,11	0,00
April	0,02	-0,99	0,17	0,00
May	0,66	0,49	0,57	0,00
Variance	1,86	1,41	0,73	0,00
% of variance	46,56	35,17	18,27	0,00
Cumulative % of var.	46,56	81,73	100,00	100,00

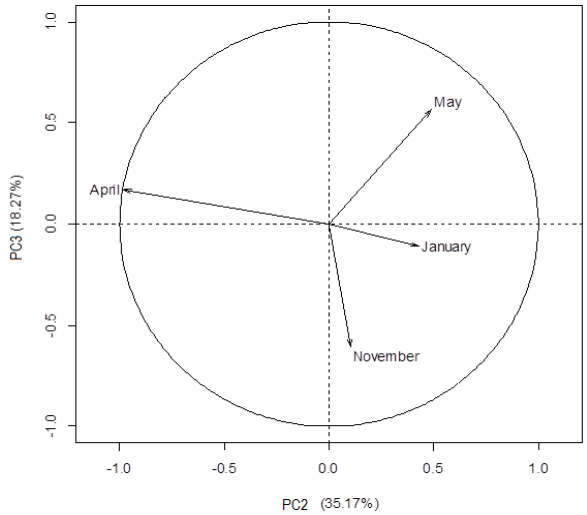

Figure 1 : Variable factor map on axes PC2 and PC3

α-Phellandrene (7) and 1,8-cineole (9), the structures of which are related, have a very high PC2 component on figures 2 and 3, and, accordingly, are greatest in April. As figure 2 shows, β-caryophyllene (24) and caryophyllene oxide (26) have also a special status, but the first is highest in January while the oxide is greatest in January and April, so that one may wonder if there is a time shift between the non-oxygenated and oxygenated products, but PCA does not evidence it.

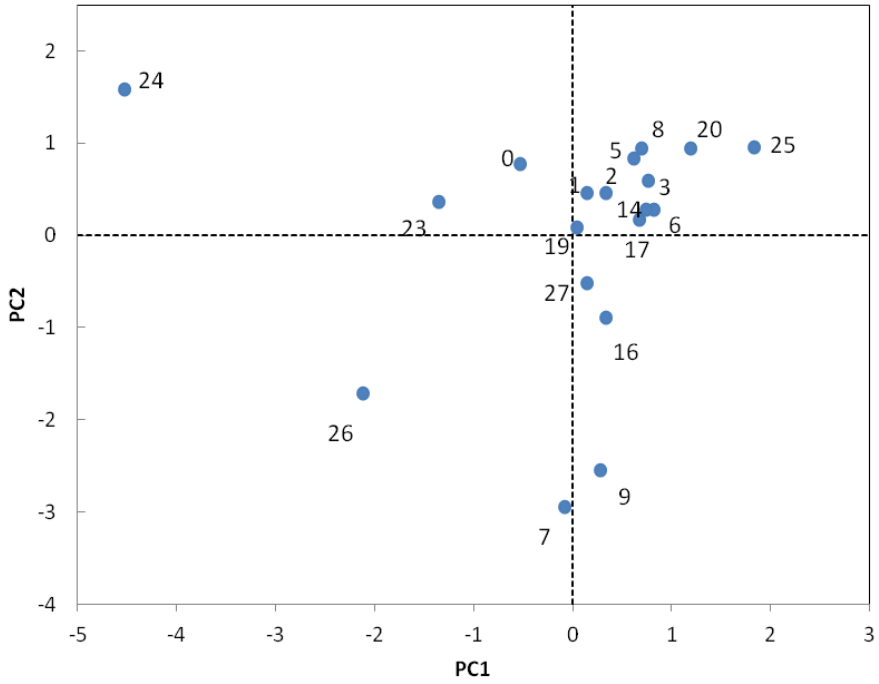

Figure 2 : Individuals factor map on axes PC1 and PC2

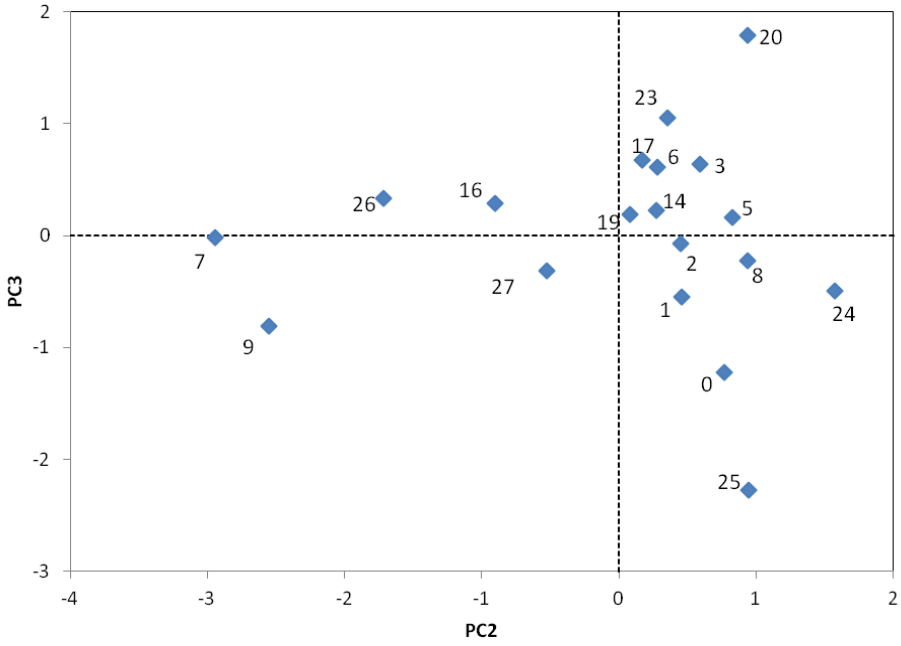

Figure 3 : Individuals factor map on axes PC2 and PC3

3.2. Extraction yield

The yield of the leaf extracts, obtained with the two different solvents (80% and 50% methanol) are presented in table 3. There is a considerable variation between different extracts corresponding on one hand to the different periods and on the other hand to the different solvent polarities ($p<0.05$). For both materials, the yield of

extractable compounds increased with the polarity (water content) of the solvent, the highest yields corresponding to extraction with methanol/water 50:50 (v/v) while the lowest yields correspond to methanol/water 80:20 (v/v) extracts. For both solvents, extraction yield is significantly better in January; the same was observed for the essential oil yield.

3.3. Total phenolic content

The Folin-Ciocalteu method is known to quantify phenols, but also some other compounds like some proteins (essentially tyrosine, which is a phenol, and tryptophan) or vitamine C; sugar response is very low [36]. We therefore considered that this test gives a reasonable idea of the total phenolic content (TPC) of our extracts. No-polar solvent(hexane), or moderate polar solvents like ethyl acetate, were used for extraction of non- polar compounds such as terpenes, carotenes , and lipids, while phenolic compounds are usually extracted using water , ethanol and methanol[37]. Water was the most prominent example of green solvent and interesting alternative to the usual organic solvents, particularity in view of its non-toxic character, low cost and polarity.TPC of the hydromethanolic extracts (methanol: water 50:50 (v/v), methanol: water 80:20 (v/v)) of *T. articulata* leaves is expressed as gallic acid equivalents, and the data are presented in table 3. Our results indicate the presence of phenolic compounds in all hydromethanolic extracts and that the extracts with 20% water contained higher total phenolic content (21.6 to 30.1 µg GAE/mg,) than the extracts obtained with 50% water (17.6 to 28.3 µg GAE/mg). The ratio TPC/ yield of extract in 20% water during the three periods ranged from 2.2 to 3.0 (units of table 3). Yields for both extractants were the richest in phenolics in January. This result is probably explained so: the higher accumulation of phenolic constituents of the two hydromethanolic extract was observed before flowering stage (January), with the value of 28.3 to 30.1 µg GAE/mg in comparison to the accumulation of phenolic compounds in the two other periods (17.6 to 26.9 µg GAE/mg).

Table 3 : Yields (% w/w), antioxidant activity (EC_{50} and OUI), total phenol content (TPC) and TPC/ yield ratio of the different extracts of *Tetraclinis articulata (*Vahl) Masters. Uncertainties are standard deviations (n=3); different letters in superscript indicate significant differences between seasons for a given solvent polarity ($p<0.05$).

Extracts/ standards	Periods	Yield (%)	TPC (µg GAE/ mg)	TPC/ yield	EC_{50} (µg/mL)	OUI (%)
α-tocopherol					21.2±0.72	100
(+)- cathechin					7.60±1 .01	98
MeOH 50%	January	19.1 ± 0.28^{A}	28.3 ± 1.31^{A}	2.8	$22 .8\pm0.28^{A}$	80.6 ± 4.33^{A}
MeOH 50%	April	17.3 ± 0.03^{B}	20.8 ± 1.08^{B}	2.1	35.3 ± 0.15^{B}	58.8 ± 0.58^{B}
MeOH 50%	November	17.5 ± 0.70^{B}	17.6 ± 0.65^{C}	1.8	36.8 ± 1.60^{B}	39.6 ± 0.54^{C}
MeOH 80%	January	16.6 ± 0.17^{a}	30.1 ± 1.04^{a}	3.0	$23.3\pm0. 35^{a}$	61.2 ± 8.65^{a}
MeOH 80%	April	14.8 ± 0.18^{b}	26.9 ± 0.41^{b}	2.7	30.9 ± 0.76^{b}	60.2 ± 4.34^{a}
MeOH 80%	November	14.0 ± 0.04^{c}	21.6 ± 1.08^{c}	2.2	30.7 ± 0.86^{b}	38.8 ± 0.57^{c}

3.4. Antioxidant capacities using the DPPH and oxygen uptake methods at different harvest times

The antioxidant activity is influenced by many factors, which cannot be fully described with one single reaction system. Thus, utilizing more than one assay to evaluate antioxidant activity tends to be necessary and may provide information on their multiple abilities in various situations. In the present study, we used an antiradical assay, DPPH radical scavenging capacity, and an inhibition of a complex oxidation by an oxygen uptake method to test the antioxidant activity of essential oil, its major compounds (α-pinene, limonene, (-)-camphene, caryophyllene), different hydro-methanolics extracts from *T. articulata* and positive controls (α-tocopherol and (-)-catechin); the results are shown in table 3.

3.4.1. Oxygen uptake method

Antioxidants are defined as substances that when present at low concentrations compared with those of an oxidizable substrate significantly delay or prevent oxidation of that substrate. In food chemistry, antioxidation is generally restricted to a chain-breaking action during the autoxidation of lipids. Even though other oxidations

are also inhibited by antioxidants, the most important reaction in lipid peroxidation is the autoxidation of unsaturated fatty acids.

In an initiation process, the unsaturated fatty acid (LH) is converted via H abstraction into lipid free radicals ($L^{\cdot}$). The $L^{\cdot}$ free radicals are in turn oxidized by molecular oxygen (O_2) to lipid peroxy radicals $LOO^{\cdot}$. $LOO^{\cdot}$ are able to abstract H atom from new LH molecules in a process referred to as chain propagation. The hydroperoxides (LOOH) are the primary products of lipid peroxidation. In the present case, the peroxidation of methyl linoleate (LH) induced by AIBN can be described by reactions (i_1) - (t_3) [22].

inducer $\rightarrow$ 2 R· (i_1)

R· + $O_2 \rightarrow RO_2$· (i_2)

RO_2· + LH $\rightarrow RO_2H$ + L· (i_3)

L· + $O_2 \rightleftarrows LO_2$· (2)(-2)

LO_2· + LH $\rightarrow LO_2H$ + L· (3)

LO_2· + LO_2· $\rightarrow LO_2L + O_2$ (t_1)

LO_2· +L· $\rightarrow LO_2L$ (t_2)

L· + L· $\rightarrow$ L2 (t_3)

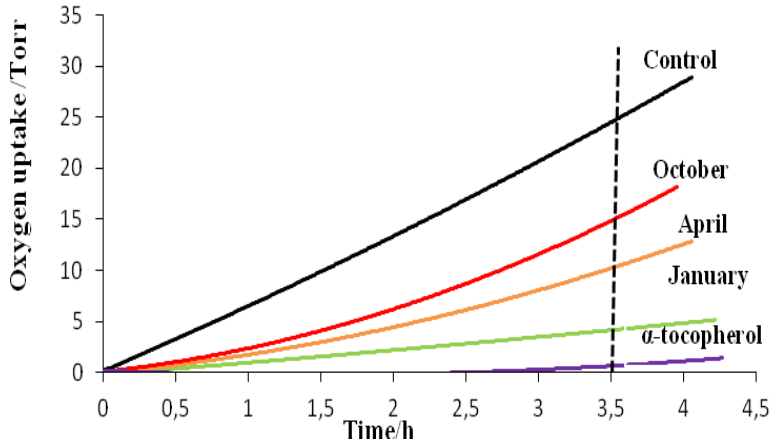

Figure 4 : Influence of the methanol/water 50:50 (v/v) extracts (0.1mg /mL) of *T. ariticulata(*Vahl) Masters on the autoxidation of methyl linoleate (1.6 M) induced by AIBN (1.810^{-2} M) at 60 ◦C in butan-1-ol; P (O2) = 140 Torr.

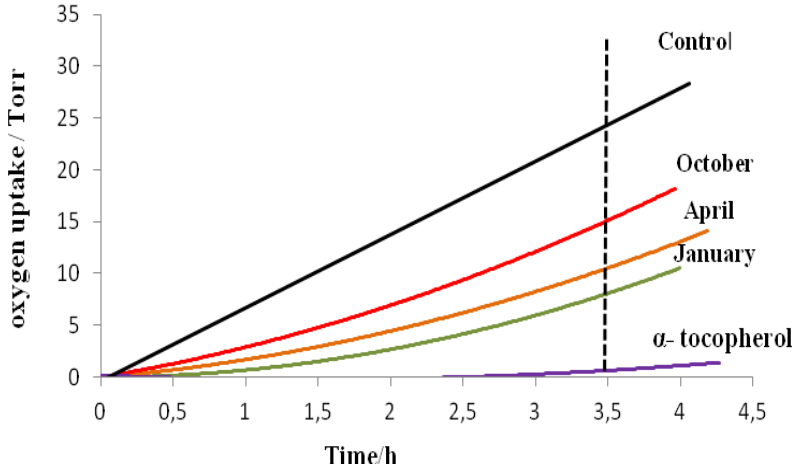

Figure 5: Influence of the methanol/water 80:20 (v/v) extracts (0.1mg /mL) of *T. ariticulata (*Vahl) Masters on the autoxidation of methyl linoleate (1.6 M) induced by AIBN (1.810^{-2} M) at 60◦C in butan-1-ol; P (O2) = 140 Torr.

Therefore, the role of an antioxidant compound (AH) is to break the oxidation chain reaction by acting in the phase of propagation. It donates its mobile H atom to free radicals (reaction 4); if the $A^{\cdot}$ radical produced is unreactive, it stops the kinetic chain of the oxidation (and therefore AH is called a chain breaking antioxidant) and it reacts only (or mainly) in new termination steps (reaction 5), so that the peroxidation is inhibited.

$LOO^{\cdot} + AH \rightarrow LOOH + A^{\cdot}$ (4).
$LOO^{\cdot} + A^{\cdot} \rightarrow LOOA$ (5).

Tetraclinis articulata leaf aqueous methanol extracts of the three periods and essential oil of January were compared with two well-known antioxidants: (-)-catechin, and α-tocopherol with regard to their antioxitative activity. The methyl linoleate oxidation assays are presented in Fig. 4 and 5 for the various extracts. In the conditions of the test OUI of catechin is 100%. It appears that the autoxidation of methyl linoleate alone (control) is almost linear. Antioxidative capacities [OUI (%)] of extracts, defined as the ratio of oxygen uptake at 3.5 h in the presence and in the absence of an extract, are reported in table 3. Let us recall that the higher OUI%, the most efficient the antioxidant. The essential oil showed a weak inhibition with an OUI near 20%, while all aqueous methanol extracts under study inhibit the oxidation of LH induced by AIBN with an OUI going from 39 to 81%. This activity was most important in comparison with the antioxidant effects of α-tocopherol and (-)-catechin (OUI = 98 and 100%, respectively).

Natural antioxidants are being used in the food industries to inhibit lipid peroxidation. Djouahri et al., [6] studied the activities of essential oil and various organic extracts of cone of *Tetraclinis articulata* against lipoxygenase and found an interesting inhibition with an EC_{50} of 8.16 ±0.44, 2.21±0.34, 7.67±0.56, µg/mL, for essential oil, 70% ethanol, and 100% ethanol extracts, respectively. It was also observed that their antioxidant capacities depend on the seasonal variations. Our extracts obtained with 50 % water are clearly more efficient in January than those obtained with 20% water (80.6 vs.67.3%, respectively, for 50 % water and 20 % water), efficiencies of both extracts are lower and not significantly different in November and April. This difference of the activity in these extracts is explained by the difference in the proportions of phenolics components due to the polarity difference of the two solvents; the same result was found by Rached et al. [17], with various species.

Based on the significant difference in the antioxidant effects of aqueous methanol extracts, the three periods are classified in the following order: January > April > November. This antioxidant property difference according to the seasonal variation would be directly attributed to their different composition and/or percentage in phenolic compounds.

3.4.2. DPPH assay

The effect of an antioxidant on DPPH radical scavenging is thought to be due to its hydrogen donating ability or radical-scavenging activity. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced non radical form 1,1-diphenyl-2-picrylhydrazine with the loss of the DPPH violet color. The degree of discoloration indicates the scavenging potential of the substance. DPPH scavenging activity is usually presented by EC_{50} defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. The antioxidant activity of the six aqueous methanol fractions corresponding to two different solvents polarity (methanol: water, 80:20 and 50:50 v/v) and three different periods of *T. articulata*, and controls (α-tocopherol and (-)-cathechin) are presented in table 3. Let us recall that the higher EC_{50}, the less efficient the antioxidant. Different fractions exhibited free radical scavenging activity as indicated by their EC_{50} values and varied significantly ($p<0.05$) with the solvents and periods.

EC_{50} values ranged from 23.3 to 30.9 µg/mL and from 22.8 to 36.8 µg/mL for methanol: water (80:20) and methanol: water (50:50), respectively. All activities were important in comparison with the antioxidant property of two commercial standards α-tocopherol and (-)-catechin (21.2 and 7.6 µg/mL, respectively). This activity can be related to the phenolic content present in this plant, results confirmed by Rached et al. [17] and by Djouahri et al.[6] for water, ethanol, 70 % ethanol, and methanol extracts because their richness in flavonoids and tannic acid, caffeic acid, catechin, rutin and quercetin, phenolic compounds identified in the extracts of this plant.

Generally, it appears that methanol/water (80:20) extracts exhibit a higher antioxidant activity than methanol/water (50:50) extracts. These data can be explained by the low phenolic content in aqueous part of extracts, result documented by Rached et al. [17], Tachkittirungrod et al. [30], and Yen et al. [31], with various species.

The methanol/water extracts of January have the lower EC_{50} values (22.8 and 23.3 µg/mL for 50% methanol and 80% Methanol, respectively) and the higher efficient antioxidant. These extracts present activity very close to that of the control α-tocopherol (EC_{50}= 21.2 µg/mL). These results contrast with those of Rached et al. [17] who observed the strongest accumulation of phenolic compounds and antioxidant activity in aqueous extract of this plant leaves during spring (April).

The present work demonstrates that the radical scavenging activities of all aqueous methanol extracts are important and the active substances are polar, since they are soluble in methanol and water, and their quantities

varied significantly with the season. The *T. articulata* aqueous methanol extracts act as electron or hydrogen donors for DPPH.
The investigated *T. articulata* essential oil of January sample and its major compounds (α-pinene, limonene, myrcene and caryophyllene) demonstrated extremely low antioxidant activities (EC_{50}> 100 mg/mL, in the DPPH test). Our finding is in agreement with Hersi et al. [8, 9] and Djouahri et al. [5,7]. Essential oils, especially when obtained by hydrodistillation, do not contain phenolic compounds, which are known to possess potential antioxidant activity [31-34].

3.5. Correlations between the various measurements
The correlation between the lipid peroxidation inhibition (OUI %) and the total content of phenol compound was performed and positive linear correlation (R^2= 0.680) is shown by fig. 6. Also, a good correlation exists between the DPPH radical scavenging activity of the extracts and their phenolic content (fig. 7, R^2= 0.851); similar result was found by Rached et al. [17], in contrast with those obtained by Ellnain-Wojtaszek et al.[35] on *Ginkgo biloba* L., who reported that DPPH scavenging activity of extracts decreases with accumulation of phenolics during the vegetative cycle.
The antioxidant activity has been attributed to various mechanisms, among which some are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. In the present case the DPPH reaction with an antioxidant AH should be regarded as the analogous to the reaction (4) of inhibition of the oxidation of linoleate and we should not be surprised that there is a correlation between EC_{50} and OUI as shown by figure 8.

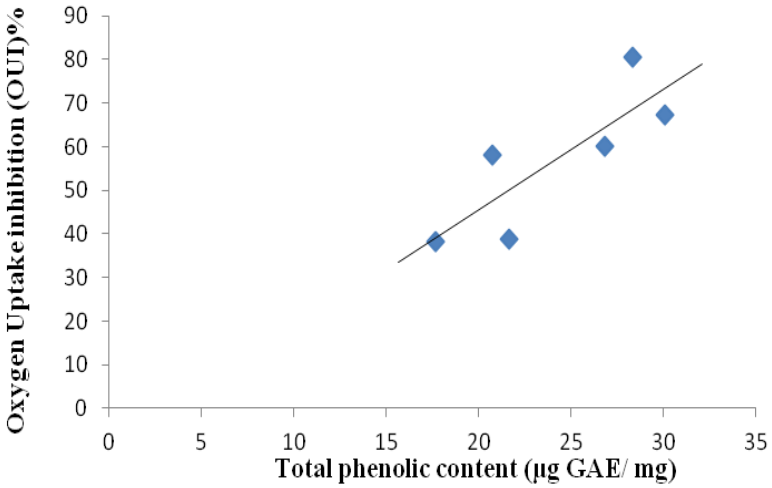

Figure 6 : Correlation between the antioxidant activity OUI % and Total phenolic content of extracts from the *T. articulata* leaves.

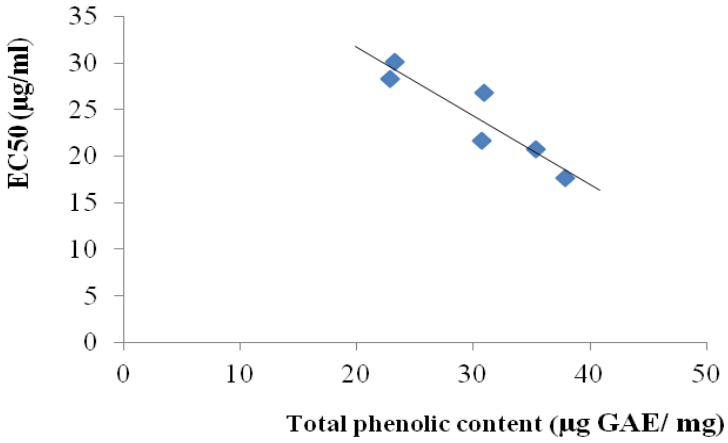

Figure 7 : Correlation between the DPPH scavenging activity and Total phenolic content of extracts from the *T. articulata* leaves.

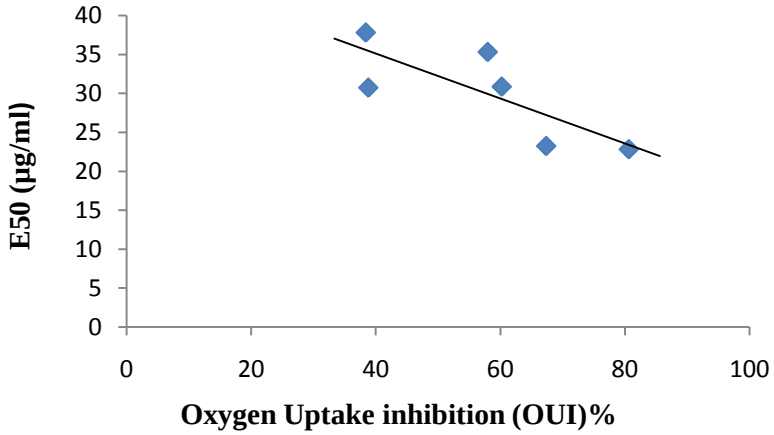

Figure 8 : Correlation between the antioxidant activity OUI % and DPPH scavenging activity t of extracts from the *T. articulata* leaves.

Conclusion

Provided that genetics has much less effect as the environment, we can affirm the first three points:

1. In general, growing season and place affected chemical composition of the essential oils of *T. articulata*.
2. Seasonal and pedological effects were also observed on the extraction yield and the antioxidant activity of leaf extracts.
3. These differences can be attributed to the changes in temperature, humidity and soil type, and therefore to different stages of plant metabolism.
4. As discussed above, aqueous methanol extracts showed good free radical-scavenging activity, a good inhibition of the autoxitation of methyl linoleate, and significant amounts of phenolic compounds, as estimated by the Folin-Ciocalteu method.
5. The present results encourage additional and more in-depth studies on the phenolic composition of the plant extracts and assessment of antioxidant activity of each compound separately. Some phenolic compounds remain to be identified and further biological tests should be conducted.

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