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Research Paper

Chemical composition, acute toxicity and antioxidant activities of *Artemisia arborescens* essential oils from the western Algeria

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Abstract

The objective of this work is the study of the chemical composition, the acute toxicity and some biological activities of *Artemisia arborescens* essential oils (EOs), collected from two regions of Algeria (Sidi Bel Abbes and Bechar). The chemical composition of EOs were established by gas chromatography (GC) and gas chromatography / mass spectrometry (GC/MS). The acute toxicity was assessed using Lorke's approach in Animal Models. The iron chelating effect was tested using ferozzin and lipid antiperoxidation was performed using β-carotene and linoleic acid. Forty-one compounds were identified, accounting for 96.0–98.8% of the total EO. The major chemical components characterizing the EOs were β-thujone, camphor and chamazulene, but the percentage of each compound varied depending on the EOs. The toxicity of Bechar's EO was higher than Sidi bel abbes's EO with LD_{50} of 1264.91 and 2154.07 mg/kg respectively. The chelating effect of ferrous ions gave a result higher than 95% and the propagation of lipid peroxidation was inhibited with a percentage higher than 50% for both samples.

Keywords: *Artemisia arborescens*, essential oil, chemical composition, acute toxicity, iron chelation, lipid antiperoxidation.

Introduction

Artemisia arborescens is a species belonging to the Asteraceae family[1]. It is an aromatic and medicinal plant growing in arid and semi-arid climates around the Mediterranean Sea in many regions of African and European countries[2-4]. This plant is known as " Echiba " in the vernacular language of the North African people. It is used in the folk□medicine for various diseases (skin, bronchial catarrh, fever, asthma and insufficient bile production[5-7]. Furthermore, *A. arborescens* exhibits antifungal, hypoglycemic, anticoagulant[8], antispasmodic, antiangiogenic[9], and antiviral activities[10]. The *A. arborescens* EO has also many properties in the treatment of inflammation, diarrhea, intestinal trouble[11] and high antioxidant activity against free radicals mainly attributed to their components[12,13]. Recently, the essential oil of *A. arborescens* has been used as an ecological pesticide in both conventional and organic agriculture and in domestic and public use. The fresh leaves of the plant are

widely used to flavor tea[14], which is consumed almost daily in North African countries, especially after meals but, this plant is not included in the European Pharmacopeia, probably due to the presence of thujones[15]. Indeed, thujones are one of the major compounds of *A. arborescens* EOs but various chemotypes were found depending on the different environments. For example, in the Mediterranean region, the chemical composition of the *A. arborescens* EO from south-west of Algeria (Bechar) was characterized by β-thujone and camphor, that from west of Algeria (Ain Sefra) only by β-thujone[16], whereas that from the north-west of Algeria (Bejaia) by chamazulene and β-thujone[3]. In Morocco, the EO of *A. arborescens* was characterized by β-thujone and camphor[17]. Finally, in the south of Italy, the chemotypes chamazulene and β-thujone are the most present[18]. According to various studies, the environmental factors have a direct effect on chemotypes so that the plant can adapt to local environmental conditions[19,20]. To our knowledge, there is no information about the toxicity, the iron chelating effect and lipid antiperoxidation of *A. arborescens* EO. Thus, the aim of this study is to show the different chemotypes of *A. arborescens* EOs from two different regions of Western Algeria, their acute toxicity and the chelating effect of ferrous ions and lipid antiperoxidation.

Materials and Methods

Plant material

A. arborescens leaves were collected from the region of Bechar (latitude: 31° 54′ 59″ N; longitude: 2° 18′ 0″ W; altitude: 870 m) and region of Sidi Bel Abbes (latitude: 35° 11′ 38″ N; longitude: 0° 38′ 29″ W; altitude: 483 m) during the flowering stage. Specimens were then dried in the open air for 15 days and stored in a cool place before extraction. A voucher specimen is deposited at the Herbarium of the University of Sidi Bel Abbes (Algeria).

Essential Oil Extraction

Dried leaves were subjected to hydrodistillation for 3 h using a Clevenger apparatus. The EO yields were 0.46 and 0.32 (% w/w) for samples from Bechar and Sidi Bel Abbes respectively. The EOs obtained were dried over anhydrous sodium sulfate and stored in a sealed vial in the dark at 4° before analysis. The *A. arborescens* EOs presented a strong characteristic odor and blue color.

Gas chromatography (GC) / mass spectrometry (GC/ MS) analysis

GC analyzes were performed on a 7890A GC (Agilent Technologies) system with a flame ionization detector (FID) equipped with an HP5 capillary column (30 x 0.25 mm, film thickness 0.25 µm). Experimental conditions were: oven temperature 2 min at 80 °C, then 80 °C to 200 °C (5 °C / min), then 200 °C to 260 °C (20 °C /min), and held at final temperature for 5 min. Injector and detector temperatures were set at 250 °C. Hydrogen was the carrier gas at a flow rate of 1.2 ml/min. One µL of diluted EO (0.05 g in 1.5 ml of CH_2Cl_2) was injected. Linear retention indices were calculated with reference to n-alkanes (C_8–C_{28}). The identification of the compounds was based on the comparison of their retention indices with those of authentic samples in literature[21].

GC/MS analysis of the EO was carried out on an Agilent Technologies GC instrument equipped with a GC 7890A gas chromatograph system, an MS 5975C VL MSD mass spectrometer detector, and an HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The data acquisition and processing were performed using the MSD Chemstation E.01.01.335 (Agilent) software. One µL of diluted EO (0.05 g in 1.5 ml of CH_2Cl_2) was injected. The experimental conditions were: solvent delay, 2 min, column temperature program, 2 min at 80°C, then 80 to 200°C (5°C/min), then 200 to 260°C (20°C/min), and held at the final temperature for 5 min, temperature injector (split ratio 60) and detector were 250°C, carrier gas was helium at a flow rate of 1.2 ml/min, ionization voltage 70 eV, electron multiplier, 1 kV. The identification of the components was based on the comparison of their mass spectra with those of the Wiley and NIST libraries.

Acute toxicity study in mice

BALB/c mice (30–40 g) were obtained from the *Pasteur Institute of Algeria.* Mice were housed, fed and kept under normal conditions with a light/dark cycle (12 h/12 h). 14 hours before experiments, food was withheld, but animals had free access to drinking water. The EOs were suspended in a vehicle (Tween 80, 0.9% in saline). The intraperitoneal injection (IP) volume is 10 mL/Kg of the animal's body weight as recommended by the American Association for laboratory animal science[22]. The EOs toxicity was determined according to Lorke's approach[23]. In the first step of this method, it is

necessary to determine the approximate extent of toxicity. This last was obtained by forming three groups of three mice, which each group receives intraperitoneally (IP) doses of 10, 100 and 1000 mg/kg body weight respectively. The second step consists of administering specific doses chosen according to the number of deaths in the first phase as shown in Table 1.

Table 1: Doses required according to Lorke's approach in mice[23]

First step doses (mg/kg body weight)			Second step doses (mg/kg body weight)			
10	**100**	**1000**				
0/3[a]	0/3	0/3	/	1600	2900	5000
0/3	0/3	1/3	600	1000	1600	2900
0/3	0/3	2/3	200	400	800	1600
0/3	0/3	3/3	140	225	370	600
0/3	1/3	3/3	50	100	200	400
0/3	2/3	3/3	20	40	80	160
0/3	3/3	3/3	15	25	40	60
1/3	3/3	3/3	5	10	20	40
2/3	3/3	3/3	2	4	8	16
3/3	3/3	3/3	1	2	4	8

[a] **:** Number of animals died/number of animals used

In this second step, only three animals were used which was divided into three groups, one animal each. In both phases, the animals were observed for two hours after the administration of the treatments. The number of mortalities, toxic effects and/or changes in general behavior were noted after 24 hours. The test is completed by determining the lethal dose for 50% of the animals (LD_{50}) using the following formula[24]:

$$LD_{50} = \sqrt{D_0 \times D_{100}},$$

D_0 = Highest dose that gave no mortality, D_{100} = Lowest dose that produced mortality.

The acute toxicity experiments were performed according to the ethical principles of the European Union Directive (2010/63/EU) on the protection of animals used for scientific experiments.

Iron Chelation Test

Ferrous ion chelation (FIC) assay of EOs was realized according to the method described by Gülçin[25]. Briefly, 1 ml of $FeSO_4$ (0.1mM) was added to 1 ml of various dilutions of *A. arborescens* EOs (1.75, 2.50 and 5.00 mg/ml), followed by 1 ml of ferrozine (0.25 mM). The reaction mixtures were incubated at room temperature for 10 min before the absorbance measurements were taken at 562 nm. Ascorbic acid, catechin, gallic acid and EDTA were used as positive controls. The percentage of inhibition of ferrozine complex formation was given in the formula below:

$$\text{Ferrous ions chelating (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 and A_1 are the absorbance of the negative control and EOs, respectively. The negative control contained 1 ml each of methanol, $FeSO_4$ and ferrozine.

***In vitro* Lipid antiperoxidation effect**

Lipid peroxidation inhibitory activity in β-carotene bleaching system was determined using the method described by Sun *et al.*[26]. It is a technique that simulates the lipid peroxidation reaction *in vitro*.

β-carotene (2 mg) that was dissolved in chloroform (10 ml) was mixed with linoleic acid (20 mg) and Tween 40 (200 mg). The chloroform was removed by a rotary evaporator at 40 °C. 50 ml of H_2O was slowly added to the residue. 0.2 ml of the Eos (2 mg/ml) or standard antioxidants (ascorbic acid, catechin and gallic acid) were mixed with 5 ml of the obtained emulsion. The mixture was incubated at 50 °C and the absorbance was read at 470 nm every 20 min until 120 min. The inhibition percentage was calculated according to the following equation[27] :

$$I\% = [1 - (A_0 - A_t) / (A_0^1 - A_t^1)] \times 100,$$

where, A_0 and A_0^1 are the absorbance measured at zero time of incubation for EO and control respectively, and A_t and A_t^1 are the absorbance measured in EO and control respectively, after the incubation for 120 min. Control contained 0.2 ml water and 5 ml emulsion.

Statistical analysis

One-way ANOVA analysis followed by Tukey's multiple comparisons procedure was used to determine significant differences between both samples of *A. arborescens* and standards analyzed. All determinations were performed in triplicate, in three separate experiments and the results are expressed as mean ± SD. All data were performed using the statistical software Minitab 18.

Results and Discussion

Essential Oil Composition

The chemical compositions of the oils were investigated using GC and GC/MS techniques (Table 2). Forty-one compounds were identified, representing 98.81% and 97.62% of the oil for Bechar and Sidi Bel Abbes respectively.

Table 2: Chemical composition of *Artemisia arborescens* essential oil from two Algerian (Bechar and Sidi Bel Abbes) sites

RI[a]	RI[b]	Compound	Origin	
			Bechar	Sidi Bel Abbes
913	924	α-Thujene	0.31	0.12
925	932	α-Pinene	1.15	0.56
945	954	Camphene	1.22	0.32
974	946	Sabinene	0.90	0.85
981	969	6-Methyl-5-hepten-2-one	0.23	0.11
982	974	β-Pinene	tr[c]	0.21
985	988	Myrcene	2.12	1.04
1010	1002	α-Phellandrene	0.15	0.12
1019	1014	α-Terpinene	0.78	0.14
1026	1020	*p*-Cymene	1.35	0.28
1030	1024	Limonene	0.4	0.29
1034	1026	Eucalyptol	0.33	0.21
1058	1054	γ-Terpinene	1.98	0.33
1074	*1065*	*cis*-Sabinene hydrate	0.31	10.84
1087	1086	Terpinolene	0.39	0.13
1095	1095	Linalool	1.77	4.57
1107	1101	α-Thujone	1.34	1.67
1115	1112	β-Thujone	30.78	0.28
1123	1118	P-menth-2-en-1-Ol	0.21	0.32
1150	1141	Camphor	22.35	56.68
1173	1165	Borneol	tr	0.76
1183	1174	Terpinen-4-ol	7.81	3.78
1196	1186	α-Terpineol	0.75	0.66
1272	1261	Chrysanthenyl Acetate	2.76	0.18
1282	1269	Perillaldehyde	0.28	0.21
1293	1284	Bornyleacetate	tr	0.11
1298	1289	Thymol	0.18	0.12
1384	1374	α -Copaene	0.24	0.15
1396	1387	β-Bourbonene	tr	0.15
1408	1403	Methyleugenol	0.27	0.12
1432	1419	β-Caryophyllene	0.61	0.46
1464	1454	α-Caryophyllene	tr	tr
1491	1484	Germacrene D	0.73	0.11
1513	1505	α-Farnesene	0.16	0.38
1524	1513	γ- Cadinene	0.52	0.8
1534	1522	δ- Cadinene	Tr	0.4
1558	1548	Elemol	0.73	0.29
1595	1582	Caryophyllene oxyde	0.12	tr
1665	1649	β-Eudesmol	tr	0.96

1738	1730	Chamazulene	15.58	8.91
		Monoterpenehydrocarbons	10.75	4.39
		Oxygenatedmonoterpene	68.87	80.39
		Sesquiterpenehydrocarbons	17.84	11.36
		Oxygenatedsesquiterpene	0.85	1.25
		Others	0.5	0.23
		Total identified	98.81	97.62

[a]Retention indices on HP-5 capillary column. [b]Retention indices in the bibliography. [c]trace.

The major compounds were oxygenated monoterpenes (35.91 – 84.79%) including *cis*-sabinene hydrate (0.31 – 10.84%), linalool (1.77 – 4.57%), β-thujone (30.78 – 0.28%), camphor (22.35–56.68%) and le terpinen-4-ol (3.78–7.81%), followed by hydrocarbon sesquiterpenes (11.36 – 17.84%) mainly represented by chamazulene (8.91 – 15.58%). Hydrocarbon monoterpenes and oxygenated sesquiterpenes were found in small quantities: α-pinene (0.56–1.12%), camphene (0.32 – 1.22%), myrcene (1.04 – 2.12%), *p*-cymene (0.28 – 1.35%), γ-terpinene (0.33–1.98%), elemol (less than 0.73%), caryophyllene oxide (less than 0.12%) and β-eudesmol (less than 0.96%).

Indeed, the majority constituents of Bechar's EO are β-Thujone (30.78%) followed by camphor (22.35%) and chamazulene (15.58%) and finally terpinen-4-ol (7.81%). However, EO from Sidi Bel Abbes has a high content of camphor (57.68%) followed by *cis*-sabinene hydrate (10.84%) and chamazulene (8.91%). According to these results, we can say that Bechar's EO is a thujone chemotype while Sidi Bel Abbes's EO is a camphor chemotype. The chemical composition of our EOs is in agreement with those previously reported in the bibliography. For example, those realized by Said *et al.*, where the EO of *A. arborescens* from the region of Bechar were mainly characterized by β-thujone (26.98%), camphor (25.70%) and chamazulene (13.78%)[16]. Other works also show that EO of the same species collected in southern Morocco and Lebanon are a thujone chemotype (36–69%)[1,17]. Another study carried out by Younes *et al.* which shows that the oils of this species collected in different regions of northwestern Algeria, including our second sampling (Sidi Bel Abbes region), are characterized by a high content of camphor (33–72%)[28]. These qualitative and quantitative differences in the chemical composition of EOs could be attributed to several factors such as environmental conditions, especially drought, which can strongly influence the composition of the plant lipophilic fraction[29-31] due to their influence on physiological mechanisms related to secondary metabolism[32,33]. In the present study, the EO chemotype from a relatively arid climate (Bechar) is different from that of a relatively semi-arid environment (Sidi Bel Abbes). These differences in aridity which have a relation with the collection sites are confirmed by the De Martonne index (Figure1) especially from October to April.

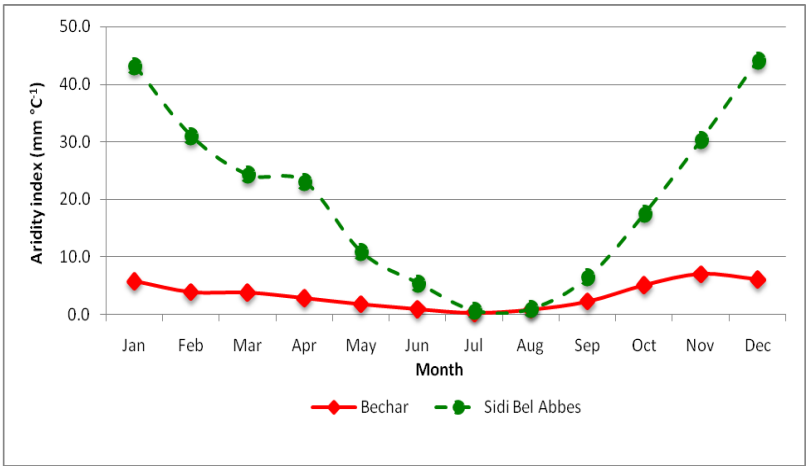

Figure 1: De Martonne index, calculated every month, of the collection sites from Algeria (Bechar and Sidi Bel Abbes). De Martonne index lower than 15, between 15 and 30, and higher than 30 indicate an arid month, semi-arid to subhumid, and humid month, respectively.

Also, Bechar showed 12 dry months according to Figure 1 and Sidi Bel Abbes five dry months (from May to September). The harvesting season, the nature of the soil, the age of the plant parts (young or

adult), the plant material (dried or fresh), the part of the plant used (leaves or flowers) and the time of collection can also influence the composition of an EO[34-37].

Acute toxicity

In vivo acute toxicity test were estimated for *A. arborescens* EOs in mice using Lorke's method. The results of the first step determining the approximate extent of toxicity by the administration of the 10 mg/kg. 100 mg/kg and 1000 mg/kg doses are presented in Table 3.

Table 3: Results of the first step of the toxicity test

Step 1			
Dose (mg/kg)	**10**	**100**	**1000**
Number of mortalities after 24 h			
Bechar's EO	0/3	0/3	1/3
Sidi Bel Abbes's EO	0/3	0/3	0/3

IP administration of *A. arborescens* EOs at doses of 10 and 100 mg/kg had no apparent effect on animals behaviour, which means that these doses are probably not toxic. However, the administration of the 1000 mg/kg dose induced a change in behavior and also the appearance of few signs of toxicity: agitation, disorientation and inconsistency. These were very remarkable in mice treated with Bechar's EO. This can be explained by the high content of β-thujone which is a neurotoxic ketone and these symptoms are often attributed to it[38-40]. Based on the number of mortalities in the first step, we were able to determine the doses to be administered in the second step according to Lorke's protocol[23] (Table 4).

Table 4: Results of the second step of the toxicity test

Step 2						LD_{50} (mg/kg)
Dose (mg/kg)	**600**	**1000**	**1600**	**2900**	**5000**	
Number of mortalities after 24 h						
Bechar's EO	0/1	0/1	1/1	1/1	/	1264.91
Sidi Bel Abbes's EO	/	/	0/1	1/1	1/1	2154,07

The results revealed that HE from the Bechar region is more toxic than that from Sidi Bel Abbes with LD_{50} values of 1264.91 and 2154.07 mg/kg respectively. Thujones induce convulsions and a lethal effect in rodents[15,41], which may explain this difference in toxicity between the two EOs.

Iron chelation

The ferrous ion chelation test of *A. arborescens* EO shows activity, which is not dose dependent (P=0.551). As shown in Figure 2, at the highest concentration (5 mg/ml), the two EO samples had the highest activity: 96.9 and 98.2% (Sidi Bel Abbes and Bechar respectively).

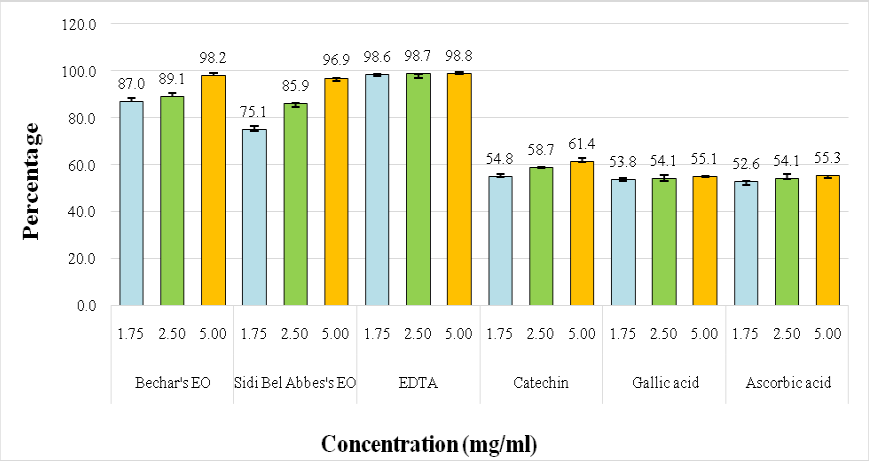

Figure 2: Percentage of ferrous ions chelation of *A. arborescens* EOs from Algerian sites (Bechar and Sidi Bel Abbes) and ascorbic acid, catechin, gallic acid and EDTA as positive controls

This chelating effect is superior to that of the reference phenolic standards (ascorbic acid, catechin, gallic acid) used with a highly significant difference (P <0.001). The differences between the majority compounds of the two EOs did not induce a significant difference in terms of activity, which indicates that there is no difference between the EOs of the two regions studied (P =0.115). Studies on *A. absinthium* EO, which is considered to be analogous to *A. arborescens*, revealed a chelating effect of 53% at a concentration of only 1 mg/ml[42]. The presence of transition metal ions in a biological system could catalyze Haber-Weiss and Fenton-type reactions, leading to the formation of hydroxyl radicals[43]. In this assay, the ferrous ion was used as a transition metal ion and the *A. arborescens* EOs showed very considerable chelating activity.

Lipid antiperoxidation effect

In vitro lipid peroxidation inhibitory activity consists in measuring the bleaching of β-carotene resulting from its oxidation by the degradation products of linoleic acid. The oxidation of the latter forms peroxyl radicals which lead to the loss of the chromophore and the characteristic orange color of β-carotene[44]. The bleaching of β-carotene is followed by the measurement of its absorbance in fixed time intervals (20 min), which made it possible to trace the kinetics curves (Figure 3).

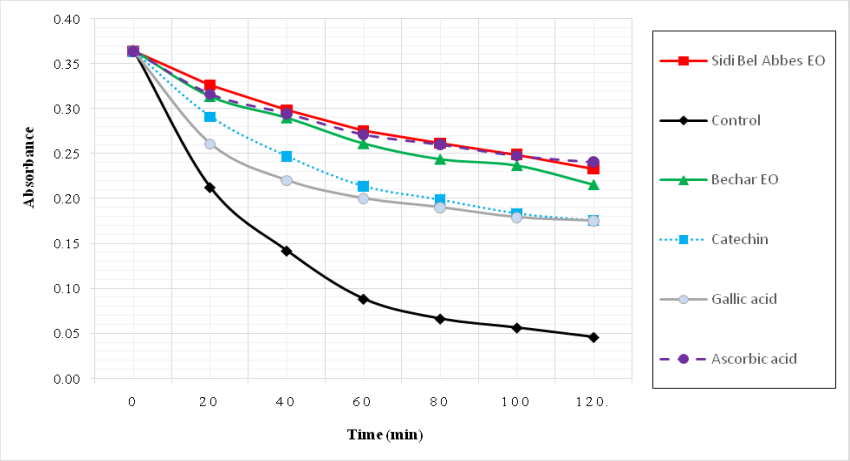

Figure 3: β-carotene bleaching kinetics of *A. arborescens* EOs from Algerian sites (Bechar and Sidi Bel Abbes), ascorbic acid, catechin and gallic acid as standards and control

At t=0 min, there was no difference between the absorbances of the samples/standards and negative control (P =0.077). From t=20 min, the difference became highly significant (P <0.001). It was found that *A. arborescens* EOs preserved the color of β-carotene in a similar manner to that of ascorbic acid (P >0.05) and superior to phenolic standards (catechin and gallic acid) (P <0.05). The inhibition percentage does not show a significant difference between the EOs of Bechar and Sidi Bel Abbes regions (Table 5).

Table 5: Percentages of antiperoxidation activity

EO and Standards	I%
Sidi Bel Abbes's EO	58.78 ±1.1
Bechar's EO	53.06 ± 2
Catechin	41.03 ±1.6
Gallic acid	40.86 ± 2.1
Ascorbic acid	61.41 ± 1.3

The addition of EOs did not block the peroxidation initiation phase, but it delayed the propagation of the reaction compared to the negative control (-) and this with percentages of activity of 53.1 and 58.8% for the EO from Bechar and Sidi Bel Abbes respectively (Table 5). So the antioxidants in the EOs can neutralize hydroperoxides and prevented the bleaching of β-carotene[45]. The comparison with other works is a delicate task because of certain limitations of the technique, such as the different concentrations of extracts used by the researchers and also the thermally induced oxidation of linoleic acid which is not controlled. Furthermore, the work of Daíse Lopes *et al.* carried out under experimental conditions similar to ours, on some species of the genus *Artemisia*, including *A. absinthium*, shows that with an EO concentration of 1 mg/ml, the percentages of peroxidation

inhibition vary between 10 and 20% in the majority of species[12]. To our knowledge, this is the first study to provide data that *A. arborescens* EO possess a significative lipid antiperoxidation effect.

Conclusion

The objective of this study was the chemical analysis of *A. arborescens* EOs from South-West and North-West Algeria, as well as the influence of the composition on acute toxicity and biological activity since this plant is used daily to flavor tea and also as a remedy in many pathological states. The results revealed two different chemotypes: Thujone for Bechar's EO (South-West) and camphor for Sidi Bel Abbes's EO (North-West). The toxicity of thujone had an impact on the results obtained since we were able to demonstrate that the EO from the first region is more toxic than that from the second with LD_{50} values of 1264.91 and 2154.07 mg/kg respectively. This difference in majority compounds did not influence the biological activities tested. According to the results of the statistical study, there are no significant differences between both samples of *A. arborescens* EO. In the first test, the chelating effect of ferrous ions was greater than 75% with a maximum activity of 96.9 and 98.2% for Sidi Bel Abbes's and Bechar's EOs respectively. In the second test, the propagation of peroxidation was inhibited by a percentage of 53.06% for the EO of Bechar and 58.78 for that of Sidi Bel Abbes, which is close to the best antioxidant standard used (61,41%). These results are promising and reveal that EO from *A. arborescens* can compensate for the oxidative stress caused by heavy metals and lipoperoxidation and its adverse effects on biological systems. The EO of this plant has great potential and other *in-vitro* and *in-vivo* biological tests are planned to explore the powers of this species.

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