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Article

Chemical Composition and Antioxidant, Anti-Inflammatory and Anticholinesterase Properties of the Aerial and Root Parts of *Centaurea acaulis* Essential Oils: Study of the Combinatorial Activities of Aplotaxene with Reference Standards

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Abstract: In recent years, growing attention has been given to essential oils. Essential oils obtained from medicinal plants show high therapeutic potential against various types of pathologies, including Alzheimer's, cancer and inflammatory diseases. The purpose of this work was to study the chemical composition of the aerial and root parts of *Centaurea acaulis* essential oils by GC/FID and GC/MS, to evaluate the anti-inflammatory, antioxidant and neuroprotective properties, and *in-vitro* combinatory effect of aplotaxene isolated from the roots with reference standards, in order to find new and more effective agents for the treatment of degenerative and inflammatory diseases. The statistical analysis clustered the essential oil aerial parts into two distinct groups. The specimens characterized by a humid climate and low altitude was mainly discriminated by the high contents of (E)-β-caryophyllene (3.4-8.4%), hexadecanoic acid (6.3-10.8%) and caryophyllene oxide (6.2-9.9%). While the second group characterized by a humid climate and higher altitudes was characterized by high levels of limonene (15.2-19.2%) and τ-muurolol (12.6-17.2%). In contrast, essential oils of roots were dominated by aplotaxene whatever the climate type. All samples showed very good antioxidant and anti-inflammatory activities. The combination of aplotaxene with references gave synergistic effects with excellent activities about 2 times higher than the synthetic references. The neuroprotective activity of essential oils and aplotaxene did not show inhibition against AChE, whereas they inhibited BChE with IC_{50} values comparable to Galantamine.

Keywords: Aplotaxene, Biological activities, *Centaurea acaulis*, Chemical variability, Essential oils.

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Introduction

Alzheimer's disease is considered the most common neuro-degenerative disorder because of the neurodegeneration that occurs during the course of this disease [1]. Although it is not possible to treat it completely, there are nowadays some drugs to slow down the clinical progression of the disease, in particular, cholinesterase inhibitors: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) in the brain [2] that represents the predominant cholinesterase in the brain in the late Alzheimer's disease stage [3]. The excessive production of free radicals, including reactive oxygen species (ROS) once formed lead to oxidative damage to integral enzymes, proteins and even DNA which leads to degenerative or pathological processes such as ageing, cancer, inflammation, diabetes and neurodegenerative disorders [4] . A study conducted by Ben Kheder *et al.* has shown that people who have an imbalance between oxidants and antioxidants in the blood have a much higher risk of developing Alzheimer's disease. The research team showed that oxidative markers, known to be involved in Alzheimer's disease, increase up to five years before the onset of the disease [5]. On the other hand, inflammation is one of the most important protective mechanisms of the body against the damages caused by injury, irritation, infections, or internal dysfunctions due to autoimmune pathologies. In addition, excessive inflammation can result in severe chronic illnesses like Crohn's disease [6] and may be partly responsible for some of the brain changes seen in dementia [7]. The commonly used drugs to reduce inflammation are NSAIDs, thus, prolonged use of these agents carries the risk of cardiovascular, gastrointestinal toxicity, and other toxicity [4]. Essential oils are the aromatic and volatile products biosynthesized by plants as secondary metabolites [8]. However, the molecular diversity of the metabolites they contain gives them biological roles and properties. Some essential oils can act as anti-inflammatory and antioxidant agents by inhibiting the release of pro-inflammatory mediators, scavenging free radicals and increasing antioxidant defenses to improve health or cure diseases in humans [6]. For this reason, in recent years, the use of extracts and volatile components obtained from plants as natural alternative treatments to synthetic drugs "has attracted" the attention of scientists for industrial applications, especially in the field of cosmetics and pharmaceuticals [9]. The genus *Centaurea* is one of the largest genera of the sub-tribe *Centaureinae*, and belongs to the biggest and important botanical family: Asteraceae. It contains more than 700 species distributed all over the world. In Algeria, it is represented by 45 species with 7 localized in the Sahara [10]. Among them, *Centaurea acaulis*, an endemic species of Algeria and Tunisia, is widely used by the Algerian population in the treatment of colds, dizziness, and headache [11]. As reported by many phytochemical and pharmacological studies, various essential oils and isolated compounds from Algerian *Centaurea* species have shown important biological activities. According to the literature, the *in-vitro* anti-inflammatory effects of the chloroform extracts of *C. hierapolitana*, *C. calolepis* and *C. cadmea* showed strong activities [10]. On the other hand, *C. cadmea* and *C. ensiformis* showed strong antioxidant activity [12]. Additionally, methanol extracts of *C. polypodiifolia* var. *pseudobehen* and *C. antalyense* showed good inhibition against AChE and BChE, which suggests this species might be a potential source of anti-cholinesterase substances [13]. The study conducted on the n-butanol extract of *C. tougourensis* showed significant antioxidant, anti-inflammatory and antimicrobial activities as well as neuroprotective effects [14]. Also, antiulcerogenic and immunologic effects of *Centaurea L*. has been discussed [15]. To our knowledge, until now, no pharmacological study has been made of *C. acaulis* essential oils from the aerial and root parts. For that purpose, the current paper reports original results about the (i) intraspecies variations of essential oils of *C. acaulis* native to western Algeria, (ii) evaluate the *in-vitro* antioxidant, anti-inflammatory and anti-cholinesterase activities of essential oils and its major component, aplotaxene and (iii) evaluate the biological activities of the combination of aplotaxene and essential oil with reference molecules in the hope of finding new drugs that are more effective and less harmful.

Materials and methods

Chemicals used in the study

Solvents and reagents used were: 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, iron (III) chloride, linoleic acid, Tween 40 and 80, ferrozine, butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline, diclofenac sodium acetylcholinesterase, butyrylcholinesterase and galantamine. All chemicals used in the present study were of analytical grade purchased from Sigma (Sigma-Aldrich).

Plant materials and essential oil extraction

The root (500 g) and aerial parts (500 g) of *C. acaulis* were harvested from eight locations (P1-P8) near Tlemcen city (Algeria) during the flowering stage (March-July). The plant was identified by the botanist Dr. BABA Ali from the Department of Ecology and Environment of the University of Tlemcen (Algeria), where reference specimens of the plant have been placed in the herbarium (N° voucher: C.a. 0118 to C.a 0818). Some information of sample locations (geographical origin, yields and altitudes), are presented in Table 1. The root and aerial parts were air-dried at room temperature. The total plant material from each population was submitted to hydrodistillation for 4-5 hours using a Clevenger apparatus according to the procedure described in the European Pharmacopeia that allows the recycling of the aqueous phase of the distillate by cohobage [16]. The procedure was repeated three times. The isolated essential oils were dried over anhydrous sodium sulfate, filtered and then the essential oil mass was determined.

Isolation of Aplotaxene

The collective essential oil (1 g) of roots was obtained by the mixture of all essential oil samples, which was then subsequently submitted to column chromatography for separation, using silica gel column chromatography (FC, silica gel 200-500 µm) and eluted with 100% Hexane.

Identification of the oil components

Gas chromatography

The gas chromatography (GC) analysis was carried out using Clarus 500 Perkin-Elmer Auto system apparatus equipped with two flame ionization detectors (FID), with a fused capillary column (50 m x 0.22 mm I.D; film thickness

Table 1. Geographical distribution of *C. acaulis* from western Algeria

Codes	Locations	N° Voucher	GPS coordinates	Climate	Alt (m)	Yields
AP1, RP1	GHAZAOUET	C.a.0118	35°06′48″N; 1°50′04″O	Humid	036	AP 0.6 RP 0.7
AP2, RP2	NEDROMA	C.a.0218	35°01′38″N; 1°09′45″O		185	AP 0.3 RP 0.6
AP3, RP3	ZENATA	C.a.0318	34°70′03″N; 1°27′30″ O		280	AP 0.6 RP 0.5
AP4, RP4	HENAYA	C.a.0418	34°58′90″N; 1°27′15″O		428	AP 0.5 RP 0.5
AP5, RP5	AIN FEZZA	C.a.0518	34°86′70″N; 1°23′30″O		900	AP 0.4 RP 0.5
AP6, RP6	ZARIFET	C.a.0618	34°52′20″N; 1°19′08″O	Humid and cooler	980	AP 0.2 RP 0.2
AP7, RP7	BENI BAHDEL	C.a.0718	34°43'00"N; 1°31'00"O		600	AP 0.3 RP 0.4
AP8, RP8	BENI SNOUS	C.a.0818	34°39′22″N; 1°33'04″O		850	AP 0.2 RP 0.3

RP: Root parts; AP: Aerial parts

0.25 μm), BP-1 (polymethyl- siloxane) and BP-20 (polyethylene glycol). The oven temperature was fixed from 60°C to 220°C at 2°C/min and then held isothermal (20 min). Injector and detector temperature were maintained at 280°C. Essential oils were injected as neat samples in the split mode (1/60), using helium as the carrier gas (0.8 ml/min); the injection volume was 0.2 μl. RIS was determined using the retention times of a series of n-alkanes with linear interpolation, with those of authentic compounds or literature data [17,18].

Gas chromatography/Mass spectrometry

Essential oils were analyzed with a PerkinElmer Turbo-Mass quadrupole analyzer, coupled to a PerkinElmer Autosystem XL, equipped with two fused-silica capillary columns and operated with the same GC conditions described above, except for a split of 1/80. EI mass spectra were acquired under the following conditions: Ion source temp. 150°C, energy ionization 70 eV, mass range 35-350 Da (scan time: 1 s) [19].

Component identification and quantification

Identification of the components was based (i) on the comparison of their GC RIs on non-polar and polar columns and (ii) on computer matching with commercial mass spectral libraries [20,21] and comparison of the spectra with those of the inhouse laboratory library. The quantification of constituents was carried out using a flame ionization detector by internal standard method using the response factor calculated with respect to the tridecane (0.7 g.100 g^{-1}) used as internal standard.

Nuclear magnetic resonance (NMR)

The NMR spectrometer used was Bruker AVANCE 400 Fourier Transform spectrometer operating at the basic frequency of 400.13 MHz for ^{1}H. The spectrometer is equipped with direct detection broadband observe (BBO) probe. All NMR measurements were acquired at 298 K (25°C). Chemical shifts are expressed in δ ppm. Scalar coupling constants (J) are given in Hertz. ^{13}C NMR: The NMR spectra were recorded on a Bruker AVANCE 400 Fourier Transform spectrometer operating at 100.13 MHz for ^{13}C, equipped with a 5 mm probe, in deuterated chloroform ($CDCl_3$), with all shifts referred to internal tetramethylsilane (TMS). ^{13}C NMR spectra were recorded with the following parameters: pulse width (PW), 4 μs (flip angle 45°); acquisition time 2.7 s for 128 K data table, with a spectral width (SW) of 24,000 Hz (240 ppm); CPD mode decoupling; digital resolution 0.183 Hz/pt. The number of accumulated scans was 3,000 (50 mg of essential oil in 0.5 mL of $CDCl_3$) [22].

Evaluation of antioxidant activity

In order to evaluate the antioxidant potential of aerial parts and roots of *C. acaulis* essential oils as well as the aplotaxene, three different methods were used, namely DPPH radical scavenging, metal ion chelating and β-carotene bleaching activity.

Free radical scavenging activity (DPPH)

Free radical scavenging activities of samples were determined spectrophotometrically. The changes in color (from deep blue to light-yellow) were measured at 517 nm with a UV-VIS spectrophotometer. The radical scavenging activity of essential oils was measured by the standard method [23]. 1000 μl Of various concentrations of samples ranging from (0.2-15 g/L) were prepared in ethanol and added 1 mL of 0.2 mM DPPH solution freshly prepared. After 30 min incubation at 37°C in the dark, the anti-DPPH activity was measured at 517 nm against blank and standard (BHT). The percentage inhibition activity was calculated by the following equation:

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

Where, $A_{control}$ is absorbance of DPPH radical (without the test sample) and A_{sample} is the absorbance of DPPH radical with the essential oil samples of various concentrations. IC_{50} values (g/L) were calculated graphically by the linear regression of plotted graphs, inhibition percentages as a function of different concentrations extracts tested [24]. Analyses were achieved in triplicates and BHT was used as a reference.

Metal chelating activity

The metal chelating ability of Fe^{2+} by samples was studied as per the developed protocol and practiced by many researchers [25]. In a test tube 100 µl of (0.6 mM) $FeCl_2$, 100 µl of 5 mM ferrozine and 900 µl of methanol were mixed together with various amounts of samples (0.2-15 g/L). The reaction mixture was mixed thoroughly and incubated for 10 min. The absorbance was measured at 562 nm using a spectrophotometer. Here, EDTA (0.01 mM) was taken as standard. Using the given equation, the inhibition percentage of metal chelating activity of all samples were calculated:

$$\text{Inhibition (\%)} = \left[\frac{(\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}})}{\text{Abs}_{\text{Control}}}\right] \times 100$$

Where, Abs control is Absorbance of control sample (without the test sample) and Abs sample is the absorbance of test sample. The amount of inhibition by the test samples was expressed as the percentage of concentration required to do 50% inhibition (IC_{50}). Analyses were achieved in triplicates and BHT was used as a reference.

β-Carotene bleaching activity

Oxidation scavenging activity of samples was performed using the β-carotene bleaching method [26]. Briefly, a stock solution of the β-carotene-linoleic acid mixture was prepared: 5 mg of *β*-carotene was dissolved in 10 mL of chloroform. 1000 µl of this solution, 50 µl of linoleic acid and 200 mg of Tween 40 were mixed. The solvent was evaporated entirely by using a rotary evaporator. 100 mL of distilled water saturated with oxygen was added and shaken vigorously until an emulsion was formed. A volume of 2.5 mL of previous emulsion was transferred into test tubes containing 350 µl of essential oil dissolved in ethanol at different concentrations. The emulsion system was incubated at 50°C for 120 min. The same procedure was repeated for (BHT) the synthetic antioxidant used as positive controls. After the incubation, the absorbance was measured at 470 nm using a spectrophotometer against a blank containing only 350 µl of ethanol. The antioxidant activity was calculated according to the following formula:

$$\beta\text{-Carotene activity (\%)} = \left[\frac{A_s(120) - A_c(120)}{A_c(0) - A_c(120)}\right] \times 100$$

Where, As (120) is the absorbance of the sample at t = 120 min, A_C (120) is the absorbance of the control at t = 120 min, and A_C (0) is the absorbance of the control at t = 0 min. Analyses were achieved in triplicates and BHT was used as a reference.

Evaluation of in-vitro anti-inflammatory activity

In-vitro anti-inflammatory activity of essential oils was determined by protein denaturation assay. For this experiment, the reaction mixture (5 mL) consisting of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL essential oil of varying concentrations (0.2 to 2.5 g/L). The mixtures were incubated at 37 °C in a BOD incubator for 15 min and then heated at 70°C for 5 min in a hot water bath. After cooling, their absorbance was measured at 660 nm. A similar volume of double-distilled water served as the control. Diclofenac sodium in the same concentrations was used as the reference drug [27]. The percentage inhibition of protein denaturation was calculated by using the following formula.

$$\text{Inhibition (\%)} = \left[\frac{V_t}{V_c} - 1\right] \times 100$$

Where, Vt is the absorbance of the test sample and Vc is the absorbance of control. Each experiment was done in triplicate and the result was determined from the average values.

Evaluation of neuroprotective activity

The evaluation of the acetylcholin-esterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities was conducted as previously described by Bensaad *et al.* [28]. Briefly, A volume of 150 µL of sodium phosphate buffer (100 mM; pH 8.0) was added to 10 µL of sample dissolved in ethanol at different concentrations (3.12, 6.25, 12.50, 25.0, 50.0 and 100 µg/mL). Then, 20 µL of AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) or solution was added to the mixture. After 15 min of incubation at 25°C, a volume of 10 µL of DTNB (0.5 mM) and 10 µL of acetylthiocholine iodide (0.71 mM) or 10 µL of butyrylthiocholine chloride (0.2 mM) were added to the previous mixture. The absorbance was measured at 412 nm by a 96-well microplate reader, and galantamine was used as a reference drug and tested at the same concentration. The percentage of inhibition was determined through the formula:

$$\% \text{ Inhibition } = \left[\frac{(E - S)}{E}\right] \times 100$$

Where, E and S were the enzyme activities without and with the test sample, respectively. The concentration of the samples that caused 50% inhibition of the AChE and BChE activities (IC_{50}) was calculated via nonlinear regression analysis.

Determination of the synergistic activity

In order to evaluate the synergistic effects, a combination of 1 mL of aplotaxene with 1 mL of the synthetic references was studied with the same concentrations for each activity described.

Statistical analysis

Data treatment was done using XLSTAT 2014.5.03 to examine the discrimination between samples and its chemical constituents. Data analyses were performed using PCA, this method aims to reduce the multivariate space in which objects (oil samples) are distributed but are complementary in their ability to present results. Indeed, PCA provides the data in which both objects (oil samples) and variables (oil major components) are plotted. The results were expressed as the mean ± standard deviation. The statistical comparisons used Student's t-test. The differences were considered significant at $P < 0.05$. The correlation coefficients (r^2) for the test parameters were established via regression analysis. Analysis of each test was performed in triplicate.

Results

Yields and chemical compositions of C. acaulis essential oils

The essential oils from the aerial part of *C. acaulis* collected from Western Algeria, afforded a light-yellow color essential oil with an average yield varying from 0.2±0.04 to 0.6±0.02% (w/w), either 0.2±0.02g/100g to 0.6±0.01g/100g, based on the dry mass of the plant. While the essential oils from the root part showed slightly higher yields (0.2-0.7%) either (0.2g/100g-0.7g/100g) (Table 1). The highest yields (0.6-0.7%) or were observed of the AP1 station with an altitude of 36 m and a humid climate, while the lowest yields (0.2%) were observed of the AP6 station (altitude 980 m with humid and cooler climate) for both organs of the plant, respectively (Table 1). All the individual essential oil samples were pooled to produce a "collective essential oil" for the roots ($Coll^r$ EO) and "collective essential oil" for aerial parts ($Coll^{ap}$ EO), separately, which was used for detailed analysis. *C. acaulis* Coll EO was characterized by eighty-one compounds accounting 93.7% of total collective essential oil (Table 2). The Coll EO of aerial parts of *C. acaulis* was characterized by the abundance of sesquiterpene compounds, in which oxygenated compounds (31.4%) were higher than hydrocarbons (22.7%). The main compounds identified in the Coll EO were: τ-muurolol (8.8%), caryophyllene oxide (5.9%), β-elemene (3.4%), germacrene D (3.5%) and viridiflorol (3.7%). The second dominant class of the collective essential oil was monoterpene compounds, whose hydrocarbons (13.7%) were higher than the oxygenated (7.1%). The principal component of this class was limonene (11.1%). However, non-terpenic compounds were present in low quantities (15.6%) such as hexadecanoic acid (4.6%) (Table 2). Routine GC and GC/MS analysis of essential oil of roots led to the identification of fifty-two components. However, our spectral libraries were found to be nonoperative in identifying the major component of the essential oil (N°46 of Table 3). One predominant compound was obtained by separation by column chromatography with an average yield of (0.5g). Column chromatography allowed us to separate the major component. The identification of aplotaxene was done by other complementary analyzes such as ^1H and ^{13}C NMR.

^{13}C-NMR

($CDCl_3$, 101,13 MHz): δ = 139.0 ppm (C-16), 132.0 ppm (C-3), 130.2 ppm (C-10), 128.8 ppm

Table 2. Percentage of compounds identified in the essential oils from aerials parts of *C. acaulis* isolated from various localities

No. [a]	Compounds	RT	lRI_a [b]	RI [c]	Coll [ap] EO	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	Identificatio [d]
1	α-Pinene	14.1	930	931	1.6	2.9	3.9	4.1	3.2	0.2	0.1	0.2	0.1	RI.MS
2	Sabinene	15.8	964	964	0.4	0.3	0.7	2.0	0.1	0.1	0.1	0.1	0.1	RI.MS
3	B-Pinene	16.1	970	972	0.9	1.4	0.5	1.2	0.4	0.4	0.2	1.3	2.1	RI.MS
4	Myrcene	17.1	981	976	1.9	3.3	4.5	3.2	1.6	0.1	0.2	0.6	0.5	RI.MS
5	2-Pentyl furan	17.3	982	983	1.9	2.0	2.6	2.2	1.1	2.6	2.3	1.4	1.0	RI.MS
6	*p*-Cymene	18.9	1014	1011	0.2	0.1	0.1	0.3	0.2	0.2	0.3	0.5	0.2	RI.MS
7	Limonene	19.5	1025	1020	11.1	5.3	1.9	5.1	3.1	19.2	22.1	17.5	15.2	RI.MS
8	Z-*β*-Ocimene	19.7	1026	1024	0.3	0.2	0.5	1.1	0.1	tr	tr	0.1	0.2	RI.MS
9	E-β-Ocimene	19.8	1037	1034	0.4	0.1	0.5	1.9	0.1	0.1	tr	0.1	0.2	RI.MS
10	β-Terpinene	20.53	1045	1047	0.4	0.2	1.0	1.0	0.1	0.1	0.2	0.2	0.1	RI.MS
11	cis-6-Nonenal	22.5	1080	1083	1.3	1.3	1.5	0.8	0.9	1.0	1.6	1.8	1.2	RI.MS
12	Nonanal	22.9	1083	1082	1.3	0.5	1.7	0.4	2.5	0.6	3.2	1.2	0.5	RI.MS
13	Linalool	23.1	1088	1087	0.7	0.3	0.3	2.0	0.1	0.1	0.2	2.1	0.6	RI.MS
14	α-Pinene-oxide	23.4	1098	1096	0.3	0.2	0.1	0.5	1.1	0.3	0.1	0.3	tr	RI.MS
15	α-Campholen aldehyde	24.9	1107	1103	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.1	tr	RI.MS
16	trans-Pinocarveol	25.5	1126	1125	0.2	0.3	0.4	0.2	0.1	0.1	0.1	0.2	0.2	RI.MS
17	cis-Verbeneol	25.8	1132	1127	2.6	2.9	1.2	3.2	2.5	2.6	3.6	2.5	2.1	RI.MS
18	Pinocarvone	26.1	1136	1136	0.1	tr	0.1	0.1	0.1	0.1	0.1	0.2	0.2	RI.MS
19	α-Pinocamphone	26.8	1141	1151	0.5	0.3	0.5	tr	0.1	0.2	0.4	0.3	2.0	RI.MS
20	cis-6-Nonen-1-ol	27.2	1152	1156	0.2	0.2	0.3	tr	0.5	0.3	tr	0.1	tr	RI.MS
21	cis-2-Nonen-1-ol	27.6	1158	1158	0.2	0.1	0.1	0.2	0.1	0.1	1.0	0.2	0.2	RI.MS
22	Terpinen-4-ol	28.1	1164	1161	0.2	0.5	0.4	tr	0.3	0.3	tr	0.1	tr	RI.MS
23	Methyl salicylate	28.5	1170	1173	0.1	0.1	tr	tr	0.3	0.1	tr	0.2	0.1	RI.MS
24	Myrtenal	28.7	1172	1173	0.5	0.2	0.5	0.1	1.1	0.1	0.6	0.5	1.2	RI.MS
25	α-Terpineol	28.9	1175	1179	0.1	0.1	0.3	tr	0.1	0.1	tr	0.2	0.3	RI.MS
26	Safranal	29.1	1177	1177	0.1	0.2	0.2	tr	0.1	0.1	tr	0.1	0.2	RI.MS
27	Myrtenol	29.3	1182	1183	0.2	0.3	0.5	0.1	0.3	0.1	0.3	0.2	0.1	RI.MS
28	Verbenone	29.4	1183	1184	0.1	tr	0.1	0.1	0.2	0.2	tr	0.2	0.1	RI.MS
29	Decanal	29.5	1185	1186	0.8	0.1	0.8	1.3	1.3	1.1	0.6	0.1	1.2	RI.MS

table 2. (continued).

No. [a]	Compounds	RT	lRI_a [b]	RI [c]	Coll [ap] EO	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	Identificatio [d]
30	α-Campholenol	29.7	1186	1187	0.3	tr	0.2	0.3	0.8	0.1	0.6	0.2	tr	RI.MS
31	trans-Carveol	30.1	1201	1196	0.1	tr	0.1	0.1	0.1	0.5	0.1	tr	0.2	RI.MS
32	Carvone	31.5	1222	1222	0.3	0.1	0.2	0.1	tr	0.6	0.5	0.4	0.3	RI.MS
33	Carvacrol methyl ether	31.9	1229	1231	0.0	tr	0.1	0.1	0.1	tr	tr	0.2	tr	RI.MS
34	Piperetone	32.4	1230	1232	0.1	0.1	0.3	0.1	0.1	0.2	0.1	tr	0.1	RI.MS
35	Tridecane	35.8	1300	1300	0.8	tr	0.5	1.6	0.3	0.2	1.2	1.6	1.5	RI.MS
36	Neryl acetate	39.8	1341	1342	0.2	0.5	0.4	0.2	0.1	0.1	0.1	0.1	0.2	RI.MS
37	Geranyl acetate	40.9	1367	1361	0.1	0.1	0.2	0.2	0.4	0.1	0.1	tr	tr	RI.MS
38	α-Copaene	41.2	1373	1379	0.6	0.6	1.1	0.6	0.4	1.7	0.6	tr	tr	RI.MS
39	β-Elemene	41.8	1385	1388	3.4	0.6	0.1	3.0	0.1	0.1	7.5	8.0	7.7	RI.MS
40	(E)-β-Caryophyllene	43.8	1418	1424	1.7	8.4	3.5	3.4	3.9	0.6	0.1	0.2	tr	RI.MS
41	α-Humulene	45.7	1449	1456	0.7	2.0	0.8	0.7	1.2	1.3	tr	tr	tr	RI.MS
42	β-Ionone	46.6	1464	1466	0.1	0.1	0.2	0.4	0.2	0.1	tr	tr	tr	RI.MS
43	β-Muurolene	47.2	1471	1471	3.4	0.3	0.5	1.3	1.2	5.4	4.3	6.5	8.5	RI.MS
44	Dodecanol	47.6	1472	1475	0.3	tr	0.4	0.8	0.6	0.9	tr	tr	tr	RI.MS
45	Germacrene D	47.9	1477	1480	3.5	8.7	1.5	2.1	1.2	1.3	5.1	1.6	6.5	RI.MS
46	β-Selinene	47.8	1481	1483	0.7	tr	1.2	2.6	0.7	0.8	tr	tr	tr	RI.MS
47	4-epi-Cubebol	47.9	1487	1486	0.3	tr	0.4	0.8	0.8	0.9	tr	tr	tr	RI.MS
48	Bicyclogermacrene	48.2	1490	1494	0.6	tr	1.0	0.7	0.6	2.5	tr	tr	tr	RI.MS
49	α-Muurolene	48.3	1491	1496	0.1	0.4	0.2	0.1	0.6	0.2	tr	tr	tr	RI.MS
50	α-Selinene	48.5	1493	1495	0.3	tr	1.3	0.3	0.9	0.1	tr	tr	tr	RI.MS
51	E.E-α-Farnesene	48.9	1506	1498	0.3	tr	0.3	0.4	0.8	0.6	tr	tr	tr	RI.MS
52	β-Cadinene	50.2	1509	1507	0.1	tr	0.1	0.2	0.9	0.5	tr	tr	tr	RI.MS
53	trans-Calamenene	51.3	1511	1512	1.2	tr	0.1	0.5	0.2	1.9	0.2	3.5	0.1	RI.MS
54	δ-Cadinene	51.5	1513	1516	0.8	1.7	1.1	0.5	0.3	0.2	1.3	1.1	0.5	RI.MS
55	α-Cadinene	51.6	1539	1535	0.4	tr	1.3	0.7	0.1	0.8	tr	tr	tr	RI.MS
56	β-Calacorene	51.7	1541	1548	0.1	tr	0.4	0.2	0.2	tr	tr	0.2	tr	RI.MS
57	E-Verolidol	51.8	1550	1564	0.6	tr	1.1	0.1	tr	tr	1.2	1.5	0.9	RI.MS
58	1.5-Epoxysalvial-4(14)-ene	51.9	1552	1560	0.4	0.6	0.5	0.2	0.5	1.2	0.1	0.2	tr	RI.MS
59	3(*Z*)-Hexanyl benzoate	52.1	1554	1552	0.0	tr	tr	0.3	tr	tr	tr	tr	tr	RI.MS

table 2. (continued).

No. [a]	Compounds	RT	lRI_a [b]	RI [c]	Coll [ap] EO	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	Identificatio [d]
60	Germacrene D-4-ol	52.4	1567	1573	0.7	0.1	0.2	1.6	1.2	0.2	0.8	0.7	0.5	RI.MS
61	Spathulenol	52.7	1568	1557	1.9	0.5	0.2	0.8	0.6	1.2	2.3	2.6	3.1	RI.MS
62	Caryophyllene oxide	52.9	1571	1576	5.9	6.8	9.9	6.2	8.4	1.8	2.2	2.5	2.6	RI.MS
63	Globulol	53.2	1578	1580	0.9	0.5	0.2	0.6	0.8	1.3	1.3	1.2	1.0	RI.MS
64	Salvial-4(14)-en-1-one	53.5	1579	1578	1.3	0.1	1.9	0.8	2	4.3	0.5	0.4	0.2	RI.MS
65	Tridecanol	53.6	1580	1856	0.1	0.1	tr	0.2	tr	0.2	tr	tr	tr	RI.MS
66	Viridiflorol	53.7	1585	1591	3.7	5.5	8.6	6.5	8.1	0.4	0.2	0.1	0.3	RI.MS
67	β-Aplopenone	54.1	1591	1593	1.3	tr	0.6	0.8	3.5	1.8	2.1	0.9	0.8	RI.MS
68	Humulene epoxide 2	54.3	1594	1601	2.0	1.5	2.1	1.7	2.9	2.8	1.6	2.2	1.5	RI.MS
69	epi-Cubebol	55.8	1623	1624	1.9	1.0	2.5	2.3	2.1	1.9	2.2	2.0	1.0	RI.MS
70	τ-Cadinol	55.9	1628	1632	0.8	1.5	1.8	1.1	0.8	0.6	0.2	0.1	0.2	RI.MS
71	τ-Muurolol	56.4	1636	1634	8.8	3.1	3.9	2.5	4.5	12.6	13.6	17.2	14.2	RI.MS
72	β-Eudesmol	56.6	1639	1644	2.2	2.8	1.6	2.2	2.0	2.8	1.9	1.5	2.1	RI.MS
73	α-Cadinol	57.1	1642	1645	1.5	5.4	4.5	3.8	4.5	0.7	0.3	0.6	0.2	RI.MS
74	α-Eudesmol	57.3	1654	1653	1.2	1.3	2.0	1.7	1.4	1.1	0.9	0.6	0.4	RI.MS
75	14-Hydroxy-9-epi-E-caryophyllene	57.5	1657	1656	0.2	0.6	tr	0.1	0.4	0.2	tr	0.1	0.1	RI.MS
76	Eudesma-4(15)-7-diene-1-β-ol	58.2	1673	1672	0.9	tr	tr	3.0	0.2	1.1	0.8	0.5	2.5	RI.MS
77	cis-Farnesol	58.9	1682	1682	0.1	0.1	tr	0.1	0.2	0.2	0.1	tr	0.1	RI.MS
78	Hexahydro harnesyl acetone	66.3	1829	1831	0.4	0.5	0.6	0.2	0.3	0.4	0.2	0.3	0.1	RI.MS
79	Hexadecenoic acid	72.2	1958	1954	4.6	10.8	7.6	7.3	6.3	1.2	1.3	1.6	1.2	RI.MS
80	Z-Phytol	77.1	2069	2080	0.9	0.1	3.2	0.2	tr	1.5	tr	0.1	1.8	RI.MS
81	E-Phytol	78.7	2100	2107	2.2	1.1	1.2	1.3	1.8	4.3	5.6	4.8	5.6	RI.MS
	Identification %				93.7	91.1	97.1	98.8	91.1	96.1	98.7	97.9	95.9	
Oxygenated monoterpenes (N°: 13-19, 22-28, 30-34, 36,37)					6.9	6.5	6.6	8.2	8.5	6,4	7.4	8.6	8.1	
Hydrocarbon monoterpenes (N°: 1-4,6-10)					17.2	13.4	13.3	19.6	8.7	20,1	22.9	20.1	18.5	
Hydrocarbon sesquiterpenes (N°: 38-41, 43, 45,46, 48-56)					17.9	23.0	14.6	17.1	14.1	18,4	19.1	21.1	23.3	

table 2. (continued).

No. [a] Compounds	Coll [ap] EO	AP1	AP2	AP3	AP4	AP5	AP5	AP6	AP7
Oxygenated sesquiterpenes (N°: 42, 47, 57,58, 60-64, 66-78)	37.1	31.4	41.7	36.3	44.1	35,9	32.3	34.9	31.7
Non-terpenic compounds (N°: 5,11,12, 20,21,29,35,44,59,65,79)	11.5	15.6	16.1	15.3	13.9	8,6	11.4	8.3	6.9
Oxygenated diterpenes (N°: 80,81)	3.1	1.2	4.8	2.3	2.6	6,7	5.6	4.9	7.4
Unidentified compounds	6.3	8.9	2.9	1.2	8.9	3.9	1.3	2.1	4.1

[a] Order of elution is given on apolar column (Rtx-1). [b] Retention indices of literature on the apolar column (lRIa) reported from the literature. [c] Retention indices on the apolar Rtx-1 column (RIa). [d] RI. retention indices; MS. mass spectrometry in electronic impact mode. Coll EO: collective essential oil. AP: Aerial parts

Table 3. Percentage of compounds identified of essential oils from roots parts of *C. acaulis* isolated from various localities

No. [a]	Compounds	RT	lRI_a [b]	RI_a [c]	Coll[r] EO	R1	R2	R3	R4	R5	R6	R7	R8	Identification [d]
1	Hexanal	8.3	768	770	0.3	0.3	0.1	0.2	0.2	0.2	0.2	0.5	0.3	RI.MS
2	α-Pinene	9.9	930	931	0.4	0.1	0.1	0.3	0.4	0.3	0.5	0.6	0.8	RI.MS
3	cis-3-Hepten-1-ol	11.4	935	939	3.7	2.6	5.5	3.7	2.9	2.7	3.0	5.6	4.3	RI.MS
4	β-Pinene	16.7	970	972	1.8	0.1	0.3	tr	3.1	tr	3.1	4.0	4.1	RI.MS
5	2-Pentyl furan	16.9	982	983	0.3	0.2	0.2	0.6	0.2	0.6	0.2	0.2	0.3	RI.MS
6	Limonene	18.6	1025	1020	0.5	0.1	0.2	0.3	0.4	0.3	0.5	0.8	1.2	RI.MS
7	trans-Pinocarveol	18.9	1126	1125	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	RI.MS
8	Cis-Verbeneol	25.4	1132	1127	0.1	0.1	0.1	0.2	tr	0.2	0.2	0.1	tr	RI.MS
9	α-pino-Camphone	26.6	1141	1151	0.3	0.8	0.1	0.5	0.1	0.5	0.1	0.1	0.4	RI.MS
10	cis-6-Nonen-1-ol	27.8	1152	1156	0.5	0.2	1.3	0.1	tr	0.1	0.1	1.5	1.1	RI.MS
11	Terpinen-4-ol	28.3	1164	1161	0.1	0.1	0.1	0.2	tr	0.2	0	0.2	0.1	RI.MS
12	Methyl salicylate	28.9	1170	1173	0.8	0.1	1.6	0.3	0.3	0.3	0.3	1.5	1.8	RI.MS
13	α-Campholenol	29.5	1186	1187	0.1	0.2	0.1	tr	tr	tr	tr	0.1	tr	RI.MS
14	trans-Carveol	30.7	1201	1196	0.2	0.1	0.2	0.3	0.2	0.3	0.2	0.2	0.1	RI.MS
15	Dec-3-en-2-one	31.7	1221	1219	0.4	0.6	0.5	0.4	0.1	0.4	0.1	0.8	0.5	RI.MS
16	Piperetone	32.1	1230	1232	0.7	0.9	0.8	0.6	0.9	0.6	0.9	0.5	0.5	RI.MS
17	Tridecane	35.6	1300	1300	0.3	0.2	0.6	0.1	0.2	0.1	0.2	0.7	0.7	RI.MS

table 3. (continued).

No. [a]	Compounds	RT	*l*RI$_a$ [b]	RI$_a$ [c]	Coll[r] EO	R1	R2	R3	R4	R5	R6	R7	R8	Identification [d]
18	Nerylacetate	39.6	1341	1342	0.2	0.3	0.4	0.1	0.2	0.1	0.2	0.3	0.2	RI.MS
19	β-Elemene	41.6	1385	1388	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	RI.MS
20	Trans Caryophyllene	43.7	1418	1424	3.4	2.5	3.6	4.8	3.0	5.4	1.8	2.8	3.0	RI.MS
21	α-Humulene	45.6	1449	1456	0.2	0.2	0.1	0.4	0.2	0.4	0.2	0.2	0.3	RI.MS
22	β-Ionone	46.4	1464	1466	1.0	0.4	0.1	1.4	2.3	1.2	2.1	0.1	0.2	RI.MS
23	γ-Muurolene	47.3	1471	1471	0.2	0.3	0.2	0.1	0.4	0.2	0.3	0.2	0.1	RI.MS
24	Germacrene D	47.8	1477	1480	0.2	0.1	0.2	0.1	0.1	0.2	0.3	0.3	0.2	RI.MS
25	β-Selinene	47.9	1481	1483	0.3	0.3	0.2	0.2	0.1	0.3	0.5	0.4	0.2	RI.MS
26	4-epi-Cubebol	48.2	1487	1486	2.6	1.9	2.8	2.6	3.2	3.1	2.8	1.8	2.9	RI.MS
27	α-Selinene	48.6	1493	1495	0.4	0.2	0.8	0.1	0.2	0.2	0.3	0.6	0.5	RI.MS
28	E.E-α-Farnesene	49.2	1506	1498	0.4	0.6	0.3	0.5	0.6	0.4	0.5	0.2	0.1	RI.MS
29	γ-Cadinene	50.3	1509	1507	0.2	0.5	0.2	0.1	0.2	0.2	0.3	0.2	0.1	RI.MS
30	α-Calacorene	50.6	1528	1531	0.4	0.1	0.9	0.3	0.3	0.2	0.1	0.7	0.5	RI.MS
31	α-Cadinene	50.9	1539	1535	0.2	tr	0.4	0.2	0.3	0.1	0.2	0.3	0.2	RI.MS
32	β-Calacorene	51.1	1541	1548	0.2	0.5	0.1	0.1	0.1	0.2	0.2	0.1	0.1	RI.MS
33	3-(Z)-Hexanyl benzoate	51.8	1554	1552	0.3	0.6	0.3	0.1	0.1	0.2	0.3	0.3	0.4	RI.MS
34	Germacrene D-4-ol	52.2	1567	1573	0.7	0.7	0.9	0.4	0.7	0.4	0.9	0.7	1.0	RI.MS
35	Caryophyllene oxide	52.8	1571	1576	10.9	14.6	13.8	8.0	6.9	7.9	7.2	14.0	14.7	RI.MS
36	Salvial-4(14)-en-1-one	53.1	1579	1578	0.8	1.9	0.1	0.2	0.1	0.1	0.4	1.8	1.5	RI.MS
37	Tridecanol	53.4	1580	1586	0.9	0.2	0.5	1.2	1.3	1.6	1.4	0.5	0.7	RI.MS
38	Viridiflorol	53.6	1585	1591	0.1	0.1	0.1	tr	0.1	0.1	tr	0.6	0.2	RI.MS
39	β-Aplopenone	53.9	1591	1593	1.1	2.6	0.1	0.4	0.3	0.4	0.3	2.5	1.8	RI.MS
40	Humulene epoxide 2	54.1	1594	1601	0.3	0.2	0.7	0.1	0.2	0.2	0.3	0.3	0.4	RI.MS
41	γ-Eudesmol	55.2	1617	1619	0.3	0.4	0.2	0.1	tr	0.2	0.1	0.6	0.8	RI.MS
42	Aromadendren epoxide 2	55.7	1619	1618	0.7	0.7	0.9	0.6	0.5	0.5	0.4	0.9	0.9	RI.MS
43	τ-Muurolol	56.2	1636	1634	0.6	0.4	1.6	0.4	0.2	0.5	0.1	0.5	0.9	RI.MS
44	β-Eudesmol	56.8	1639	1644	1.2	1.1	1.1	1.3	1.2	0.9	1.6	1.0	1.3	RI.MS
45	α-Cadinol	57.2	1642	1645	0.5	0.5	0.1	0.1	0.6	tr	0.5	0.8	1.2	RI.MS
46	Aplotaxene	57.6	/	1663	51.6	46.3	43.3	61.6	60.6	59.2	58.3	42.5	40.6	RI. MS. NMR
47	Eudesma-4(15)-7-dien-1-beta-ol	57.9	1669	1672	0.3	0.1	0.2	0.1	0.5	0.1	0.2	0.1	0.6	RI.MS

table 3. (continued).

No. [a]	Compounds	RT	lRIa [b]	RIa [c]	Coll' EO	R1	R2	R3	R4	R5	R6	R7	R8	Identification [d]
48	Pentadecane	59.2	1700	1700	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.3	RI.MS
49	Tetradecanoic acid	62.1	1748	1754	1.8	1.6	1.5	2.1	2.0	3.4	1.8	1.1	0.9	RI.MS
50	Hexadecanoic acid	72.2	1958	1954	1.1	1.4	1.2	1.4	1.2	0.6	0.5	1.2	1.3	RI.MS
51	Z-Phytol	76.3	2069	2080	1.7	1.8	1.8	1.6	1.2	1.5	1.8	1.8	2.0	RI.MS
52	E-Phytol	82.3	2100	2107	0.5	0.2	0.1	0.4	0.3	0.1	0.1	2.0	1.1	RI.MS
Identification %					94.8	89.2	90.8	97.3	96.1	95.5	93.7	99.2	97.0	
Oxygenated monoterpenes (N°: 7-9, 11-14,16,18)					2.7	1.8	1.8	1.5	1.4	1.5	1.6	1.6	1.0	
Hydrocarbon monoterpenes (N°: 2.4,6)					2.7	0.3	0.6	0.6	3.9	0.6	4.1	5.4	6.1	
Hydrocarbon sesquiterpenes (N°: 19-21, 23-25, 27-32)					6.4	5.4	7.1	7.0	5.6	7.9	4.8	6.2	5.4	
Oxygenated sesquiterpenes (N°: 22, 26,34-36,38-45,47)					19.5	25.2	22.6	14.3	14.5	14.4	14.8	25.6	28.2	
Non-terpenic compounds (N°: 1,3,10,15,17,33,37,46,48-50)					61.3	54.5	56.8	71.9	69.2	69.5	66.5	56.6	53.2	
Oxygenated diterpenes (N°: 51,52)					2.2	2.0	1.9	2.0	1.5	1.6	1.9	3.8	3.1	
Unidentified compounds					5.2	10.8	9.2	2.7	3.7	4.5	6.3	0.8	3.0	

[a] Order of elution is given on apolar column (Rtx-1). [b] Retention indices of literature on the apolar column (lRIa) reported from the literature. [c] Retention indices on the apolar Rtx-1 column (RIa). [d] RI. retention indices; MS. mass spectrometry in electronic impact mode; Coll EO: collective essential oil. RP: Root parts

(C-6,7), 128.2 ppm (C-4), 127.1 ppm (C-9), 115.2 ppm (C-17), 35.0 ppm (C-5,8), 33.8 ppm (C-11), 33.9 ppm (C-15), 30.0 ppm (C-12,14), 28.0 ppm (C-13), 20.5 ppm (C-2), 14.2 ppm (C-1).

The ^{13}C NMR spectrum showed the presence of 17 carbon atoms, including eight olefinic carbons (two carbons come from a terminal vinyl; δ 115.2-139.0). The proton-coupled ^{13}C-NMR spectrum confirmed the presence of seven olefinic CH groups, one methylidene group, eight alkyl CH_2 groups and one methyl group (δ 14.2-35.0). The values of olefinic chemical shifts suggest the absence of conjugated double bonds and oxygenated organic function [29,30].

^{1}H NMR

($CDCl_3$, 400.13 MHz) δ 0.98-1.02 (3H, t, J = 7.5 Hz), 1.23-1.27 (1H, s), 1.23-1.46 (8H, m), 2.0-2.14 (6H, m), 2.74-2.87 (4H, m), 4.89-4.97 (1H, ddt, J = 1.2, 2.3, 10.2 Hz), 4.94-5.04 (1H, ddt, J = 1.5, 2.2, 17.1 Hz), 5.26-5.46 (4H, m), 5.74-5.88 (1H, ddt, J = 6.7, 10.2, 16.9 Hz).

The ^{1}H NMR spectrum displays three 1,2-disubstituted alkene units and one terminal double bond (CH_2=CH-CH_2-) corresponding to signals 5.26-5.46 (6H) and 5.74-5.88 (3H) ppm which appeared as a doublet of triplets with J(H, H) = 16.9; 10.2; 6.7 Hz. Two doubly allylic CH_2 groups and three monoallylic CH_2 groups corresponding to 2 multiplets to signals δH=2.0-2.14 ppm (6H) and δH = 1.23-1.27 ppm (6H). The triplet at δH = 0.98-1.02 ppm (3H, J=7.5 Hz) was consistent with a CH_3CH_2- group attached to an olefinic center. The comparison of the data with described in the literature [29,30] led to the identification of aplotaxene (Fig. 1).

Chemical variability of C. acaulis essential oils

A principal component analysis (PCA) was applied to identify possible relationships between the major components and the environmental indices. According to the results of Table 3, the essential oils of the root parts showed no variability in the chemical compositions. The principal components of this essential oil were aplotaxene that varied 40.6-61.6% and caryophyllene oxide of 6.9-14.7% (Table 3).

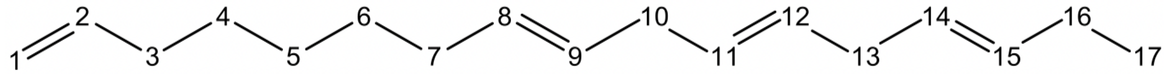

Figure 1. Structure of aplotaxene

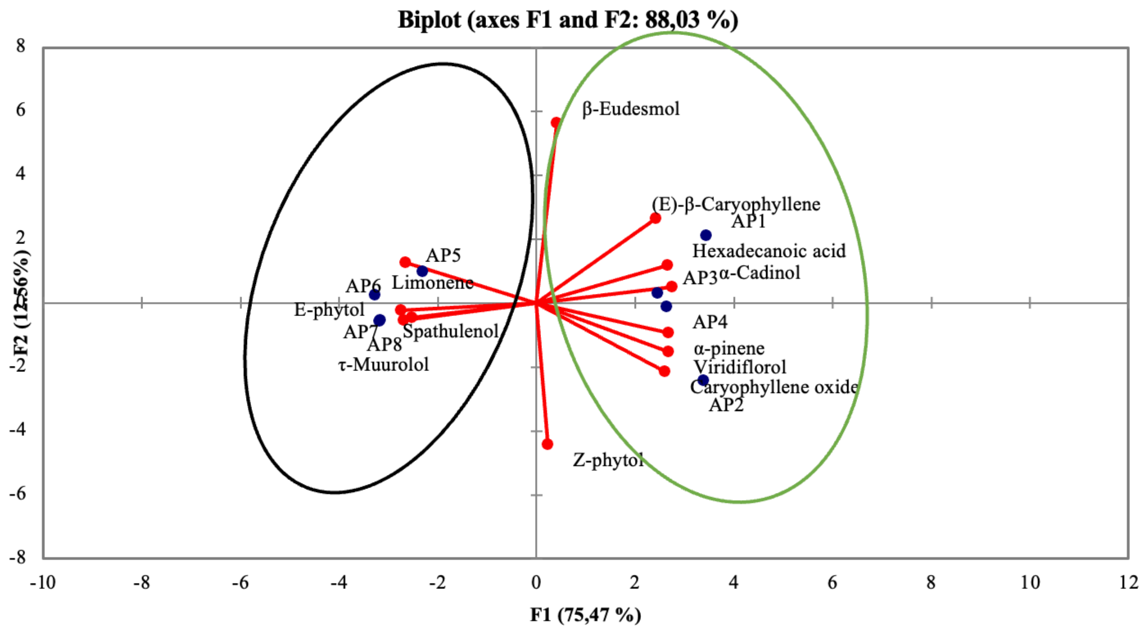

Figure 2. Principal component analysis (PCA) of chemical compositions of *C. acaulis*

On the other hand, the results of PCA (Fig. 2) suggested that there are two main clusters of essential oils of aerial parts of *C. acaulis*. The first two axes accounted for 75.45% and 12.56% of the total variance, respectively. The general structure of the PCA clustering confirmed the existence of two main groups. Group 1 included all samples of essential oils from Ghazaouet, Nedroma, Zenata and Henaya (AP1 to AP4), and Group 2 included all samples of essential oils from Ain Fezza, Zarifet, Benibahdel and Beni snous (AP5 to AP8). Group 1 characterized by a humid climate and low altitude was mainly discriminated by the high contents of (E)-β-caryophyllene (3.4-8.4%), hexadecanoic acid (6.3-10.8%), α-pinene (2.9-4.1%), viridiflorol (5.5-8.6%) and caryophyllene oxide (6.2-9.9%). While, the second group II characterized by a humid climate and higher altitudes was characterized by high levels of limonene (15.2-19.2%), τ-muurolol (12.6-17.2%), E-phytol (4.3-5.6%) and spathulenol (1.2-3.1%) (Fig 2).

Antioxidant activities

DPPH free radical scavenging assay

The free radical scavenging activity of *C. acaulis* essential oils, aplotaxene and the combination between aplotaxene and BHT were analyzed using DPPH assay. The results are shown in Fig. 3. The scavenging ability of all samples has shown antioxidant activity. Comparison of the DPPH scavenging activity of the investigated essential oils and aplotaxene with those expressed by BHT showed that aplotaxene exhibited the strongest activity (IC_{50}=0.24 g/L) better than reference BHT (0.26 g/L). While, the essential oils of aerial and root parts showed low antioxidant activity with IC_{50}s of 2.4 g/L and 1.7 g/L, respectively (Fig. 3). On the other hand, aplotaxene-BHT combination had given a very interesting synergistic effect with excellent antioxidant activity in quenching of DPPH radical, with an IC_{50} of 0.12 g/L, more interesting than the control BHT alone (Fig. 3).

Metal chelating assay

The formation of a complex with free ferrous

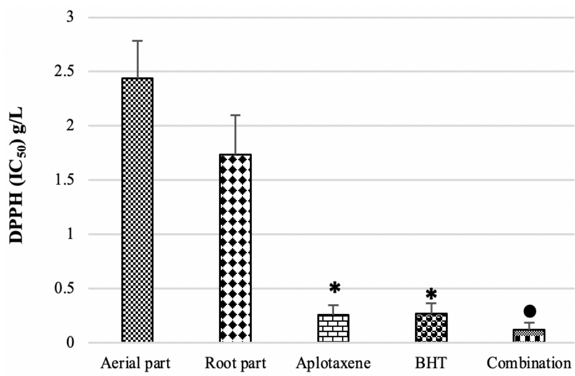

Figure 3. Antioxidant activity (IC_{50} g/L) of essential oil of *C. acaulis* by DPPH method (*significant p≤0.01 compared to aerial and root parts; ●significant p≤0.01 compared to aplotaxene and BHT)

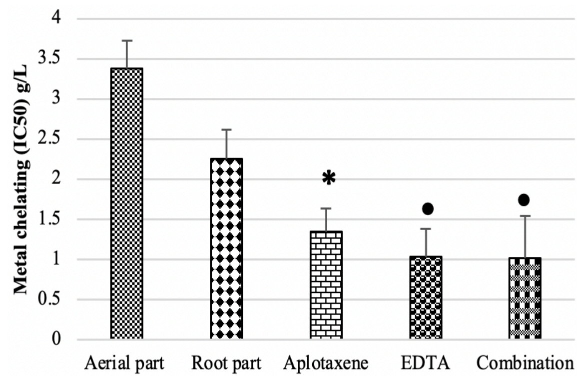

Figure 4. Antioxidant activity (IC_{50} g/L) of essential oil of *C. acaulis* by Metal Chelating Method (*significant p≤0.01 compared to aerial and root parts; ●significant p≤0.01 compared to aplotaxene and EDTA).

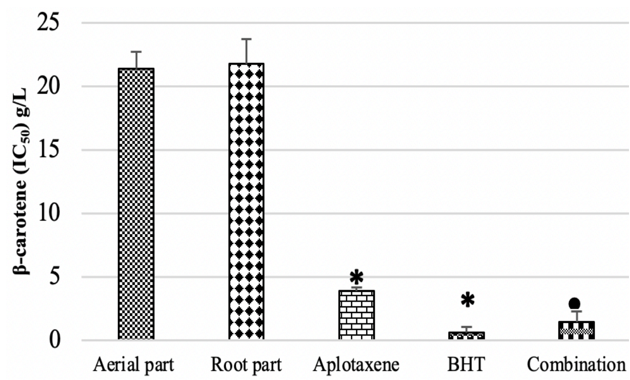

Figure 5. Antioxidant activity (IC_{50} g/L) of essential oil of *C. acaulis* by *β*-carotene method (* significant p≤0.01 compared to aerial and root parts ●significant p≤0.01 compared to aplotaxene and EDTA)

(Fe^{2+}) ions that leads to a change in the color of the test samples with increasing the amount of oil (0.2 to 15 g/L) showed very good antioxidant activity. The half-maximal inhibitory concentration (IC_{50}) providing 50% inhibition of metal chelating assay in the test solution was calculated (see Fig. 4). The comparison of the metal chelating assay of *C. acaulis* essential oils, aplotaxene and the combination of aplotaxene with EDTA showed that the combination between aplotaxene (IC_{50}= 1.34 g/L) and EDTA (IC_{50}= 1.03 g/L) had given a synergistic effect with a better chelating effect (IC_{50}= 1.01 g/L) almost equal to the synthetic antioxidant used as a reference, followed to roots (IC_{50}=2.25 g/L) and aerial parts (IC_{50}=3.37 g/L) of essential oils. (Fig. 4).

β-Carotene bleaching assay

β-carotene-linoleic acid bleaching assay is based on the loss of the yellow color of β-carotene when it is attacked by the radicals produced by linoleic acid oxidation in an emulsion [31]. The IC_{50} value of aerial and root parts essential oils were 21.3 g/L and 21.7 g/L, whereas the IC_{50} of BHT was 0.59 g/L. However, the combination between aplotaxene (IC_{50}= 3.85 g/L) and BHT had given a synergistic effect with important antioxidant activity in lipid peroxidation (IC_{50}= 1.40 g/L) but which less than the reference antioxidant BHT (Fig. 5).

In-vitro anti-inflammatory activity

The *in-vitro* anti-inflammatory activity of essential oils, aplotaxene, diclofenac sodium and combination was done using the protein denaturation method. Protein denaturation is a process of loss of biological properties of protein molecules by application of external stress or compound such as an organic solvent or heat [32]. The essential oils of *C. acaulis* protected the albumin against heat-induced denaturation. The results showed a concentration-dependent inhibition of protein (albumin) denaturation by samples (0.2 to 2.5 g/L). Sodium diclofenac was used as the reference drug at the same concentration (Table 4). The results showed that the essential oil of roots and aplotaxene have a very good inhibitory effect, with percentages of 82.7% and 77.3%, at a concentration of 2.5 g/L comparatively to diclofenac (80.3%) respectively. However, the combination between aplotaxene and diclofenac showed high inhibition (90.3%) at the same concentration (2.5 g/L) (Table 4).

The samples/drug concentration for 50% inhibition (IC_{50}) was determined by plotting percentage inhibition with respect to control against treatment concentration. The best anti-inflammatory activity was observed with the combination of aplotaxene and diclofenac with IC_{50} of 0.84 g/L compared to diclofenac (IC_{50}= 1.01 g/L) (Fig. 6).

Neuroprotective Activity

Given the research interest of our group for this medicinal plant, we tested the essential oil of *C. acaulis* as an inhibitor of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) two enzymes responsible for Alzheimer's disease. The results of the AChE and BChE inhibitory

Table 4. Percentages of inhibition of protein denaturation of *C. acaulis* essential oils, Aplotaxene, Sodium diclofenac and their combination at different concentrations

Concentrations	Percentage of Inhibition				
(g/L)	Diclofenac Sodium (%)	Aerial parts	Root parts	Aplotaxene	Combination
0.2	20.0±0.1	20.4±0.0	17.2±0.2	11.7±0.0	20.0±0.0
0.4	37.9±0.1	33.5±0.0	26.8±0.7	25.5±0.0	39.3±0.1
0.6	43.4±0.2	41.6±0.2	37.2±0.1	33.1±0.0	48.9±0.1
1.0	56.5±0.8	45.2±0.4	48.2±0.1	47.5±0.3	65.5±0.1
2.0	75.5±0.1	55.4±0.6	68.2±0.7	65.2±0.4	78.6±0.2
2.5	80.3±0.1	67.8±0.1	82.7±0.2	77.3±0.5	90.3±0.1

Samples and positive control were done in triplicates (n=3), SD=standard deviation, Combination: Aplotaxene+diclofenac

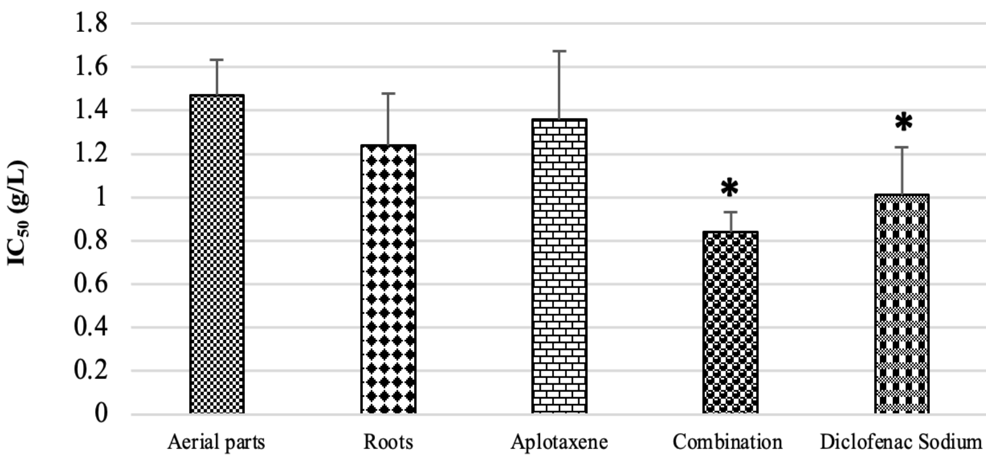

Figure 6. IC_{50} values of *C. acaulis* essential oils, aplotaxene, sodium diclofenac and their combination

activities of the samples are summarized in Table 5. The essential oil and aplotaxene did not show inhibition against AChE, while they selectively inhibited BChE at moderate levels (greater, than 55%) at the concentration of 100 mg/L (Table 5).

In addition, the essential oil of *C. acaulis* and aplotaxene showed good inhibitory activity of BChE with an IC_{50} values of 58.3 and 81.5 mg/L, but lower than galantamine (36.40±1,99 mg/L) in the anti-BChE assay (Fig. 7). Therefore, no activity was shown for the combination of the essential oil with galantamine.

Discussion

Natural products known for their medicinal properties are an inexhaustible source of phytochemicals with pharmacological effects [33]. To the best of our knowledge, the results obtained in this study are the first published data concerning the antioxidant, anti-inflammatory and enzyme inhibitory properties of essential oils of *C. acaulis* species. The results showed that the essential oils of aerial parts were rich in hydrocarbon and oxygenated sesquiterpenes, while the root parts were predominated by a good source of alkatetraenes derivatives (Aplotaxene). The previous work on other *Centaurea* species has shown that germacrene D was the main component in *C. hadimensis, C. drabifolia* subsp. *detonsa* and *C. rupestris*. While *C. iconiensis* contained a higher concentration of Undecene [34-37]. The results of the present investigation showed

Table 5. Enzyme inhibitory activity of the essential oil of *C. acaulis*

Essential oils mg/L	3.12	6.25	12.50	25.00	50.00	100.0
			AChE inhibitory activity			
EO	Na	Na	Na	Na	Na	Na
Aplotaxene	Na	Na	Na	Na	Na	Na
Galantamine	35.9±2.3	43.7±0.0	68.5±0.3	80.7±0.4	85.8±1.6	91.8±0.2
Combination	Na	Na	Na	Na	Na	Na
			BChE inhibitory activity			
EO	13.3±0.1	18.3±0.2	26.3±1.2	35.1±2.1	47.5±1.1	69.6±1.9
Aplotaxene	Na	Na	10.3±0.3	28.4±0.6	43.2±2.6	58.6±1.6
Galantamine	3.3±0.6	6.93±0.6	24.3±2.9	45.1±2.6	63.9±2.8	73.6±0.8
Combination	Na	Na	Na	Na	Na	Na

Values expressed are means ±S.D of three parallel measurements. Na: no absorbance. EO; Essential oils. Combination: Essential oil+ Galantamine

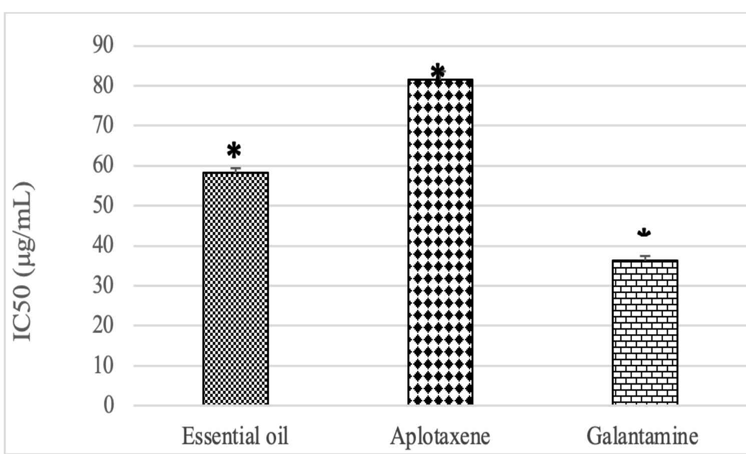

Figure 7. IC_{50} values of *C. acaulis* roots, aplotaxene and galantamine in the anti-BChE assay

that the combination between aplotaxene and the synthetic antioxidant exhibited remarkable antioxidant properties in quenching of DPPH radical which was about 2 times higher than the synthetic antioxidant used as a reference. The antioxidant activity evaluated by the method of metal chelating showed that the combination of aplotaxene with EDTA was better than aplotaxene, 3 times more active than the essential oil of the aerial part and 2 more active than that of the roots. While in β-carotene bleaching assay, the combination between aplotaxene and BHT had given a synergistic effect but which less than the reference antioxidant BHT. The combination of essential oils, extracts or natural bioactive compounds with synthetic antioxidants may lead to new natural preservatives. Indeed, a synergistic antioxidant effect between methanol extract of rosemary and BHT was demonstrated, allowing a decrease from 4.4 to17 folds in the amounts of the synthetic compound used [38]. Potential synergistic activity was found in combinations of natural antioxidants isolated from *spinach* leaves and synthetic antioxidants. On the other hand, the combination of ferulic acid, caffeic acid, and epigallocatechin-3-gallate with commercial antioxidants showed synergistic antioxidant activity [39]. However, if we review the results of antioxidant activities, we can notice that aplotaxene showed very interesting activities. Indeed, it has been suggested that apolar components have antioxidant properties because they are concentrated within the lipid-water interface, allowing the oxidation of *β*-carotene and the prevention of lipid radical formation [40]. The *in-vitro* anti-inflammatory activity showed that aerial and root parts essential oils have significant anti-inflammatory activity towards the denaturation of fresh hen's egg albumin protein, but the combination of aplotaxene and the standard drug showed better activity compared to the activity of diclofenac sodium alone. The anti-inflammatory molecules of medicinal plants, belonging to the most diverse chemical classes, have already demonstrated proven anti-inflammatory activity [41]. Among them, alkaloids, terpenes [42,43] and phenolic compounds such as tannins and flavonoids [44-46]. According to the literature, methyl eugenol rich lemongrass essential oil showed good activity in *in-vitro* anti-inflammatory test [47]. The *in-vitro* anti-inflammatory effects of *Centaurea hierapolitana*, *Centaurea calolepis* and *Centaurea cadmea* exhibited strong anti-inflammatory activities [48]. The essential oil had better BChE inhibitory activity. Neuroprotective effect against the key enzyme involved in alzheimer disease and more precisely against BChE was observed. Thus, the best anti-BChE activity of the essential oil could be attributed on one hand to aplotaxene, and other hand, to the synergistic effect of mixture of some terpene compounds that are identified as major or minor constituents. Indeed, α-pinene was potent inhibitor of AChE. While the trans-caryophyllene inhibited BChE with IC_{50} values of 78.6 mg/L. The caryophyllene oxide as a major compound of essential oil of *Salvia verticillata* subsp. *amasiaca* showed significant anticholinesterase capacity as well [49]. Recently, the activity of butyrylcholinesterase (BChE) has been a focus of many researches because in the late stages of alzheimer disease, the concentrations of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, declines dramatically by up to (85%) while the BChE level remains the same or is even up-regulated where it represents the predominant cholinesterase in the alzheimer disease patient brain [50]. For this purpose, such researches have targeted BChE as a new approach to intervening in the management of alzheimer disease [51-53].

These properties make *C. acaulis* specie a potential alternative natural to use in the food and pharmaceutical industries for the treatment of diseases that involve oxidative stress and in the treatment of inflammations. Consequently, these results can be considered as preliminary in order to show the importance of this species. In any case, further studies need to be conducted to evaluate the efficacy of essential oil and aplotaxene on industrial scale.

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