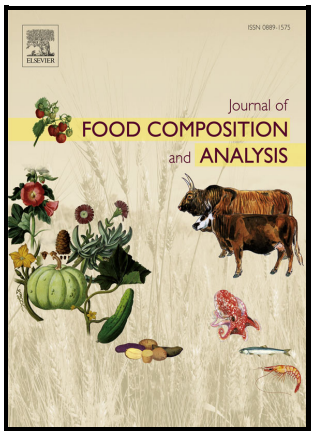

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Cistus libanotis from Algeria: Phytochemical Analysis by GC/MS, HS-SPME-GC/MS, LC-MS/MS and its anticancer activity

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Abstract

In this paper, *Cistus libanotis* (*Cistus clusii* Dunal) from Algeria was investigated. This plant is utilized in numerous industries, mostly in perfumery and, more recently, as a raw material for dietary supplements. For the first time, the present study evaluated the anticancer potential of Algerian *C. libanotis* by performing a comprehensive phytochemical investigation. The volatile fraction of the fresh plant material of *C. libanotis* was investigated using headspace solid-phase microextraction (HS-SPME) coupled to GC-MS, and the chemical composition of the essential oil (EO) of *C. libanotis* was examined using GC-MS technique; additionally, the phenolic compounds were identified and quantified using liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). A healthy cell line (L929) and two cancer cell lines (CAPAN-1 and dld-1) were used to test the ethanolic extract's cytotoxicity. HS-SPME-GC/MS analysis identified sabinene (25.3 %) and α-thujene (23.8 %) as principal components. In EO the major ingredients were γ-terpinene (17.4 %), camphene (16.13 %), α-phellandrene (15.36 %), and 4-carene (13.54 %). Nevertheless, 24 components were identified by LC-ESI-MS/MS analysis and shikimic acid, luteolin and hesperidin were most abundant. Further, the ethanolic extract of *C. libanotis* showed a significant cytotoxic effect against dld-1 and CAPAN-1 cell lines, while it was less active against the healthy cell line L929.

Keywords: HS-SPME; GC-MS analysis; LC-MS-MS analysis; cytotoxicity.

1. Introduction

The Cistaceae family, which includes eight genera and roughly 180–200 species of shrubs, is mainly made up of heliophyte shrubs, subshrubs, and herbs that grow in open spaces with little soil nutrients (Guzma´n and Vargas, 2009). Adapted to fires in Mediterranean forests, these plants are pyrophylic and resilient to wildfires, with their seeds able to rapidly repopulate destroyed areas in the subsequent season (Alonso et al., 1992; Ferrandis et al., 1999). Known for their fragrant and sweet-smelling qualities, many members of the Cistaceae family are highly valued in the perfume industry and as ornamental plants. Some of the genera in this family that are noteworthy are *Cistus*, which has between 16 and 28 species depending on the source, *Helianthemum*, and *Halimium* (Guzmán & Vargas, 2005). *Cistus*, a small yet complex genus, is characterized by its polymorphic species and hybridization between related varieties, with around 20 species of perennial shrubs found in dry or rocky soils across Europe (Tabacik and Bard, 1971; Ellul et al., 2002). Of these, seventeen are common members of the Mediterranean Flora, frequently found in dense numbers close to the coast (Guzman et al., 2009; Nicoletti et al., 2015). Shrubs within the *Cistus* genus is distinguished by its white flowers and viscous stems and leaves that provide a scented oleoresin (Guimarães et al., 2009). Some species of *Cistus* are endemic to specific regions, while others are widely distributed across the Iberian Peninsula, Canary Islands, Northwestern Africa, Italy, Greece, and Turkey (Andrade et al., 2009). The genus *Cistus* is classified into three subgenera: *Cistus*, *Halimioides*, and *Leucocistus*, with the former featuring plants bearing purple flowers, and the latter two comprising species with white flowers, as indicated by previous phylogenetic and taxonomic studies (Guzmán et al., 2009). *C. libanotis*, characterized by briefly pubescent peduncles, pedicels, and sepals, exhibits non-silvery sepals that are oval, acute, or obtuse, barely extending beyond the capsule. Typically, it is an erect, highly branched shrub measuring 50-100 cm in height. Its leaves are linear, obtuse, and revolute, with dimensions of 1-2.5 cm in length and 1-2 mm in width, featuring a glabrous upper surface and a tomentose underside, along with a distinct midrib and sessile attachment. The inflorescence resembles that described earlier, with obovate-cuneiform petals. Its capsule is ovate-oblong, included, and puberulous, while its seeds are ferruginous and granular (Quezel and Santa,

1963). According to APD (2024), *C. libanotis* auct. is synonymous with *C. clusii* Dunal, with its native habitat primarily spanning the Western and Central Mediterranean regions, where it thrives as a shrub within the subtropical biome (POWO, 2024). *Cistus* is valued for its anti-inflammatory, anti-ulcerogenic, wound-healing, antibacterial, cytotoxic, and vasodilator qualities in traditional folk medicine (Barrajón-Catalán et al., 2011; Jemia et al., 2013). In Eastern Morocco, the local population refers to *C. libanotis* as "boubala" (Zidane et al., 2013). Due to the apparent health benefits of their essential oil and tea, *Cistus* species are currently enjoying a resurgence in popularity. As a result, there is a sizable market for herbal goods, including botanical food supplements (Jemia et al., 2013). Recent studies showed light on potential bioactive compounds responsible for these effects, with new activities such as antimicrobial, antioxidant, antiproliferative, antinociceptive, and analgesic effects being attributed to *Cistus* extracts (De Andres et al., 1999; Küpeli & Yesilada, 2007; Barrajón-Catalán et al., 2010). Furthermore, the Food and Drug Administration (FDA) has approved *Cistus* essential oil as a flavoring and food additive, highlighting its safety and adaptability (Loizzo et al., 2013). Rich in various interesting compounds, these oils are also utilized as raw materials for botanical food supplements and in perfumery. Nevertheless, their widespread use necessitates thorough scientific validation (Venditti et al., 2015). *Cistus* species, particularly *C. incanus*, are utilized as dietary supplements, with 'Cistus tea' being a popular choice due to its antioxidant properties aimed at preventing chronic diseases (Riehle et al., 2012). Previous studies on the phytochemistry of different species of *Cistus* have demonstrated the existence of terpenes, like labdanum, which are used in perfumery, along with phenolic compounds like flavonoids and tannins, renowned for their antioxidant properties and other therapeutic effects (Weyerstahl et al., 1998; Miyazawa & Hisama, 2003). Recent studies have focused on isolating and identifying bioactive compounds from *Cistus* species, including clerodane terpenes, flavonoids, and bornyl derivatives, such as *p*-coumarate and caffeate (Zidane et al., 2013; Ben Jemia et al., 2013). According to a report on the chemical composition analysis of *C. libanotis* essential oils and aqueous extracts, there is a high concentration of hydrocarbons, oxygenated monoterpenes, diterpenes, and sesquiterpenes (Barrajón-Catalán et al., 2011; Zidane et al., 2013). Despite the extensive research on

well-known species like *C. monspeliensis* and *C. ladaniferus*, several other species, including *C. libanotis*, require further investigation. As part of our exploration into Algerian medicinal plants, we used cutting-edge analytical methods including GC–MS, HS-SPME–GCMS, and LC-MS/MS to perform a thorough phytochemical analysis of ethanol extracts and essential oil from *C. libanotis.* Additionally, we evaluated their antioxidant and antiproliferative activities through various *in vitro* assays, aiming to elucidate their mechanisms of action and therapeutic potential.

2. Materials and methods

2.1. Chemicals and Reagents

Ultra-pure methanol (67-56-1, ≥99%), ammonium formate (540-69-2, ≥99%), formic acid (64-18-6, ≥98%), and hexane (110-54-3, ≥99%) were obtained from Merck (Darmstadt,Germany). High-purity standards of shikimic acid (138-59-0, ≥98%), gallic acid (149-91-7, ≥99%), protocatechuic acid (99-50-3, ≥97%), epigallocatechin (970-74-1, ≥95%), catechin (154-23-4, ≥98%), chlorogenic acid (327-97-9, ≥95%), hydroxybenzaldehyde (123-08-0, ≥98%), vanillic acid (121-34-6, ≥97%), caffeic acid (331-39-5, ≥98%), caffeine (58-08-2, ≥99%), vanillin (121-33-5, ≥99%), salicylic acid (69-72-7, ≥99%), trans-ferulic acid (537-98-4, ≥99%), o-coumaric acid (614-60-8, ≥97%), isoquercitrin (21637-25-2, ≥90%), hesperidin (520-26-3, ≥97%), kaempferol-3-glucoside (480-10-4, ≥97%), fisetin (528-48-3, ≥98%), trans-cinnamic acid (140-10-3, ≥98%), quercetin (117-39-5, ≥95%), naringenin (480-41-1, ≥95%), kaempferol (520-18-3, ≥90%), luteolin (491-70-3, ≥98%) and biochanin A (491-80-5, ≥97%) were obtained from Sigma Aldrich (Steinheim, Germany). Pure water for chromatographic purposes was prepared using the Milli-Q system from Millipore. All other chemicals and solvents were of analytical grade.

2.2. Plant material

The flowering aerial parts (stems, leaves, and flowers) of *Cistus libanotis* auct. (Figure 1), were harvested in April 2022 from the Soubella region (M'sila province) in northeastern Algeria (coordinates 35°43'27 N and 05°09'48 E, altitude 929 m). Taxonomic authentication of the plant material was conducted by Prof. K. Rebbas of the University of Msila by via the Algeria Flora (Quezel and Santa, 1963) and the literature that was available. A voucher specimen (Number N° KR0129) was deposited to the University of Msila herbarium. The collected samples were cleaned, allowed to air dry for several days at room temperature (25 °C) out of the sun, and then blended into a fine powder. After that, the powder was kept at 4 °C until needed again.

Fig. 1. Illustration of aerial parts (stems, leaves, and flowers) of *Cistus libanotis* auct., photos Pr. REBBAS K. 2022

2.3. Essential oil extraction

Fresh samples weighing 50 g were initially cut and ground using a Waring blender. Subsequently, hydro-distillation was carried out for a duration of 3 hours utilizing approximately 100 g of distilled water, using the standardized technique outlined in the European Pharmacopoeia (2020). The obtained essential oils (EOs) were then dehydrated using anhydrous sodium sulfate and transferred into closed containers further down a nitrogen atmosphere. These vials were kept at -20 °C, ensuring preservation for successive GC-MS analyses. The yield of essential oil obtained from the samples was determined to be 0.04 % (w/w).

2.4. Crude Extracts Preparation

Twenty grams of powdered plant material that had been air-dried and dried was macerated for 48 to 72 hours at room temperature (25 °C) using 200 mL of 99.99 percent ethanol or 200 mL of petroleum ether three times. The extracts were concentrated by rotatory evaporation after being filtered through filter paper, and they were then stored at 4 °C until needed (Ertas et al., 2021).

2.5. HS-SPME-GC-MS Analysis of volatile content

The HS-SPME technique, as described by Ascrizzi et al. (2017) and Bendif et al. (2017), was employed in this study. A Supelco SPME (Solid Phase Micro-Extraction) device coated with polydimethylsiloxane (PDMS, 100 µm) was used to sample the headspace of the powdered aerial parts. Two grams of fresh plant material were sealed in a 4 mL glass vial and allowed to acclimate at ambient temperature for one hour. The vial was then covered with aluminum foil and left to equilibrate at room temperature for 30 minutes, followed by an additional hour in an oil bath at 40 °C. Blanks were run before each initial SPME extraction and randomly during the series to ensure accuracy. Quantitative comparisons of relative peak areas were conducted between the same compounds across different samples. SPME sampling was carried out using a new fiber preconditioned according to the manufacturer's instructions for all analyses. After the equilibration period, the fiber was exposed to the headspace for 15 minutes. Following sampling, the fiber was retracted into the needle and transferred to the injection port of the GC/MS system. The analysis was performed using a Varian CP-3800 gas chromatograph coupled with a Varian Saturn 2000 mass spectrometer (GC/EI-MS) equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 µm). The analytical conditions were as follows: injector temperature at 220 °C, transfer line temperature at 240 °C, oven temperature programmed from 60 °C to 240 °C at a rate of 3 °C/min, and helium as the carrier gas at a flow rate of 1 mL/min in splitless mode. Identification of the constituents was based on a comparison of the retention times with those of authentic samples, linear retention indices (LRI) relative to the C6-C28 series of n-hydrocarbons, and computer matching against commercial (Adams, 2017; NIST, 2014) and custom-built library mass

spectra from pure substances, as well as comparing their retention times with those of reference pure compounds and calculating their linear retention indices (LRI) in relation to the C6–C28 series of n-hydrocarbons.

2.6. GC-MS analysis of essential oil

The methodology for the essential oil analysis was as described by Badalamenti et al. (2023). Shimadzu QP 2010 plus gas chromatograph with AOC-20i autoinjector, flame ionization detector (FID), and capillary column (DB-5 MS) measuring 30 m × 0.25 mm i.d. with 0.25 μm film thickness was used for the GC-MS analysis. The oven program was to start at 40 °C and hold it there for 5 minutes. After that, it ramped up to 260 °C at a rate of 2 °C per minute and remained isothermal for 20 minutes. The carrier gas was helium, flowing at a rate of 1 mL/min. The temperatures of the injector and detector were adjusted to 250 °C and 290 °C, respectively. With a mass spectrometer (MS) range of 40-600, a 1 μL solution of the essential oil (3 % EO/hexane v/v) was injected in split mode 1.0. To determine the percentages shown in Table S1, the total ion chromatogram (TIC) from MS was utilized. Ionization voltage of 70 eV, electron multiplier energy of 2000 V, transfer line temperature of 295 °C, and solvent delay of 3.5 min were the parameters specified for the device. By matching each compound's mass spectrum with the internal mass spectral databases of NIST 11, Wiley 9, and FFNSC 2, the compounds were identified. Compounds that showed at least 90 % similarity to the library were accepted. The linear retention indices (LRI), which were computed using a range of n-alkanes spanning from C8 to C40, and published mass spectra were used to further corroborate these identifications.

2.7. LC-ESI-MS/MS analysis

With a few minor modifications, the LC-ESI-MS/MS analysis was conducted in accordance with the Griffith et al. (2019) protocol. In a 2 mL Eppendorf tube, 50 mg of the ethanolic extract was first diluted in a solution containing 1 mL of methanol and 1 mL of n-hexane. The solution was then centrifuged at 15.033 x g. for 10 minutes at 4 °C using a Hettich Universal 320 R centrifuge (Germany), after being vortexed for 2 minutes at 4 °C using a Bioprep-24 homogenizer. After that, the methanol phase was

separated and made 1:9 dilution in distilled water. The resultant samples were filtered using a 5.12 µL injection volume Captiva premium syringe filter, which was outfitted with a nylon membrane with a diameter of 25 mm, a polypropylene shield, and a pore size of 0.45 µm. Using a tandem mass spectrometer and an Agilent 1260 Infinity II LC System, the LC-ESI-MS/MS analysis was carried out. The procedure ran for a total of thirty minutes at a flow rate of 0.5 mL/min, with an oven temperature of 25 °C. A degasser (1260 Degassing), a column furnace (1260 TCC), and dual pumps (1260 Thousand Pumps) were integrated into a reversed-phase Agilent Poroshell 120 EC-C18 analytical column (100 mm × 3.0 mm, 2.7 µm), which was used for chromatographic separation. Eluent A (water with 5 mM ammonium formate) and eluent B (acetonitrile with 0.1 % formic acid) made up the mobile phases, which were kept at an isocratic 75 % A and 25 % B ratio.

An Agilent 6460 Triple Quadrupole Mass Spectrometer System with electrospray ionization (LC–ESI–MS/MS), functioning in both positive and negative ionization modes, was utilized for mass spectrometric detection. Phytochemical substances were precisely identified and quantified by the use of Multiple Reaction Monitoring (MRM) technique in Agilent Mass Hunter Software data processing. To provide the best possible fragmentation and transmission of the intended ions, collision energies (CE) were tuned. Operating conditions for the mass spectrometer included a gas temperature of 350 °C, a capillary voltage of 4000 V, a nitrogen (N2) drying gas flow of 15 mL/min, and a nitrogen nebulizing gas flow of 11 mL/min (Köktürk et al., 2022). Limit of Detection (LOD), Limit of Quantification (LOQ), and Linearity Range—three validation parameters for the method—were examined and computed in accordance with Yilmaz's technique (2020).

The limits of detection (LOD) and quantification (LOQ) for each phytochemical analyte were determined by progressively diluting standard solutions and analyzing them under the specified LC–MS/MS conditions until the lowest detectable concentration (S/N ratio 3:1) was observed. Once the minimum detectable concentrations for each analyte were established, ten identical standard solution

mixtures (including internal standards) were prepared at these concentrations and injected into the LC–MS/MS system. The LOD and LOQ values were then calculated using the following equations :

LOD = Mean + 3 × SD (SD: Standard Deviation)

LOQ = Mean + 10 × SD

Linearity was assessed by generating calibration curves for each analyte across 8 concentration levels, with each level analyzed in triplicate. These curves were created by plotting the ratio of the analyte concentration to the internal standard concentration (x) against the ratio of the analyte area to the internal standard area (y). The method proved to be linear for all compounds within the tested concentration ranges, with correlation coefficients higher than 0.99.

2.8. Anticancer activity

Cell lines such as L929 fibroblast, CAPAN-1 pancreatic cancer, and dld-1 colorectal adenocarcinoma were received from the laboratory of Prof. Dr. Mustafa Türk at Kırıkkale University (Turkey) in order to assess the anticancer potential. Multi-well and culture plates from Corning, USA were used for all cell culture operations. The frozen cells were quickly thawed at 37 °C before being moved to a sterile laminar flow hood and put into a 15 mL cell culture tube. Before seeding into 25 cm^2 flasks, 3 mL of the corresponding medium—which contained 10 % fetal bovine serum and 1 % antibiotic—was added to the tube after it had been centrifuged at 250 G for five minutes. After that, the flasks were kept in an incubator set at 37 °C with 5 % CO_2 (Karan and Erenler, 2018). The ethanolic extract of *Cistus libanotis* was combined with nutritional medium to create concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL, and these mixtures were then carefully vortexed to prepare the sample. The application for the cell phone was completed quickly and without any delay. Based on live cell counts, the number of cells in each well was adjusted to $10x10^3$. To enable cell adherence to the well plate surface, 100 µL of cell suspension in complete media was applied to each well and incubated for 24 hours. The test substances of *Cistus libanotis* ethanolic extract were introduced to the wells at

concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL after cell adhesion was confirmed. The medium was then withdrawn. 10 % DMSO was given to the positive control group and just full medium was given to the negative control group. After incubating for 24 hours, the medium was changed to 1 mg/mL of MTT (tetrazolium salt) solution in each well, and the mixture was incubated for an additional 2 to 2.5 hours at 37 °C. The MTT solution was then treated with 100 µL of MTT solvent (isopropanol), and the cell viability was assessed by measuring the optical density of the 96-well plate at 570 nm using an ELISA plate reader. The cell viability percentage for each group was calculated using the formula below, assuming the control cell viability to be 100 %.

Cell viability % =A_sample/A_control x 100

The optical density of a sample is called a sample, while the optical density of a reference is called a control. At a density of 15x103 cells per well, cells were seeded onto a 48-well plate and incubated for one day under carefully monitored conditions. After the incubation period, the medium was removed and 200 µL of samples containing 0.5 mg/mL were added to each well, with the exception of the negative control well, which was filled with cells only. After that, the plate was put back in the incubator for a full day. The plate was then covered, 70 µL of a double staining solution was applied to each well, and it was left in the dark for 15 minutes (Aydin et al., 2016).

2.9. Statistical analysis

The mean ± standard error (SE) is used to express the anticancer outcomes. Tukey's post-hoc test was performed after group means were compared using one-way analysis of variance (ANOVA) for the cell viability percentage analysis. All statistical analyses were conducted using SPSS 22.0 (Systat Software Inc., San Jose, CA, USA), with a significance level of $p < 0.05$. Three separate experiments' worth of duplicate samples were used for the data analysis.

3. Results and Discussion

3.1. HS-SPME-GC-MS Chemical composition of aerial parts

The advancement of rapid and environmentally sustainable techniques for extracting volatile compounds from plant materials, such as HS-SPME, holds significant importance. This method allows for swift sampling, thereby shortening the overall analysis time, while also minimizing environmental impact by eliminating the need for organic solvents. When applied to the aerial parts of *C. libanotis*. A combination of GC-MS and HS-SPME analysis identified 26 volatile compounds that together made up 99.3 % of the volatile extract. Table 1 provides information about the detected compounds, including relative proportions and retention indices. To the best of our knowledge, the present work is the first to report the volatile profile of untreated aerial parts of *C. libanotus* The only previous paper that describes the content of odoriferous substances by HS-SPME-GC/MS, concerns the dry material of *C. creticus* L. collected in Poland and the results showed an high percentage of carvacrol.

Table 1. Aroma profile (%) of *Cistus libanotis* aerial parts obtained using HS-SPME–GC–MS.

N°	Constituents[a]	LRI lit[b]	LRI[c]	*Cistus libanotis* (%)[d]
1	hexanal	801	802	0.5
2	*α*-thujene	932	933	23.8
3	*α*-pinene	939	941	0.7
4	camphene	953	955	4.3
5	sabinene	976	977	25.3
6	*α*-terpinene	1022	1020	0.5
7	*p*-cymene	1029	1028	9.4
8	*β*-phellandrene	1031	1033	0.7
9	5-methyldecane	1058	1058	0.4
10	*γ*-terpinene	1063	1062	1.8
11	cis-sabinene hydrate	1071	1070	4.8
12	trans-sabinene hydrate	1099	1099	5.5
13	trans-pinocarveol	1142	1141	0.8
14	camphor	1144	1145	1.3
15	sabina ketone	1156	1158	1.5
16	pinocarvone	1163	1163	0.4

17	borneol	1166	1167	7.4
18	4-terpineol	1177	1179	1.8
19	*p*-cymen-8-ol	1184	1185	0.5
20	myrtenol	1194	1194	0.8
21	verbenone	1205	1205	0.9
22	cumin aldehyde	1240	1241	0.5
23	*α*-terpinyl acetate	1351	1353	1.1
24	*trans*-calamenene	1524	1523	1.5
25	dihydroactinidiolide	1532	1532	0.4
26	caryophyllene oxide	1582	1581	2.2
	Total identified			99.3
	Monoterpene hydrocarbons			67.0
	Oxygenated monoterpenes			27.3
	Sesquiterpene hydrocarbons			1.5
	Oxygenated sesquiterpenes			2.2
	Apocarotenes			0.4
	Non-terpene derivatives			0.9

[a] Compounds are classified in order of linear retention time of the apolar column (DB-5 MS); [b] Experimental linear retention index on a DB-5 MS apolar column; [c] Linear retention index reported for DB-5 MS column reported in the literature; [d] Area is the peak volume percentage of compound in the sample. LRIs based on literature (https://webbook.nist.gov/).

3.2. Chemical composition of essential oil by GC-MS

The table 2 presents the volatile compounds of *C. libanotis* essential oil along with their Kovats indices and respective percentages. A total of twenty-nine (29) compounds were identified in the essential oil of *C. libanotis* through GC-MS analysis. Among these compounds, *γ*-Terpinene (17.4 %), camphene (16.13 %), *α*-Phellandrene (15.36 %), and 4-Carene (13.54 %) were identified as the major constituents. A comparison of our findings with the existing literature reveals significant qualitative and quantitative variations in composition. Only a few analyses have been published on the composition of *C. libanotis* essential oils. In a previous work, a different chemical composition has been reported *for C. libanotis* from Morocco; terpienen-4-ol (17.4 %) was the most abundant component followed by *γ*-terpinene (12.4 %), camphene (12.2 %) and sabinene (11.2 %) whose relative percentages were

comparable (Zidane et al., 2012). On the other hand, Loizzo et al. (2013), reported camphene (25.2 %), *α*-pinene (21.6 %) and β-pinene (10.8 %) as the main components of *C. libanotis* collected in Tunisia. These differences in the chemical makeup of essential oils can be caused by a number of variables, including climate, geography, and season (de Sá Filho et al., 2022).

Table 2. Chemical composition (%) of C. libanotis EO collected wild in Algeria.

No.	Compounds[a]	LRI (Exp)	LRI (Lit)	*Cistus libanotis* (%)
1	Tricyclene	925	924	0.61
2	*α*-Phellandrene	932	986	15.36
3	Camphene	949	951	16.13
4	Sabinene	972	976	10.06
5	*β* -Phellandrene	1000	1005	1.03
6	4-Carene	1012	1002	13.54
7	*O*-cymene	1020	1022	8.45
8	D-sylvestrene	1024	1027	3.17
9	*γ*-Terpinene	1055	1058	17.4
10	cis-Sabinenehydrate	1061	1075	1.35
11	Terpinolen	1083	1083	3.37
12	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1α,2β,5α)-	1091	1073	0.39
13	Nonanal	1100	1104	0.1
14	Thujone	1109	1105	0.21
15	4-Isopropyl-1-methyl-2-cyclohexen-1-ol	1114	1115	0.14
16	Isopinocarveol	1130	1183	0.19
17	Camphor	1134	1149	0.65
18	Ethanone,1-(1,4-dimethyl-3-cyclohexen-1-yl)	1142	1152	0.18
19	7-Tetradecyne	1148	-	0.36
20	2(10)-Pinen-3-one	1153	1165	0.39
21	Borneol	1157	1156	1.15
22	Terpineol	1170	1180	3.96
23	α-Thujenal	1175	1189	0.08
24	Myrtenal	1185	1192	0.23
25	Bornyl formate	1219	1220	0.07
26	Cumaldehyde	1229	1238	0.15

27	Acetic acid, 1,7,7-trimethyl-bicyclo [2.2.1] hept-2-yl ester	1278	-	0.15
28	Terpinyl acetate	1343	1349	0.13
29	Diethyl phthalate	1584	1591	0.13
	Total			99.06
	Monoterpenes Hydrocarbons			89.12
	Oxygenated Monoterpenes			9.07
	Other Compounds			0.87

[a] Compounds are classified in order of linear retention time of the apolar column (DB-5 MS); [b] Experimental linear retention index on a DB-5 MS apolar column; [c] Linear retention index reported for DB-5 MS column reported in the literature; [d] Area is the peak volume percentage of compound in the essential oil sample. LRIs based on literature (https://webbook.nist.gov/).

3.3. Ethanolic extract analysy by LC-ESI-MS/MS

This study employs LC-ESI-MS/MS analysis, a robust analytical technique, to delve deeper into the chemical composition of plant extracts. LC-MS-based methods are particularly valuable for exploring the biochemical diversity of plants, especially given the abundance of semi-polar compounds present, including various secondary metabolites. These compounds are more effectively separated and detected using LC-MS approaches. Table 3 provides an overview of the chemical constituents identified in the ethanolic extract of *C. libanotis* through LC-ESI-MS/MS analysis. By utilizing the extract's high-resolution mass, retention duration, and MS fragmentation patterns, a total of 24 compounds were successfully identified. Additionally, quantification of the identified compounds was conducted. Among the components detected, Shikimic acid emerged as the most abundant, with a concentration of 4553.72 µg/g, followed by Luteolin at 990.45 µg/g and Hesperidin at 880.22 µg/g. Conversely, Biochanin A was present in lower quantities, with a concentration of 0.56 µg/g.

Other species have been investigated previously such as *C. creticus* and *C.salviifolius* growing in Croatia which have been assessed with UPLC-MS/MS providing a high number of phenolic compounds detected in the aqueous extracts (Carev et al., 2020). Also *C. incanus* growing on seashore dunes in Southern Tuscany was investigated to describe the polyphenolic content (Gori et al., 2016). Also, *C. screticus* and *C. salvifolius* from Syria showed a total content of phenolics and flavonoids (Waed et al., 2016).

The limit of detection (LOD) in this study ranged from 1.1609 to 237.5 ug/L, while the limits of quantification (LOQ) varied between 4.5238 and 265,9 ug/L. These values were close to those reported by Yilmaz, (2020). A recent research highlights that LOD values for LC-MS methods can vary depending on instrument conditions and estimation methods. As such, it is practical to use a conservative approach to estimate LOD/LOQ and apply these values broadly across different matrices. Modern LC-MS instruments are generally sensitive enough that concentrations below the LOD are often negligible (Sulyok et al., 2020).

Table 3. Phenolic compounds of *C. libanotis* ethanolic extract by LC-ESI-MS/MS analysis

No	Compound	Concentration (µg/g)	RT (min)	Ion sosurce	Ion Transitions	Ion Mode	Equqtion	R^2	LOQ (ug/L)	LOD (ug/L)	Linearity Range (ug/L)
1	Shikimic acid	4553.72	1.41	ESI	173.0 -> 93.0	Negative	y=0.070125+1.8616×	0.9978	50.4	15.7	1250-20000
2	Gallic acid	236.54	3.21	ESI	169.0 -> 125.1	Negative	y=0.0548597+20.8732×	0.9986	18.543	7.265	31.25-500
3	Protocatechuic acid	49.90	5.52	ESI	153.0 -> 109.0	Negative	y=0.243373+12.8862×	0.9969	13.1729	3.151	15.625-250
4	Epigallocatechin	46.97	6.84	ESI	307.0 ->139.0	Negative	y=−0.00494986+0.0483704×	0.998	265.9	237.5	1250-20000
5	Catechin	370.67	7.23	ESI	288.9 -> 245.1	Negative	y=−0.00370053+0.431369×	0.9946	7.5013	1.7055	343.750-5500
6	Chlorogenic acid	41.38	7.42	ESI	353.0 -> 191.0	Negative	y=0.289983+36.3926×	0.9981	25.9023	11.589	31.25-500
7	Hydroxybenzaldeyde	29.67	7.72	ESI	121.0 -> 92.0	Negative	y=0.06762+5.48767×	0.9993	12.8651	4.9742	15.625-250

8	Vanillic acid	109.26	7.82	ESI	167.0 -> 151.8	Negative	y=−0.0480183+0.779564×	0.9958	164.421	141.042	1250-20000
9	Caffeic Acid	10.82	7.89	ESI	178.9 -> 135.1	Negative	y=0.120319+95.4610×	0.9994	24.34	6.9505	31.25-500
10	Caffein	0.83	8.45	ESI	195.0 -> 137.9	Positive	y=0.045467+1.03157×	0.9986	15.23	6.9099	18.75-300
11	Vanillin	8.42	8.70	ESI	153.0 -> 125.0	Positive	y=0.00185898+20.7382×	0.9949	40.76	14.456	62.5-1000
12	o-coumaric acid	16.61	9.53	ESI	163.0 -> 119.1	Negative	y=0.00837193+11.2147×	0.9996	7.578	4.0983	15.625-500
13	Salicylic Acid	18.18	9.89	ESI	137.0 -> 93.1	Negative	y=0.239287+153.659×	0.9981	82.465	47.6695	112.5-1800
14	Trans-ferulic acid	20.18	10.20	ESI	193.1 -> 133.9	Negative	y=−0.0735254+1.34476×	0.995	11.675	6.1184	31.25-1000
15	Hesperidin	880.22	11.95	ESI	611.0 -> 302.9	Positive	y=-0.111120+4.10546×	0.9957	17.687	4.1396	31.25-500
16	Isoquercitrin	75.50	11.97	ESI	464.9 -> 302.8	Positive	y=-0.111120+4.10546×	0.9982	11.268	9.9382	18.75-300
17	Kaempferol-3-glucoside	28.90	13.33	ESI	448.8 -> 286.9	Positive	y=0.035035+17.16947×	0.9997	4.5238	1.1609	7.8125-125
18	Fisetin	1.57	13.33	ESI	287.0 -> 137.0	Positive	y=0.0365705+8.09472×	0.9954	44.3662	10.8961	15.625-250
19	Trans-cinnamic acid	3.07	14.46	ESI	149.0 -> 131.1	Positive	y=0.01461+1.0634×	0.9999	22.132	11.1853	31.25-500
20	Quercetin	15.10	15.03	ESI	300.8 -> 151.0	Negative	y=0.005232+3.39417×	0.9964	16.834	4.6558	27.5-440
21	Naringenin	7.64	15.21	ESI	270.9 -> 119.1	Negative	y=-0.00393403+14.6424×	0.996	3.9	2.6	31.25-500
22	Kaempferol	77.37	16.46	ESI	284.9 -> 116.9	Negative	y=-0.00459557+3.13754×	0.9997	5.414	1.8683	312.5-10000

23	Luteolin	990.45	18.04	ESI	285.0 -> 133.1	Positive	y=-0.0541723+30.7422×	0.9962	21.4535	20	31.25-500
24	Biochanin A	0.56	17.96	ESI	284.9 -> 116.9	Negative	y=0.02342+6.54636×	0.9997	5.4004	1.8683	312.5-10000

RT: Retention Time. R^2: Coefficient of determination. LOD/LOQ (µg/L): Limit of detection/quantification.

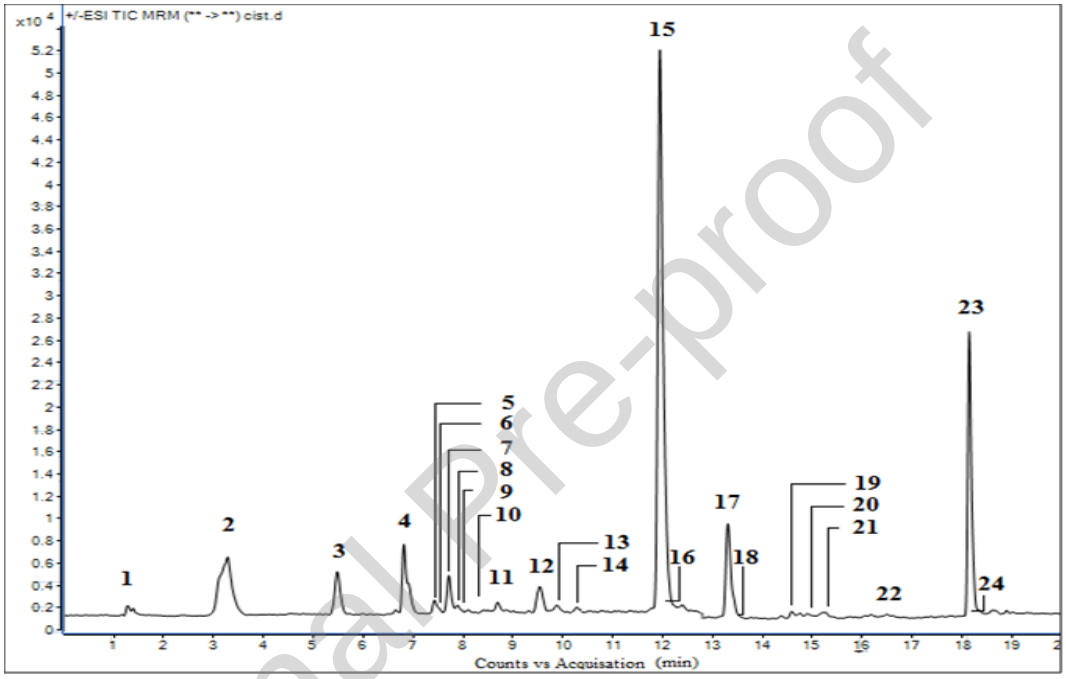

Fig. 2. Chromatogram of phenolic compounds of *Cistus libanotis* ethanolic extract by LC-ESI-MS/MS analysis

1.Shikimic acid; 2. Gallic acid; 3.Protocatechuic acid; 4.Epigallocatechin; 5.Catechin; 6.Chlorogenic acid; 7.Hydroxybenzaldeyde; 8.Vanillic acid; 9.Caffeic Acid; 10.Caffein; 11.Vanillin; 12.o-coumaric acid; 13.Salicylic Acid; 14.Trans-ferulic acid; 15.Hesperidin;16.Isoquercitrin; 17.Kaempferol-3-glucoside; 18.Fisetin; 19.Trans-cinnamic acid; 20. Quercetin; 21. Naringenin; 22. Kaempferol; 23.Luteolin; 24.Biochanin A.

3.4. Anti-cancer activity

Using the MTT assay, the cytotoxic potential of the ethanolic extract derived from *C. libanotis* was assessed against three distinct cell lines: dld-1 (colorectal adenocarcinoma cell line), CAPAN-1 (pancreatic cancer cell line), and L929 (fibroblast cell line). Table 4 provides a summary of the findings. The results show that the ethanolic extract of *C. libanotis* has a significant cytotoxic effect on the dld-1 and CAPAN-1 cell lines at different doses. Particularly, at 1 mg/mL of extract, the greatest effect was seen, leading to 40.51 % and 70.79 %, respectively, of cell line viabilities for dld-1 and CAPAN-1.

Similar to this, the extract showed notable cytotoxicity at 0.5 mg/mL, with cell line viabilities for dld-1 and CAPAN-1 of 57.97 % and 66.84 %, respectively. However, at lesser concentrations of 0.25 and 0.125 mg/mL, the cytotoxic effect was not as noticeable. On the other hand, the L929 cell line, which is regarded as the reference healthy cell line, showed very little toxicity when exposed to the ethanolic extract. The cell viability was determined at 102.11 % at a concentration of 1 mg/mL for the extract (Figure 3).

ANOVA and Tukey's post-hoc tests revealed significant differences in cell viability across dld-1, CAPAN-1, and L929 cell lines, when treated with the ethanolic extract of *C.libanotis* at concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL, with a *p* –value lower than 0.05. The findings clearly indicate that increasing the concentration of the ethanolic extract leads to a significant reduction in cell viability, highlighting its potential as an effective anticancer agent.

Table 4. Cytotoxic effect of *C. libanotis* ethanolic extract on cancer cell lines

Concentration (mg/mL)	Cell viability %		
	dld-1	**CAPAN-1**	**L929**
1	40.51 ± 0.21^{a}	70.79 ± 0.43^{a}	102.11 ± 0.87^{a}
0.5	57.97 ± 0.52^{b}	66.84 ± 0.21^{a}	241.28 ± 1.34^{b}
0.25	99.73 ± 0.61^{c}	174.07 ± 1.98^{b}	401.72 ± 2.24^{c}
0.125	110.07 ± 0.59^{d}	195.87 ± 1.76^{c}	420.19 ± 3.45^{c}
Control absorbance	0.239725	0.24222	0.1223175

The values with different superscripts (a, b, c or d) in the same columns are significantly different ($p< 0, 05$)

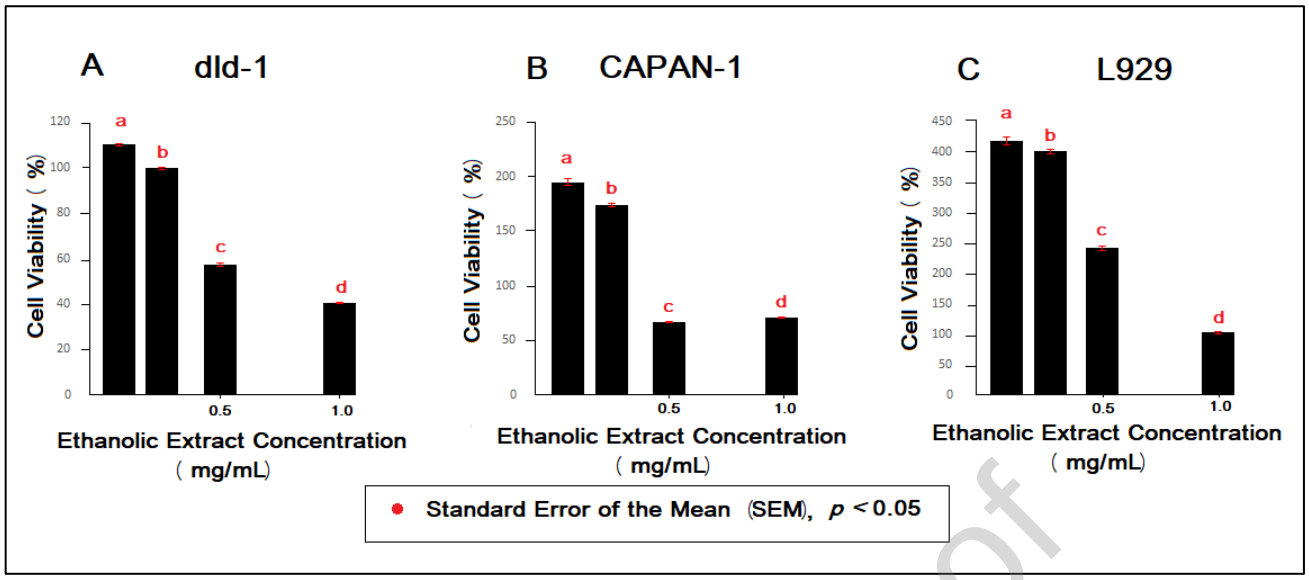

Fig. 3. Cytotoxicity of *Cistus libanotis* ethanolic extract against dld-1, CAPAN-1, and L929 cell lines

Several hexane extracts of *C. libanotis* from Tunisia were used to assess the antiproliferative activity against A-375 human melanoma cells and MCF-7 breast cancer cells. The results demonstrated that the extracts had no effect on any of the cell lines that had been evaluated (Ben Jemia et al., 2013). *C. laurifolius* showed anticancer efficacy against pancreatic cancer cell lines MIA PaCa-2, according to recent investigations on the cytotoxic activities of other *Cistus* species (Guzelmeric et al., 2023). In a different study, the extracts from Albanian *C. incanus* L. demonstrated strong antiproliferative activity *in vitro* against V79 cell culture and caused apoptosis (Moreira et al., 2017).

The presence of various bioactive molecules, such as shikimic acid, which was the predominant compound in the ethanolic extract of *C. libanotis* in the current study, may enhance its anticancer potential. In fact, shikimic acid is employed in traditional medicine for the treatment of several diseases due to its pharmacological properties (Batory and Rotsztejn, 2022). Additionally, numerous studies have documented that shikimic acid is the most prevalent precursor in the metabolism of phenolic compounds in plants, and serve as a supportive agent in cancer treatment indirectly influencing cell proliferation (Saltveit, 2017 ; Lee et al., 2020). According to Lee et al., (2020), shikimic acid effectively protects against cisplatin-induced nephrotoxicity by reducing renal damage and improving function, indicating its potential as a supportive treatment to mitigate cisplatin-related renal side effects in cancer therapy.

Furthermore, luteolin and hesperidin were detected in the ethanolic extract of *C. libanotis* by considerable concentration. In fact, luteolin is a flavonoid found in various plants, including medicinal herbs, inhibits carcinogenesis by disrupting cell transformation, metastasis, and angiogenesis through mechanisms such as modulation of the cell cycle and induction of apoptosis (Birt et al., 2001). It induces G1 phase arrest in various cancer cell lines and specifically suppresses CDK2 activity in colorectal and melanoma cells (Imran et al., 2019). Furthermore, hesperidin is a flavonoid known for its anticancer properties, that has been reported to exert effects on gastric, colon, and breast cancers through both intrinsic and extrinsic pathways by inducing apoptotic cell death in cancer cells (Aggarwal et al., 2020 ; Devi et al., 2015). Furthermore, compounds such as naringenin, vanillin, fisetin, caffeic acid, o-coumaric acid, salicylic acid, isoquercitrin, kaempferol-3-glucosideand and protocatechuic acid were identified and quantified by LC-ESI-MS/MS analysis in the ethanolic extract of *C. libanotis*. These compounds have demonstrated significant antiproliferative properties, contributing to their potential as therapeutic agents in cancer treatment (Kakkar and Bais, 2014 ; Patel et al., 2018 ; Naveed et al., 2018 ; Derbak et al., 2024). The diverse anticancer effects of these active compounds highlight the substantial promise of *C. libanotis* ethanolic extract in advancing therapeutic strategies and enhancing cancer treatment efficacy. However, comprehensive studies are essential to fully elucidate and substantiate the anticancer potential of this plant ethanolic extract.

4. Conclusions

This is the first study to examine the chemical composition and potential biological activities of *Cistus libanotis* from Algeria. The identification of major compounds in the essential oils, such as γ-terpinene, camphene, α-phellandrene, and 4-carene, highlights the richness of secondary metabolites present in *C. libanotis* oil. HS-SPME-GC/MS analysis allowed to detect 26 volatile compounds, comprising primarily monoterpene hydrocarbons and oxygenated monoterpenes, collectively representing 99.3 % of the total volatiles. Notably, α-thujene and sabinene emerged as major constituents. LC-ESI-MS/MS provided valuable insights into the plant's chemical composition of

ethanolic extract, with 24 identified components, among which shikimic acid emerged as the most abundant, followed by luteolin and hesperidin. Conversely, biochanin A was present in lower quantities. The antiproliferative activity of the plant extracts suggests promising medicinal properties. Future research on *C. libanotis* should delve deeper into its mechanisms of action, therapeutic potential, and possible synergistic effects with other compounds. Overall, the study lays a solid foundation for future exploration of *C. libanotis* and its role in promoting human health and well-being.

Author contributions

Samiha Ameur: investigation, writing—original draft preparation. Mohamed Toumi: Conceptualization and methodology. Hamdi Bendif: Conceptualization, methodology, validation, writing—review and editing and supervision. Larbi Derbak: writing—original draft preparation and formal analysis. İlyas Yildiz: formal analysis. Khellaf Rebbas: software and formal analysis. Ibrahim Demirtas: validation, resources, visualization, and project administration. Guido Flamini: software, validation, investigation. Maurizio Bruno: writing—review and editing, supervision, project administration, and funding acquisition. Stefania Garzoli: formal analysis, writing—original draft preparation and supervision. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Data availability

Data will be made available on request.

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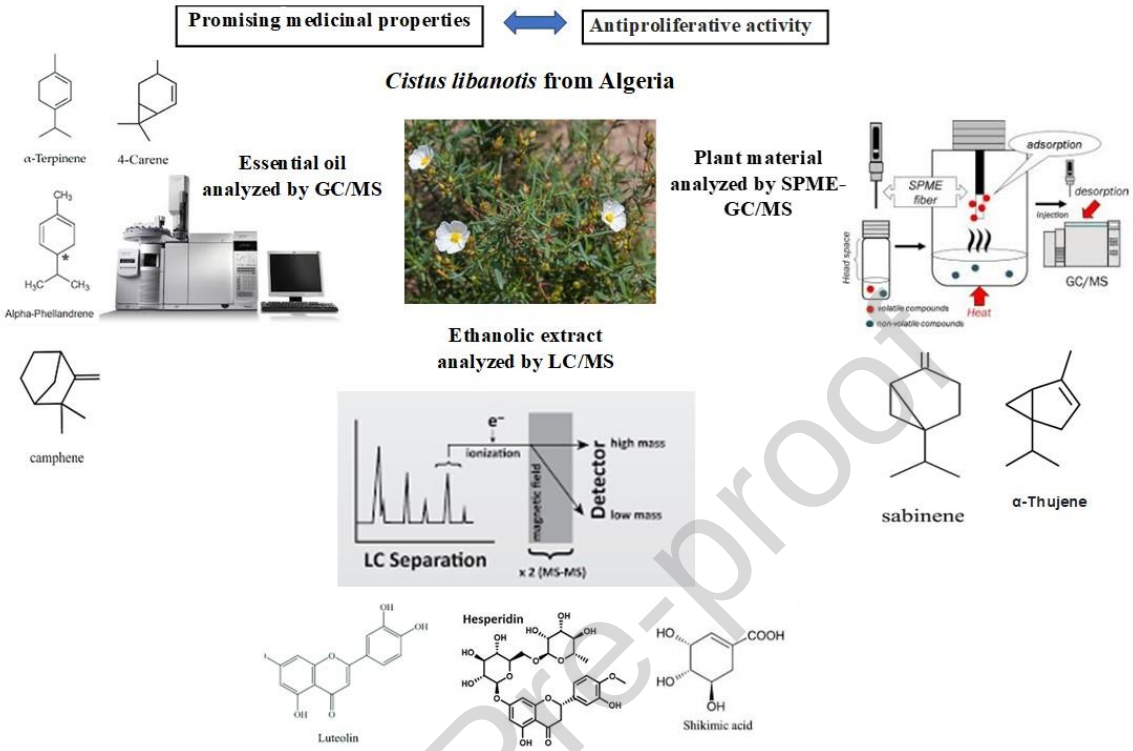
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- Sabinene and α-thujene were the major components found in the volatile fraction of *C. libanotis*.
- *C. libanotis* EO was rich in monoterpenes such as γ-terpinene, camphene and α-phellandrene.
- Shikimic acid, luteolin and hesperidin were the principal detected phenolic compounds.
- The ethanolic extract of *C. libanotis* showed cytotoxic effect against dld-1 and CAPAN-1 cell lines