Isolation of essential oil from *Ailanthus altissima* (Mill) stems: Chemical composition, separation and biological activities

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Abstract: The essential oil from *Ailanthus altissima* stems, an aromatic and medicinal plant, is widely used in the Chinese folk medicine**.** The essential oil, obtained by hydrodistillation, was fractionated using a silica gel column, yielding six different fractions. The chemical compositions of the volatile oil together with its fractions were investigated using Gas chromatography/Flame Ionization Detector (GC/FID) and Gas chromatography-Mass Spectrometry (GC-MS). The stems EO was a complex mixture of fifty-six compounds, mainly composed by sesquiterpene hydrocarbons (77.9%), among which γ-muurolene (41.1%), β-caryophyllene (23.5%) and α-humulene (6.0%) were the most represented. The comparison of this composition (may 2015) of the essential oil of the same plant harvested in the same locality and at the same times of the year (May 2012), showed significant differences that may be explained by the variation of the climatic conditions. The essential oil and its fractions were screened for the first time for their *in vitro* anti-5-lipoxygenase, anti-acetylcholinesterase and xanthine oxidase inhibitory properties. Results indicated that fraction 1 and fraction 6 displayed potent anti-5-lipoxygenase activity, with an inhibition percentage of 57.95 ± 2.65 and 60.09 ± 1.09%, respectively. These findings suggest that the essential oil of *A. altissima* stems and its active fractions might have a potential therapeutic application for the treatment of inflammatory diseases after confirmation of this activity by *in vivo* studies and clinical trials.

Keywords: *Ailanthus altissima*, stems essential oil, fractionation, chemical variability, biological activities.

INTRODUCTION

Medicinal and aromatic plants (MAPs), also known as herbal drugs can be defined as botanicals that provide people with medicines to prevent disease, combat pain and suffering, maintain health or cure ailments. They benefit almost everyone on earth through nutrition, toiletry, bodily care, incense and ritual healing [1]. They are also the starting materials for value-added processed natural ingredients, such as essential oils, dry and liquid extracts and oleoresins. In fact, as a traditional Chinese herbal plant, *Ailanthus altissima* has, been used since ancient times not just as a bitter aromatic drug, but also in the treatment of dysentery, gonorrhea, hemorrhoids and as a remedy for cough, gastric and intestinal diseases [2]. This species contains a wide variety of active compounds, such us proteins, flavonoids, alkaloids, quassinoids, terpenylated coumarins, lipids and fatty acids, tetracyclic triterpenoids and volatile oils, among others. It is endowed with antibacterial, antiviral, antioxidant, cytotoxic,

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antidiarrheal, anti-inflammatory, antipyretic, analgesic, antituberculosis, antihistaminic, antiparasitic, insect repellent, anti-progestogenic and many other pharmacological proprieties [2,3]. In recent years, the investigations on the essential oils from natural herbs have drawn considerable attention by the researchers, and some of the EOs have been broadly applied in the fields of pharmaceutical, agricultural, sanitary and cosmetic industries [4]. The oil composition of *A. altissima* organs was studied by several authors [3][5-7]. To the best of our knowledge, no previous study of the anti-5-lipoxygenase (anti-5-LOX), anti-acetylcholinesterase (anti-AChE) and anti-xanthine oxidase activities (anti-XOD) of the essential oil of *A. altissima* stems have ever been conducted.

Hence, in order to study the effect of soil conditions, significantly changed from 2012 to 2015 in Tunisia, the chemical composition of the essential oil obtained from the stems of the plant *A. altissima* growing in Tunisia, taken as a model, was studied; furthermore, a chromatographic fractionation of the oil was performed for a better identification. Then, the isolated essential oil and its fractions were screened for their anti-5-LOX, anti-AChE and anti-XOD activities.

RESULTS AND DISCUSSION

1. Chemical composition of the essential oil

The content and chemical compositions of all the investigated essential oil fractions are listed in Table I. Overall, fourteen compounds were

Table I: Chemical composition of the fractionated essential oil of *Ailanthus altissima* stems.

N.	Compounds	LRI [a]	Relative content [%] [b]							
			EO	F1	F2	F3	F4	F5	F6	EO [c]
1	Phenylacetaldehyde	1045	- [d]	-	-	1.3	1.2	-	-	-
2	Linalool	1101	-	-	-	0.4	2.8	-	-	1.0
3	Nonanal	1102	-	-	-	0.7	-	-	-	-
4	Isopentylisovalerate	1105	-	-	-	-	2.7	-	-	-
5	*α*-Terpineol	1190	-	-	-	-	-	1.8	6.8	-
6	Nerol	1227	-	-	-	-	-	0.8	-	-
7	Geraniol	1256	-	-	-	-	-	4.6	1.8	-
8	Thymol	1292	-	-	-	2.2	-	-	-	-
9	(*E*,*E*)-2,4-decadienal	1316	-	-	-	1.6	-	-	-	-
10	7-Epi-silphiperfol-5-ene	1345	-	0.2	-	-	-	-	-	-
11	*α*-Cubebene	1352	0.5	0.6	-	-	-	-	-	0.4
12	Eugenol	1358	-	-	-	2.8	-	-	-	-
13	*α*-Copaene	1377	1.0	1.9	-	-	-	-	-	-
14	(*E*)-*β*-Damascenone	1382	-	-	-	0.9	-	-	-	-
15	*β*-Cubebene	1391	-	0.3	-	-	-	-	-	-
16	*β*-Elemene	1392	-	0.3	-	-	-	-	-	-
17	*β*-Caryophyllene **(1)** [e]	1419	23.5	28.1	-	-	-	-	-	18.9
18	2,5-Dimethoxy-p-cymene	1424	-	-	0.9	-	-	-	-	-
19	*α*-Humulene **(2)**	1455	6.0	8.7	-	-	-	-	-	6.5
20	(*E*)-Geranylacetone	1455	-	-	-	1.1	-	-	-	-
21	*γ*-Muurolene **(3)**	1478	41.1	50.2	-	-	3.8	-	-	0.9
22	Unknown	1486	-	-	23.1	-	-	-	-	-

N.	Compounds	LRI [a]	Relative content [%] [b]							
			EO	F1	F2	F3	F4	F5	F6	EO [c]
23	Epi-cubebol	1494	-	-	-	-	1.8	-	-	-
24	Bicyclogermacrene	1495	1.6	1.7	-	-	-	-	-	-
25	2-Tridecanone	1496	-	-	-	2.4	-	-	-	-
26	*α*-Muurolene	1499	-	0.2	-	-	-	-	-	0.6
27	(*E*,*E*)-*α*-farnesene	1508	2.1	3.4	-	-	-	-	-	-
28	Cubebol	1515	-	-	-	-	4.4	-	-	-
29	*δ*-Cadinene	1524	2.1	3.5	-	-	-	-	-	3.1
30	(*E*)-Nerolidol	1564	-	-	-	3.8	-	-	-	-
31	Spathulenol	1577	-	-	-	-	5.0	-	-	-
32	Dendrolasin	1580	-	0.2	-	-	-	-	-	-
33	Caryophylleneoxide **(4)**	1582	1.8	-	-	34.8	-	-	-	8.3
34	Globulol	1584	-	-	-	-	-	2.6	-	0.5
35	Viridiflorol	1591	-	-	-	-	3.2	-	-	-
36	Humulene epoxide II	1607	-	-	-	13.1	-	-	-	2.2
37	Tetradecanal	1614	1.4	-	8.6	6.7	-	-	-	1.6
38	Humulane-1,6-dien-3-ol	1615	-	-	-	10.6	-	-	-	-
39	Epi-*γ*-eudesmol	1615	-	-	-	-	2.6	-	-	-
40	*1*-Epi-cubenol	1629	-	-	-	-	-	2.3	-	1.5
41	*γ*-Eudesmol	1632	-	-	-	-	2.3	-	-	-
42	Caryophylla-4(14),8(15)-dien-5-ol	1636	-	-	-	-	2.5	-	-	1.0
43	*T*-Cadinol	1640	2.0	-	-	-	34.6	-	-	2.8
44	Cubenol	1642	-	-	-	7.7	-	-	-	-
45	*α*-Muurolol	1647	-	-	-	-	-	7.3	-	-
46	*T*-Muurolol	1649	-	-	-	-	-	6.1	-	1.0
47	*β*-Eudesmol	1650	-	-	-	-	6.8	-	-	-
48	*α*-Cadinol**(5)**	1655	2.1	-	-	3.0	12.1	48.2	6.9	4.1
49	1-Heptadecene	1692	-	-	-	-	8.2	3.2	-	-
50	Eudesma-4(15),7-dien-1*β*-ol	1693	-	-	-	-	-	18.2	3.7	-
51	Pentadecanal	1716	1.4	-	10.4	-	-	-	-	3.0
52	Mintsulfide	1740	-	-	0.9	-	-	-	-	-
53	Tetradecanoic acid	1765	-	-	-	-	-	-	75.5	-
54	1-Octadecene	1793	-	-	-	-	-	1.7	-	-
55	2-Ethylhexyl salicylate	1811	-	-	1.6	-	-	-	-	-
56	Hexadecanal **(6)**	1817	7.6	-	51.5	-	-	-	-	4.5

Compounds	LRI [a]	Relative content [%] [b]							
		EO	F1	F2	F3	F4	F5	F6	EO [c]
Oxygenatedmonoterpenes		0.0	0.0	0.9	2.6	2.8	7.2	8.6	1.0
Sesquiterpenehydrocarbons		77.9	99.1	0	0	10	0	0	54.1
Oxygenatedsesquiterpenes		5.9	0.2	0	73	69.1	84.7	10.6	25.6
Apocarotenes		0	0	0	2	0	0	0	-
Phenylpropanoids		0	0	0	2.8	0	0	0	-
Sulfurderivatives		0	0	0.9	0	0	0	0	-
Others		10.4	0	72.1	12.7	12.1	4.9	75.5	0.5
Total identified %		94.2	99.3	73.9	93.1	94.0	96.8	94.7	91.0

[a] linear retention index as determined on a *HP-5MS* column using homologous series of *n*-alkanes (C_8-C_{30}).
[b] The contents were determined on a *HP-5* capillary column.
[c] Chemical composition of the EO studied by El-Ayeb-Zakhama et al. [8].
[d] -: Not detected.
[e] Number of the compound in **Fig. 1**.

identified (94.2% of the total oil). Sesquiterpene hydrocarbons and oxygenated sesquiterpenes were the main chemical classes and were represented respectively by 77.9% and 5.9% of the total essential oil, while other compounds attained 10.4%. As mentioned before, in order to identify more compounds, the essential oil was fractionated by column chromatography into six fractions. The study of the chemical composition of the collected fractions allowed the identification of additional forty-two components. Considering the main chemical groups of *A. altissima* volatiles, the six fractions were characterized by their high content of sesquiterpene hydrocarbons (10.0-99.1%), oxygenated sesquiterpenes (0.2-84.7%) and non-terpene compounds (4.9-75.5%), while oxygenated monoterpenes expressed the lowest content (0.9-8.6%).

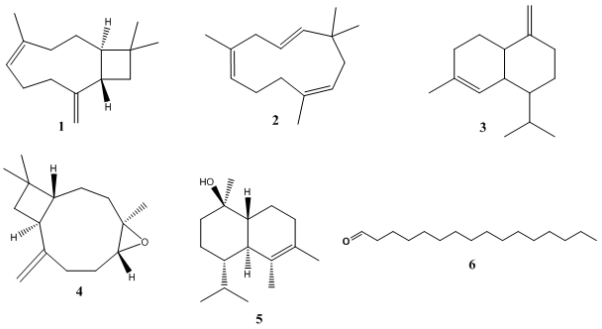

Figure 1: Chemical structures of some major constituents of the essential oil from stems of *Ailanthus altissima*. **1**, β-caryophyllene; **2**, α-humulene; **3**, γ-muurolene; **4**, caryophyllene oxide; **5**, α-cadinol; **6**, hexadecanal.

The chemical structures of some of the major constituents are shown in Figure 1. In fraction 1, thirteen compounds were identified, representing 99.3% of the total composition with a predominance of sesquiterpene hydrocarbons (99.1%), among which *γ*-muurolene (50.2%), *β*-caryophyllene (28.1%) and *α*-humulene (8.7%) were the primarily represented constituents. For fraction 2, 73.9% of the composition was identified while one compound (23.1%) remained unidentified. In this fraction, the content of aliphatic volatile compounds was 70.5% and the main components were hexadecanal (51.5%), pentadecanal (10.4%) and tetradecanal (8.6%). Fraction 3 represented 93.1% of all the identified compounds and contained a significant amount (73%) of oxygenated sesquiterpenes, with caryophyllene oxide (34.8%) and humulene epoxide II (13.1%) being the most abundant ones. For fraction 4, 94.0% of the total composition was identified, with *T*-cadinol (34.6%) as the major constituent. In fraction 5, 96.8% of the composition was characterizedand oxygenated sesquiterpenes exhibited the highest contribution (84.7%), with *α*-cadinol (48.2%) and eudesma-4-(15)-7-dien-1*β*-ol (18.2%) as the dominant compounds. Moreover, five identified compounds constitute the fraction 6 that accounted for 94.7% of the essential oil.

Essential oils of different parts, including stems, were studied by El-Ayeb-Zakhama et al. [7]. The plant material was collected from the same locality (Zaghouan, Tunisia) and in the same season of the present study (spring 2015). This previous investigation showed that the essential oil isolated in 2012 from *A. altissima* stems was dominated by *β*-caryophyllene, (*Z*)-caryophyllene and germacrene *D*. The two latter were not found in our stems essential oil. Among the thirty-one compounds identified in the study described by El-Ayeb-Zakhama et al. [7], only 19 were found in our oil. Besides, in our essential oil, the content of the major compounds, such as *γ*-muurolene (41.1%) and *β*-caryophyllene (23.5%) was higher than those reported by El-Ayeb-Zakhama et al. [7], which reached 0.9% and 18.9%, respectively. Moreover, caryophyllene oxide (1.8%), *δ*-cadinene (2.1%), *α*-cadinol (2.1%) and pentadecanal (1.4%) were obtained in lowest amounts compared to the study of El-Ayeb-Zakhama et al. [7], where they reached 8.3, 3.1, 4.1 and 3.0%, respectively. Furthermore, we identified other compounds not reported in the previous study. The content of these compounds varied between 1.0 and 2.1%, such as *α*-copaene (1.0%), bicyclogermacrene (1.6%) and (*E,E*)-*α*-farnesene (2.1%). On the other hand, we noted that the fractionation of our essential oil allowed the identification of many other components, among which humulene epoxide II (13.1%, F3), humulane-1,6-dien-3-ol (10.6%, F3), 1-heptadecene (8.2%, F4), cubenol (7.7%, F3), *α*-muurolol (7.3%, F5), *β*-eudesmol (6.8%, F4) and geraniol (4.6%, F5) were the most significant. These compounds were not reported in the aforementioned study of El-Ayeb-Zakhama et al. [7], except humulene epoxide II.

These differences in the contents of the dominant compounds and in the chemical composition between our results and those of El-Ayeb-Zakhama et al. [7] may be due to several factors. Considering that the harvests were conducted at the same locality, from the same tree and at the same season of the year, May 2012 (average T = 19.8 °C; average rainfall = 14.0 mm) and May 2015 (average T = 23.6 °C; average rainfall = 11.0 mm), it is probable that the climatic conditions of the harvest year may play a primary role for the significant variation found in the essential oil contents. As mentioned by Moraes [8], the harvest time may reorient the metabolic pathway, leading to the biosynthesis of different compounds and causing an increase in the levels of certain compounds in detriment of others, as observed in our study. It was believed that such changes might be due to differences in climate conditions (temperature, solar exposure, dryness, humidity, wind exposure, etc.). In fact, many authors concluded that the contents of essential oil and its chemical composition are extremely dependent on weather conditions and environmental factors [9-12].

On the other hand, storage methods, drying methods, light, contaminations, oxidation, etc., could also affect the amounts of secondary metabolites [13].

2. Anti-5-lipoxygenase activity

The *in vitro* anti-inflammatory activity (anti-5-LOX) of stems essential oil of *A. altissima* and its fractions was evaluated at the concentration of 50 mg/L and was compared to the NDGA, used as positive control. As shown in Table II, the EO exhibited a good anti-inflammatory activity, with an inhibition percentage (IP) value of 45.63 ± 1.92% and among the tested fractions, 1 and 6 (Fig. 2) were found to possess strong anti-inflammatory activity with IP values of 57.95 ± 2.65 and 60.09 ± 1.09 %, respectively, whereas fraction 5 showed moderate inhibitory activity (IP = 42.64 ± 0.75%).

The anti-inflammatory activity of fraction 1 has allegedly been attributed to the high content of sesquiterpene hydrocarbons and especially to their major constituents, such as *β*-caryophylene and *γ*-muurolene. In fact, the anti-inflammatory activity of *β*-caryophyllene is well documented [14-16].

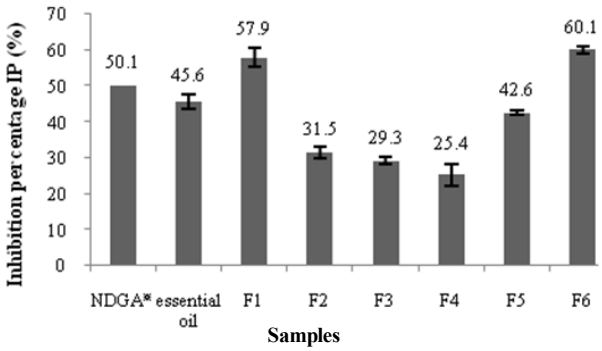

Figure 2: Anti-5-lipoxygenase activity of the essential oil and its fractions from *A. altissima* stems tested at 50 mg/L.
NDGA: nordihydroguaiaretic acid.
* tested at 2 mg/L.

Table II: Anti-5-lipoxygenase, anti-acetylcholinesterase and anti-xanthine oxidase activities of stems essential oil of *A. altissima*.

Samples	Anti-5-lipoxygenase [a]	Anti-acetylcholinesterase [a]	Anti-xanthine oxidase [a]
Essential oil (EO)	45.63 ± 1.92	9.32 ± 1.61	13.54 ± 2.02
F1	57.95 ± 2.65	23.74 ±1.10	12.52 ± 2.80
F2	31.48 ± 1.49	22.23± 0.31	7.59± 2.43
F3	29.32 ± 0.86	33.33 ± 1.18	13.15 ±2.22
F4	25.35 ± 3.06	6.88±1.98	8.71 ± 0.96
F5	42.64 ± 0.75	na [c]	5.65 ± 2.78
F6	60.09 ± 1.09	2.93 ± 0.83	12.87 ± 1.24
NDGA [b]	1.81 ± 0.05	-	-
Galanthamine [b]	-	1.17 ± 0.06	-
Allopurinol [b]	-	-	1.13 ± 0.78

[a] Inhibition percentage at a concentration of 50 mg/L.
[b] IC_{50} (mg/L).
[c] na: not active.
NDGA: nordihydroguaiaretic acid.

Moreover, γ-muurolene was reported to have anti-inflammatory properties in the essential oil from *Garcinia brasiliensis* [17]. In addition, the highest inhibitory effect of fraction 6 could be due to the high content of tetradecanoic acid. Indeed, it has been reported that fatty acids possess various important pharmacological activities, including anti-inflammatory effect [18].

Nevertheless, it's hard to attribute the anti-inflammatory activity in this investigation to one or few major components since synergistic or antagonistic effects of compounds in minor percentage of mixture should be taken into consideration.

Also, it should be pointed out that this is the first time that the anti-inflammatory activity of the volatile oil from *A. altissima* stems was proved.

3. Anti-acetylcholinesterase activity

In the attempts to evaluate the capacity of *A. altissima* stems EO to inhibit AChE, the key enzyme in the breakdown of acetylcholine, the EO and its fractions were screened at the concentration of 50 mg/L.

Unfortunately, the results summarized in Table II, revealed that the essential oil and the fractions exhibited a weak potency of AChE inhibition, and this may largely be attributed to the relatively high concentration in sesquiterpenes. The best activity was found for fraction 3 (IP = 33.33 ± 1.18%), which may be attributed to the presence of thymol, even if present at in low amounts (2.2%) in the composition of this fraction. Reports on AChE inhibition mentioned that thymol (monoterpenol) is known for its anticholinesterase activity [19,20]. It is within the interest of our study to indicate that this weak activity of *A. altissima* stems EO could presumably be due to the low content of oxygenated monoterpenes or the absence of monoterpene hydrocarbons. In this regard, many authors reported that AChE inhibition may be due to monoterpene hydrocarbons such as α-pinene [21,22]. Additionally, previous studies have shown that bicyclic monoterpene hydrocarbons containing an allylic methyl group were potent inhibitors of AChE activity [23], but they also demonstrated the importance of the position of the double bond for the activity [24].

As far as we know, there are no previous studies on the anti-AChE activity of *A. altissima* stems essential oil.

4. Anti-xanthine oxidase activity

The xanthine oxidase (XOD) inhibitory activity of the essential oil of *A. altissima,* together with its fractions was investigated at the concentration of

50 mg/L. As reported in Table II, the EO and its fractions did not present a good XOD inhibitory activity. Fraction 3 only possessed a slight XOD inhibitory activity (IP = 13.15 ± 2.22%).

We should notice that only few researchers studied the xanthine oxidase inhibition using essential oils. Although, Wang et al. [25] reported that cinnamaldehyde, which belongs to the family of phenylpropanoids, contributes to the XOD inhibitory activity in the EO of *Cinnamomum osmophloeum* (IC_{50} = 8.4 µg/mL). Nevertheless, as can be observed from Table I, fraction 3 contains phenylpropanoids, but in low percentage (2.8%), and the slight activity displayed by this fraction could be explained by the presence of these components.

To the best of our knowledge, so far there are no studies or reports on the activity of *A. altissima* essential oil against xanthine oxidase.

EXPERIMENTAL

1. Chemicals and reagents

All chemicals were of analytical grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France).

2. Plant Material

Stems of *Ailanthus altissima* were harvested in spring (May, 2015) from old trees (ca. 20 years) cultivated in the garden of the High Institute of Agriculture of Mogran, (36°21′N latitude, 10°06′E longitude, altitude 156 m above sea) (average T° = 23.6 °C; average rain fall = 11.0 mm; May 2015), Zaghouan, in the north east of Tunisia. The plant material was identified by Professor Fethia Harzallah-Skhiri at the Laboratory of Bioresources: Integrative Biology & Valorization, Higher Institute of Biotechnology of Monastir, University of Monastir, Tunisia. A voucher specimen of *A. altissima* has been deposited at the Laboratory of Heterocyclic Chemistry, Natural Products and Reactivity, Faculty of Science of Monastir, Tunisia (*A.al*-Zag/15).

3. Essential oil Extraction and Fractionation

Fresh stems were subjected to conventional hydrodistillation for 3h using a *Clevenger*-type apparatus. The obtained distillate was extracted with anhydrous diethyl ether until the distilled liquid was colorless. The essential oils were subsequently collected, dried over anhydrous sodium sulfate and then stored in airtight containers at 4°C, prior to analyses. The yield of extraction was expressed in % (v/w) of the fresh material.

After that, the crude essential oil (130 mg) was chromatographed on a silica gel column (50 mm diameter, 800 mm length) by gradient elution with *n*-pentane and diethyl ether (the proportion of two solvents changed from 100:0-0:100). The collected fractions were monitored by TLC and those with similar profile were combined giving six main fractions (F1= 40 mg, F2= 18 mg, F3= 10 mg, F4= 18 mg, F5= 8 mg, F6=15 mg).

4. Gas chromatography-FID analysis

Gas chromatography analyses were performed on a *HP 5890-series II* gas chromatograph equipped with a flame ionization detector (FID) and a fused silica capillary *HP-5* column (30m x 0.25 mm i.d., 0.25 µm film thickness). The oven temperature was programmed to rise from 50°C (1 min) to 280°C at 5°C/min (1 min). The injector and detector temperatures were maintained at 250°C and 280°C, respectively. The flow rate of the carrier gas (Nitrogen) was 1.2 mL/min and the injection volume for all samples was 0.1 µL of 1% hexane solution.

5. Gas Chromatography/Mass Spectrometry analysis

GC-MS analyses were carried out using *Varian CP -3800* gas chromatograph equipped with a *HP-5* capillary column (30 m x 0.25 mm, 0.25 µm film thickness) and a *Varian Saturn 2000* ion trap mass detector. The oven temperature was programmed at 60°C then rising from 60°C to 240°C at 3°C/ min. Injector and transfer line temperatures were set at 220°C and 240°C, respectively. The carrier gas was helium at flow rate of 1 mL/min, the split ratio was 1:30 and the injection volume was 0.2 µL (10% hexane solution).

6. Compound identification

The volatile compounds were identified based on comparison of their retention times with those of authentic samples and by matching their linear retention indices (*LRI*s) relative to (C_8-C_{30}) *n*-alkanes and their recorded mass spectra with those stored in the *NIST 2014* mass-spectral library, those published in Adams, [26] or in a home-made library mass spectra built up from pure substances and components of known essential oils and MS literature data [27-29].

7. Anti-5-lipoxygenase activity

5-lipoxygenase (5-LOX) inhibition activity was determined spectrophotometrically by measuring the increase in absorbance at 234 nm for the *in vitro* oxidation of linoleic acid to a conjugate diene [30]. 20 µL of essential oil was mixed individually with 150 µL of sodium phosphate buffer solution (pH = 7.4), 60 µL of linoleic acid and 20 µL of 5-LOX enzyme solution, yielding a final volume of 250 µL. The blank does not contain the substrate, replaced by 20 µL of buffer solution. The absorbance was recorded at 234 nm after 10 min of incubation under controlled temperature (25°C). Nordihydroguaiaretic acid (NDGA) was used as positive control.

8. Anti-acetylcholinesterase activity

The acetylcholinesterase inhibitory activity was measured using Ellman's method [30]. In brief, 25 µL of essential oil solution (50 µg/mL), 50 µL of sodium phosphate buffer A (0.1 M, pH = 8), 125 µL of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) solution (3 mM) prepared in sodium phosphate buffer C (100 mM, pH = 7) and 25 µL of the acetylcholinesterase (AChE) enzyme solution (1.4 U/mL) dissolved in buffer B (Na_2HPO_4, $12H_2O$ (20 mM, pH = 7)) were mixed and left to incubate for 15 min at 25°C. Subsequently, acetylthiocholine iodide solution (15 mM, in Buffer A) was added. The absorbance at 412 nm was read after 10 min of incubation. Galanthamine was used as positive control.

9. Xanthine oxidase inhibition (XOD) assay

The essential oil and its fractions were assessed for their in vitro inhibitory activity against commercial enzyme xanthine oxidase spectrophotometrically by measuring the formation of uric acid at 295 nm [31]. The assay mixture consisted of 60 µL of phosphate buffer solution (70 nM, pH = 7.5), 50 µL of sample solutions (50 µg/mL in well) and 30 µL of xanthine enzyme solution (0.1 U/mL). After pre-incubation for 15 min, 60 µL of xanthine solution (150 µM) were added. The total mixture was then homogenized and incubated for 5 min under controlled temperature (25°C) and the absorbance was recorded against a blank without the sample. Allopurinol, a known XO inhibitor, was used as a positive control.

10. Statistical analysis

All measurements were carried out in triplicate and results are expressed as mean values ± standard deviation (SD). Data were subjected to combined analysis of variance (ANOVA) using the SPSS 13.0 software package. Differences at $P < 0.05$ were considered statistically significant.

CONCLUSION

In summary, this study reports a fractionation approach of the essential oil of the Tunisian *A. altissima* stems obtained by hydrodistillation in the aim to identify additionally components listed in a previous study. Our investigation was performed with the aim to verify the effect of climatic conditions on the chemical composition of the same tree harvested in the same locality and during the same season of the year (May of 2012 and 2015).

Our data showed that the qualitative and quantitative composition of the volatile oil varies according to the harvesting year of the plant material. Furthermore, the oil and its isolated fractions exhibited significant anti-5-LOX activity, which may be related to some components such as *β*-caryophyllene, *γ*-muurolene and tetradecanoic acid. On the other hand, the anti-AChE and anti-XOD activities were weak. The chemical identification of the different compounds characterizing the *A. altissima* stems EO revealed that it was mainly composed by sesquiterpenes hydrocarbons, in particular *β*-caryophyllene and *γ*-muurolene, which can act as anti-inflammatory agents.

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