

## Chemical Composition and Assessment of Antimicrobial, Antioxidant and Antiproliferative Activities of Essential oil from *Clinopodium sericeum*, a Peruvian Medicinal Plant

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**Abstract:** *Clinopodium sericeum* is widely used in Peruvian folk medicine in the form of infusion to treat stomach distress, indigestion and antflu. In this study, the essential oil from *C. sericeum* was obtained by hydrodistillation, analyzed by GC and GC/MS, and 73 compounds were identified. Major components of the oil were  $\beta$ -germacrene-D (15%),  $\beta$ -caryophyllene (13.8%), and sabinene (11.2%). Furthermore, we assessed the *in vitro* biological activities displayed by the oil obtained from the aerial parts of *C. sericeum*, namely the antioxidant, antimicrobial and antiproliferative activities. The antioxidant activities of the essential oil were evaluated by FRAP, CUPRAC, ABTS and DPPH radical scavenging activity. The essential oil displays antibacterial activity against Gram-negative and Gram-positive bacterial strains (MIC 50-200  $\mu$ g/mL) in a dose-range close to standard antibiotics. Such activity may be related to the presence of terpene compounds. The antiproliferative activity of the essential oil was measured *in vitro* using the MTT colorimetric assay in healthy non-tumorigenic cells (HEK-293) and in three human cancer cell lines (T24, DU-145, and MCF-7). The calculated IC<sub>50</sub> values were around 0.2 mg/mL. Since the essential oil was almost devoid of antioxidant activity, its anti-proliferative action is unlikely related to oxidative stress and relies on other unknown mechanisms.

**Keywords:** Folk medicine; *Clinopodium sericeum*; antioxidant; antimicrobial; antiproliferative; cancer cells.

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### 1. Introduction

Essential oils are volatile compounds extracted from the leaves, flowers, fruits, stalks, roots and resins of plants. Plant essential oils and their components have longtime been used for their numerous

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biological activities such as antimicrobial [1,2,3], antioxidant [3,4,5] and cytotoxic activities [3,5,6]. The components of essential oils include terpene hydrocarbons, alcohols, aldehydes, ketones, and esters [7,8]. Such chemical constituents are widely used in aromatherapy, perfumes, cosmetics, and food industry [9].

During the last decade, we have explored the biological properties of several medicinal plants commonly used by the Chilean and Peruvian Andean highlands communities [10-14]. In this work, we focus on the genus *Clinopodium*, Lamiaceae family, one of the richest species among the Angiosperms that includes 135 perennial herbs [15] and is frequently used for its medicinal properties. *Clinopodium sericeum* (C. Presl ex Benth.) Govaerts, a plant collected in the region of Cajamarca (Perú), was selected because its use by natives in folk medicine in the form of infusion for digestive disorders (stomach distress and indigestion) and antifu. This herb is known by the vernacular name “romerito de campo” [16].

To our knowledge, the essential oil chemical composition of *Clinopodium sericeum* has not been investigated. Therefore, using a double approach gas chromatography-mass spectrometry (GC-MS), the present study aimed at determining the essential oil composition of *Clinopodium sericeum*. In addition, we assessed the potential biological activities of the essential oil. To this end, first, we investigated the antimicrobial capacity of *Clinopodium sericeum* essential oil against a panel of human pathogenic bacteria: two Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*), and three Gram-negative (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*). Second, we measured the polyphenolic content total phenolic (TP) and the antioxidant activities: ferric-reducing antioxidant power (FRAP), cupric-reducing antioxidant power (CUPRAC), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of essential oil. Finally, in order to assess its antiproliferative activity, the *in vitro* cytotoxicity of essential oil was tested against a panel of three human-derived cancer cell lines and non-tumor fibroblast kidney cells.

## 2. Materials and Methods

### 2.1. Plant Material

*Clinopodium sericeum* (C.Presl ex Benth.) Govaerts., plants were collected in January 2019 at 2648 m above sea level. Once collected, the specimens were identified from the “Herbarium Truxillense de la Facultad de Ciencias Biológicas de la Universidad Nacional de Trujillo”. A voucher sample under accession HUT 59478 was deposited in this herbarium.

### 2.2. Essential Oil Isolation

Fresh aerial parts (50 g) were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The obtained oil was dried over anhydrous sodium sulfate. Afterwards, it was filtered, stored under protection at +4°C until analysis and further testing.

### 2.3. Gas chromatography Analysis (GC)

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (St. Louis, MO, USA), Merck (Darmstadt, Germany) and were used as supplied.

The essential oil was analyzed on a Perkin Elmer Clarus 400 gas chromatograph. It was equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were set. A DB-1 fused-silica column (polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc.]. Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a pentane-oil solution (1:1). The percentage composition of the oil was calculated by the normalization method from the GC peak areas, calculated as mean values of two injections from oil, without using correction factors.

#### 2.4. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis of the essential oil was conducted on a Perkin Elmer Clarus 600 gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25  $\mu\text{m}$ ; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Clarus 600T mass spectrometer (software version 4.1, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 m/z; scan time, 1 sec. The identity of the components was assigned by comparison of their retention indices, relative to C<sub>9</sub>-C<sub>21</sub> n-alkane indices and GC-MS spectra from a homemade library, constructed based on the analyses of reference oils, laboratory-synthesized components and commercial available standards.

#### 2.5. Antimicrobial Activity

Two techniques were used to test the antimicrobial activity of the *Clinopodium sericeum* oil: the paper disc diffusion and the dilution broth methods. The minimum inhibitory concentration (MIC) was determined by the latter method.

##### 2.5.1. Bacterial Strains

The microorganisms used were *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922).

##### 2.5.2. Paper Disc Diffusion

The antimicrobial activity was carried out according to the disc diffusion assay [17], the bacterial strains were maintained in agar at room temperature. Two point five mL of every bacteria inoculum were incubated in Mueller-Hinton agar at 37°C for 18 hours. The bacterial inoculum was adjusted to the McFarland N° 0.5 turbidity standard (10 cfu/mL). Every inoculum was spread over plates containing Mueller-Hinton agar and a filter paper disc. The plates were left for 30 min at room temperature and they were further incubated for 24 h at 37°C. The inhibitory zone around the disc was measured and it was expressed in mm. A positive control was also included to check the sensitivity of the tested organisms using the antibiotics ceftazidime and clindamycin. A negative control was also included in the test using a filter paper disc saturated with dimethylsulfoxide (DMSO) to check possible activity of this solvent against the bacteria assayed. The experiments were performed at least three times.

##### 2.5.3. Determination of the Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the essential oil was determined by the broth dilution method [17]. The following concentrations were tested: 200, 100, 50, 25, and 12.5  $\mu\text{g/mL}$ . After incubation, the microbial growth was examined. The results are expressed in Minimum Inhibitory Concentration (MIC), the lowest concentration of essential oil yielding no visible growth. The bactericidal/bacteriostatic activity was determined by sub-cultivation of the samples in normal culture media at appropriate temperature and incubation times. The MIC of each essential oil was performed in triplicate. The essential oil was dissolved in DMSO (Merck). Ceftriaxone was used as a positive control, at the same essential oil concentration.

#### 2.6. Total Phenolic Contents (TPC)

The total phenolic content of the essential oil was estimated using the Folin-Ciocalteu method adapted from Singleton and Rossi [18]. To 25  $\mu\text{L}$  of diluted essential oil, was added followed by 125  $\mu\text{L}$  of Folin-Ciocalteu solution. After 20 minutes, 100  $\mu\text{L}$  sodium carbonate (7%) was added. The whole mixture was incubated at 45°C for 10 minutes. A deep blue coloration developed whose absorbance was read at 760 nm. Results are expressed as mg of gallic acid equivalent (GAE)/mL of essential oil.

## 2.7. Antioxidant Capacity Assays

The FRAP and CUPRAC assays were carried out as previously described [19] with some modifications. Briefly, the FRAP the stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-S-triazine) solution in 40 mM HCl, and 20 mM FeCl<sub>3</sub> x 6H<sub>2</sub>O solution. The working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL FeCl<sub>3</sub> x 6H<sub>2</sub>O solution and then warmed at 37°C before using. The stock solution of 0.5 mM of the Trolox® was prepared. From this stock solution, serial dilutions (0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mM) were made. Essential oil (8 µL) were allowed to react with 200 µL of the fresh FRAP solution for 30 min in the dark. Readings of the colored product ferrous tripyridyltriazine complex were then taken at 593 nm (n = 3). The standard curve was performed with the standard antioxidant Trolox®. The CUPRAC assay was carried by mixing 250 µL ammonium acetate (pH 7.0) with 250 µL CuCl<sub>2</sub> and 250 µL Neocuproine solution in a 1:1:1 proportion. Antioxidant standard solution/sample (10 µL) and 265 µL H<sub>2</sub>O were added to the initial mixture. The reaction was stopped, and the reduction of the cupric ion was measured at 450 nm after 30 min incubation in the dark. In the FRAP and CUPRAC assays, the results were expressed as mg of TE (Trolox® equivalents)/mL of essential oil.

The scavenging of the ABTS<sup>+</sup> radical was carried out according to Re et al. [20]. Briefly, Trolox® solution was first dissolved in ethanol (EtOH) to a final concentration of 1 mg/mL and then, it was kept in the dark. The stock solutions were serially diluted in 96-well microplates to final concentrations of 50, 100, 200, 300, 400, 600, 700 and 800 µM. Then, 10 µL of each dilution mixed with 300 µL ABTS radical cation solution, and the reaction mixture was measured at 750 nm in Fisherbrand accuSkan GO UV/Vis Microplate Spectrophotometer (Hampton, USA). For the essential oil analysis, the same procedure was carried out, changing the 10 µL of Trolox® for 10 µL of essential oil. Afterwards, a curve of % ABTS<sup>+</sup> radical versus concentration was plotted and IC<sub>50</sub> values were calculated. IC<sub>50</sub> denotes the concentration of sample required to scavenge 50% of ABTS radical cation.

Free radical scavenging activity of compounds were determined by using a stable free radical, namely DPPH (2,2-diphenyl-1-picrylhydrazyl), according to a slightly modified method of of the literature [21]. Trolox® solution was first dissolved in EtOH to a final concentration of 1 mg/mL and it was further kept in the dark. The stock solutions were serially diluted in 96-well microplates to final concentrations of 1, 0.8, 0.6, 0.4, 0.2 and 0.1 mM. Then, 20 µL of each dilution mixed with 300 µL DPPH radical solution, and the reaction mixture was incubated for 30 min at room temperature and absorbance was measured at 517 nm in Fisherbrand accuSkan GO UV/Vis Microplate Spectrophotometer (Hampton, USA). For the essential oil analysis, the same procedure was carried out, changing the 20 µL of Trolox® for 20 µL of essential oil. Afterwards, a curve of % DPPH bleaching activity versus concentration was plotted and IC<sub>50</sub> values were calculated. IC<sub>50</sub> denotes the concentration of sample required to scavenge 50% of DPPH free radicals. All assays were performed in triplicate and are reported as mean values ± SD.

## 2.8 Cellular Assays

### 2.8.1. Cell Lines and Cell Cultures

Human cancer cell lines bladder (T24), prostate (DU-145), breast (MCF-7) and non-tumor HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cultures were maintained at a density of 1-2 x 10<sup>5</sup> cells/mL and the medium was changed at 48- to 72-h intervals. They were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). All cultures were kept at 37°C in 95% air/5% CO<sub>2</sub> at 100% humidity. Phosphate-buffered saline (PBS) was purchased from Gibco. Cells were incubated at the indicated times at 37°C with or without essential oil at various concentrations.

### 2.8.2. Cell Survival Assays

The cytotoxicity of the essential oil was assessed by following the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan blue [22]. Cells were seeded into 96-well plates at a density of 10 000 cells/well for 24 h and then incubated for 48 h with or without the essential oil. Doxorubicin was used as standard chemotherapeutic agent (positive control). Cells were first washed twice with warm PBS and subsequently incubated with MTT (0.5 mg/mL) for 2 h at 37°C. Blue formazan crystals were solubilized by adding 100  $\mu$ l DMSO/well, and the optical density of colored solutions was subsequently read at 550 nm. Results are expressed as % of MTT reduction compared to untreated control conditions. The IC<sub>50</sub> values were calculated using the GraphPad Prism software (San Diego, CA, USA).

## 3. Results and Discussion

*Clinopodium sericeum* (C. Presl ex Benth) Govaerts plants were collected in the community of Chugur in the province of San Marcos, Department of Cajamarca, Peru. The essential oil of *C. sericeum* was obtained from hydrodistillation of plant (leaves and stems) and its yield was calculated based on a moisture-free basis as 0.25% (w/w).

### 3.1. Chemical Composition of *Clinopodium sericeum*

Table 1 shows the gas chromatography (GC) and GC-MS analysis of the essential oil of the aerial parts from *Clinopodium sericeum* (C. Presl ex Benth) Govaerts. We identified the essential oil components by comparing the GC retention indices (RI) on polar and non-polar columns. The components were determined according to the retention time of a series of n-alkanes with linear interpolation with those standards and our essential oils database. The GC analysis of the essential oil revealed seventy-three compounds, accounting for 90.8% of the total composition (Table 1). Sesquiterpene hydrocarbons were the major constituents (39.2%), while the monoterpene hydrocarbons were present in concentrations of 22.5%. The oxygen-containing monoterpenes were prevalent (20.7%) as compared to oxygen-containing sesquiterpenes (7.6%). In addition, phenylpropanoids were present in low concentrations in oil (0.8%).

The major constituents of the essential oil of *Clinopodium sericeum* are a natural bicyclic monoterpene, namely sabinene (11.2%), and the sesquiterpenes,  $\beta$ -caryophyllene (13.8%) and germacrene-D (15%).

Historically, natural products have been used as sources to treat, cure and prevent diseases [23]. The greatest contribution of these natural products occurs through plants, which can be classified according to their chemical constitution, and this classification is defined as chemotaxonomy [24]. The Lamiaceae family has been demonstrated to be full of chemotaxonomic markers at every level: family, subfamily, genus, and species. This statement is mainly true for the polar fraction metabolites.

The essential oil of Lamiaceae species is particularly rich in volatile monoterpenes, sesquiterpenes, and diterpenes, which are constituted with 10, 15, and 20 carbon atoms, respectively. Among monoterpenes, the main compounds are  $\alpha$ -pinene,  $\beta$ -pinene, 1,8-cineole, menthol, limonene, and  $\gamma$ -terpinene [25-30]. Indeed, germacrene D, caryophyllene, and spathulenol represent the main sesquiterpene compounds [31-34]. In fact, concerning essential oils, none of the previously described compounds can really represent a chemotaxonomic marker. This is due to two important reasons: the first one, concerning phytochemistry, reflects how the presence of those compounds has been reported in other families [35-38], and the second one, concerning ecology, underlines that the essential oil content of a species depends largely on environmental factors, and thus does not permit absolute and definitive results [39,40]. On the other hand, the polar fraction metabolites present more specific amounts from the chemotaxonomic standpoint. Actually, under this aspect, the chemotaxonomy of Lamiaceae is very complex.

**Table 1.** Percentage composition of the Essential Oil isolated from *Clinopodium sericeum* collected in Cajamarca, Peru

Compounds	RI <sup>a</sup>	Relative content (%)	Identification method	RI data <sup>b</sup>
$\alpha$ -Thujene	924	0.4	RI, MS	905-948
$\alpha$ -Pinene	930	1.6	RI, MS	909-956
Camphene	938	0.4	RI, MS	929-978
Sabinene	958	11.2	RI, MS	944-980
$\beta$ -Pinene	963	4.1	RI, MS	952-986
Myrcene	975	0.2	RI, MS	962-993
$\alpha$ -Phellandrene	995	t	RI, MS	985-1019
$\alpha$ -Terpinene	1002	0.1	RI, MS	1003-1024
$\beta$ -Phellandrene	1005	0.1	RI, MS	1007-1045
Limonene	1009	0.8	RI, MS	995-1044
<i>p</i> -Cymene	1013	2.7	RI, MS	992-1072
1,8-Cineole	1015	0.1	RI, MS	1007-1046
<i>cis</i> - $\beta$ -Ocimene	1017	0.1	RI, MS	1022-1051
<i>trans</i> - $\beta$ -Ocimene	1027	0.5	RI, MS	1032-1054
$\gamma$ -Terpinene	1035	0.2	RI, MS	1030-1089
<i>trans</i> -Sabinene hydrate	1037	0.1	RI, MS	1043-1116
<i>n</i> -Octanol	1045	0.2	RI, MS	1051-1082
Terpinolene	1064	0.1	RI, MS	1071-1097
<i>cis</i> -Sabinene hydrate	1066	t	RI, MS	1043-1116
Linalool	1074	5.2	RI, MS	1078-1107
Isopentyl isovalerate	1094	0.1	RI, MS	1094-1105
<i>trans-p</i> -2-Menthen-1-ol	1099	0.1	RI, MS	1083-1146
<i>trans</i> -Pinocarveol	1106	0.1	RI, MS	1113-1155
<i>cis-p</i> -2-Menthen-1-ol	1110	0.1	RI, MS	1106-1122
<i>trans</i> -Verbenol	1114	0.1	RI, MS	1114-1163
Menthone	1120	0.1	RI, MS	1124-1142
Isomenthone	1126	t	RI, MS	1132-1159
Borneol	1134	1.0	RI, MS	1140-1188
Neomenthol	1139	0.5	RI, MS	1153-1176
Terpinen-4-ol	1148	2.8	RI, MS	1141-1206
Myrtenal	1153	0.6	RI, MS	1151-1197
$\alpha$ -Terpineol	1159	0.5	RI, MS	1144-1203
Myrtenol	1178	0.1	RI, MS	1170-1198
<i>cis</i> -Piperitol	1182	0.1	RI, MS	1176-1197
<i>trans</i> -Carveol	1189	0.1	RI, MS	1188-1220
<i>trans</i> -Piperitol	1189	0.3	RI, MS	1181-1212
Octanol acetate	1189	0.4	RI, MS	1190-1215
Carvone	1210	0.4	RI, MS	1210-1246
Piperitone	1221	0.2	RI, MS	1223-1268
2-Phenyl ethyl acetate	1228	0.1	RI, MS	1224
Bornyl acetate	1265	0.6	RI, MS	1261-1297
Thymol	1275	0.1	RI, MS	1264-1302
<i>trans</i> -Pinocarvyl acetate	1278	1.6	RI, MS	1295-1300
Carvacrol	1286	1.4	RI, MS	1279-1317
Myrtenyl acetate	1290	0.1	RI, MS	1322
<i>trans</i> -Carvyl acetate	1325	0.2	RI, MS	1312-1342
$\delta$ -Elemene	1332	t	RI, MS	1324-1386
Citronellyl acetate	1343	t	RI, MS	1336-1356
Carvacrol acetate	1348	4.1	RI, MS	1353-1391
Geranyl acetate	1370	0.1	RI, MS	1354-1392
$\alpha$ -Ylangene	1371	0.1	RI, MS	1350-1406

Compounds	RI <sup>a</sup>	Relative content (%)	Identification method	RI data <sup>b</sup>
$\alpha$ -Copaene	1375	1.0	RI, MS	1351-1407
$\beta$ -Bourbonene	1379	1.0	RI, MS	1346-1396
$\beta$ -Cubebene	1385	0.1	RI, MS	1360-1400
$\beta$ -Elemene	1388	1.3	RI, MS	1362-1410
$\beta$ -Caryophyllene	1414	13.8	RI, MS	1411-1421
$\beta$ -Copaene	1426	0.6	RI, MS	1428-1437
Geranyl acetone	1434	t	RI, MS	1442-1468
$\alpha$ -Humulene	1447	2.7	RI, MS	1428-1489
<i>allo</i> -Aromadendrene	1456	0.2	RI, MS	1442-1474
$\gamma$ -Muurolene	1469	t	RI, MS	1449-1502
Germacrene-D	1474	15.0	RI, MS	1451-1519
$\beta$ -Selinene	1476	t	RI, MS	1436-1497
Valencene	1484	0.5	RI, MS	1458-1495
Bicyclogermacrene	1487	0.5	RI, MS	1470-1533
<i>trans,trans</i> - $\alpha$ -Farnesene	1500	0.5	RI, MS	1499-1522
$\gamma$ -Cadinene	1500	0.2	RI, MS	1480-1531
<i>trans</i> -Calamenene	1505	t	RI, MS	1509-1529
$\delta$ -Cadinene	1505	1.7	RI, MS	1486-1563
<i>trans</i> -Nerolidol	1549	1.3	RI, MS	1535-1569
Spathulenol	1551	2.6	RI, MS	1552-1622
$\beta$ -Caryophyllene oxide	1561	2.7	RI, MS	1549-1617
Humulene epoxide	1580	1.0	RI, MS	1600-1605

<sup>a</sup>RI - Retention index as determined on the DB-1 column using the homologous series of n-alkanes (C<sub>9</sub>-C<sub>21</sub>); t – trace (< 0.05). <sup>b</sup>RI data - Retention index data reported in plants essential oils on non-polar column (www.webbook.nist.gov)

### 3.2. Antibacterial Activity of *C. sericeum* Essential Oil

We assessed whether essential oil from *C. sericeum* possesses antimicrobial activity. To this end, the antibacterial activity of *C. sericeum* essential oil, obtained from the leaves and stems, was evaluated against human pathogens, two gram-positive (*S. aureus* and *E. faecalis*) and three gram-negative (*K. pneumoniae*, *P. aeruginosa* and *E. coli*). Since these microorganisms are different on the morphological and phylogenetic levels, we assume that the results obtained with the essential oil will be representative of its intrinsic antibacterial activity.

Table 2 shows the essential oil antibacterial activity against hazardous bacteria, both Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*K. pneumoniae*, *P. aeruginosa* and *E. coli*). Note that antibiotic resistant bacteria have been observed with increasing frequency over the past several decades. Indeed, *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. coli* have been involved in several human infections and they developed resistance against standard commercial antibiotic [41].

The effect by essential oil on microorganisms was assessed by estimating the size of bacterial growth inhibition diameter, using the paper disc diffusion test. The inhibitory effect of essential oil was less important than that showed by standard antibiotics. Specifically, the diameter obtained by the essential oil at 3 mg/mL was in the range of 6 - 7 mm in Gram-positive bacterial strains as compared with 14 - 16 mm obtained by 30  $\mu$ g Clindamycin (CLI). Regarding Gram-negative bacteria, an inhibitory diameter of 6 - 8 mm was observed in case of essential oil as compared with 12 - 32 mm with 2  $\mu$ g Ceftazidime (CAZ). Interestingly, Table 2 also shows that the minimum inhibitory concentration (MIC) values of the essential oil was in the range of 50 - 200  $\mu$ g/mL. These values are 2 - 4 times lower in bacterial strains either Gram-positive or Gram-negative when compared with those of Ceftriaxone (CRO).

**Table 2.** Paper disc diffusion and minimum inhibitory concentration (MIC) of the essential oil (EO) of *Clinopodium sericeum*

Microorganism	Gram +/-	Diameter of the Inhibition Zones (mm) <sup>a</sup>			MIC ( $\mu\text{g/mL}$ )	
		EO <sup>b</sup>	CAZ	CLI	EO	CRO
<i>Staphylococcus aureus</i>	G+	6.0 $\pm$ 0.50	-	16.0 $\pm$ 0.50	200	400
<i>Enterococcus faecalis</i>	G+	7.0 $\pm$ 0.50	-	14.0 $\pm$ 0.44	100	N.T.
<i>Klebsiella pneumoniae</i>	G-	6.0 $\pm$ 0.50	12.0 $\pm$ 0.44	-	200	N.T.
<i>Pseudomonas aeruginosa</i>	G-	8.0 $\pm$ 0.50	32.0 $\pm$ 0.44	-	100	N.T.
<i>Escherichia coli</i>	G-	7.0 $\pm$ 0.50	16.0 $\pm$ 0.33	-	50	200

<sup>a</sup>The diameter of the inhibition zones (mm), are given as mean values  $\pm$  SD of triplicate experiments. <sup>b</sup>Diameter of the inhibition zones of the EO of *C. sericeum* (tested volume, 3 mg/mL). Positive control: CAZ, Cefazidime (30  $\mu\text{g}$ ); CLI, Clindamycin (2  $\mu\text{g}$ ); CRO, Ceftriaxone; N.T.: not tested.

We do not have a suitable explanation for such differences in the responses of the tested microorganisms to the essential oils as measured by either the paper disc diffusion test or the MIC calculation. While the first one is frequently used to estimate the bacterial susceptibility against a given compound, the second one (MIC) denotes the lowest concentration of that compound preventing visible bacterial growth. Nonetheless, one potential explanation is that the size of the inhibitory diameter may be influenced, other than the intrinsic antibacterial activity, by the diffusion of the compound within the agar medium due to its physical-chemical properties and molecular configuration.

Gram-negative bacteria are bounded by a cytoplasmic membrane and an outer cell membrane defining a periplasmic compartment, a cell wall structure composed by lipopolysaccharide and outer membrane organization [42,43]. Such structural arrangement forms a hydrophilic permeability barrier that provides protection against the effects of toxic agents [44]. Consequently, Gram-negative bacteria may be more resistant to antibiotics than Gram-positive because these latter bacteria do not have a periplasmic compartment. However, the effect of essential oil in both bacterial classes was rather similar.

Although the mechanism of action of terpenes is not fully understood, it is thought to involve membrane disruption by the lipophilic compounds in Gram-negative bacterial strains [45]. Regarding Gram-positive bacteria, essential oil may unfold its antimicrobial effectiveness by cellular metabolic disturbances after its cellular uptake. Alterations such as denaturation of proteins and enzymes, loss in the equilibrium of  $\text{K}^+$  and  $\text{H}^+$  ions, affect the entire cell morphology, leading to microorganism death [46-48]. While the precise mechanism remains poorly understood, such antibacterial activity may be sustained by sabinene,  $\beta$ -caryophyllene and germacrene-D, as well as other minor components of *C. sericeum* essential oil. Since essential oils are quite complex mixtures containing a large variety of constituents, the observed antimicrobial effect is may result from the activity of several active compounds as well as to potential synergistic effects between them. Thus, this complexity in chemical composition makes it often difficult to explain the biological activities shown by essential oils [49].

### 3.3. Total Phenolic Content and Antioxidant Capacity of *C. sericeum* Essential Oil

A second purpose was to investigate the putative antioxidant activity of the essential oil. The total phenolic (TP) content and antioxidant activity of essential oil of *C. sericeum* are summarized in Table 3. The TP content of the essential oil of the *C. sericeum* was expressed in Gallic acid equivalent (GAE) units (mg/mL of essential oil), and the result revealed low TP content of the essential oil (54.38 mg GAE/mL).

In addition, the antioxidant capacity of essential oil of *C. sericeum* were determined by using several chemical-based methodologies. These assays are based on different strategies providing complementary information about the interaction between radicals and essential oil. In the present work, we evaluated the antioxidant activity of the essential oils using four different assays, based on three different chemical mechanisms: including the reducing capacity of the extracts ferric-reducing antioxidant power (FRAP) and cupric ion-reducing antioxidant capacity (CUPRAC) assays, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radical scavenging and the scavenging of free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH).

**Table 3.** Polyphenolic content and antioxidant activities of essential oil of *Clinopodium sericeum*

Samples	TP	FRAP	CUPRAC	ABTS	DPPH
	(mg GAE/mL)	(mg TEAC/mL)	(mg TEAC/mL)	IC <sub>50</sub>	IC <sub>50</sub>
Essential oil	54.38 ± 1.28	1.40 ± 0.05	30.17 ± 1.60	106.06 ± 7.92	473.03 ± 14.11
Quercetin	-	1800.0 ± 0.02	3980.0 ± 0.35	0.05 ± 0.04	0.08 ± 0.01
Trolox®	-	-	-	0.09 ± 0.06	0.09 ± 0.02

TP = total phenolic; FRAP = ferric-reducing antioxidant power; CUPRAC = cupric-reducing antioxidant power; ABTS = 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH = 2,2-diphenyl-1-picrylhydrazyl radical; GAE = Gallic acid equivalent; TEAC = Trolox® equivalent antioxidant capacity. Results are expressed as means values ± SEM (n=3).

The four assays we have employed show that essential oil is rather devoid of an effective antioxidant activity. For instance, in FRAP assay, the results revealed lower reducing activity of the essential oil (TEAC= 1.40 mg/mL) than the values of reference standards, Quercetin (TEAC= 1800 mg/mL). In the CUPRAC assay, weak activity (TEAC= 30.17 mg/mL) was observed as compared of reference standard, Quercetin (TEAC= 3980 mg/mL). In the ABTS cation radical scavenging assay, the essential oil of *C. sericeum* displayed weak activity IC<sub>50</sub> of 106 mg/mL. The results of DPPH assay demonstrated that the *C. sericeum* essential oil showed a low radical scavenging activity with IC<sub>50</sub> of 473 mg/mL, which was much higher than the IC<sub>50</sub> values of reference standards, Quercetin (IC<sub>50</sub> of 0.08 mg/mL) and Trolox® (IC<sub>50</sub> of 0.09 mg/mL). As previously noted with antibacterial activity, essential oils are quite complex mixtures composed of a great variety of compounds, a complexity making difficult to explain their activities. Therefore, many reports on the antioxidant potentials of essential oils often refer to concepts such as synergism, antagonism, and additivity [50]. In our situation, we have determined that essential oil display a weak antioxidant activity.

#### 3.4. Antiproliferative Activity of *C. sericeum* Essential Oil

In addition to ethno-pharmacologic uses and biological activities (i.e. antimicrobial, fungicide, anti-inflammatory), we investigated the potential antiproliferative of essential oils. Table 4 shows the growth inhibitory effect by the essential oil of *C. sericeum* on three human tumor cell lines (T24, DU-145 and MCF-7), and non-tumorigenic fibroblasts (HEK-293). By using the MTT assay, the antiproliferative activity of essential oils was determined and expressed as IC<sub>50</sub> values. The essential oil shows no particular sensitivity against cancerous or non-tumor cells with a mean IC<sub>50</sub> value around 200 µg/mL, a number remote from that Doxorubicin, a currently used antitumor drug. Nevertheless, it is comparable to IC<sub>50</sub> values reported for other essential oils [51,52].

**Table 4.** The cytotoxic activity of the essential oil from *Clinopodium sericeum*

Treatments	Cytotoxic activity IC <sub>50</sub> (µM)			
	T24	DU-145	MCF-7	HEK-293
Essential oil	213.40 ± 4.14	202.50 ± 0.18	197.80 ± 5.19	195.90 ± 7.46
DOXO	0.46 ± 0.08	0.70 ± 0.02	0.05 ± 0.003	4.27 ± 0.34

Cells were seeded into 96-well plates at a density of 10 000 cells/well for 24 h and then incubated for 48 h with or without the essential oil. At the end of the incubation, aliquots of cells suspension were taken and the MTT test was performed as described in the Experimental section. Results are expressed as means values ± SEM (n =3). DOXO =doxorubicin.

A precise mechanism of action of the essential oil in our study remains to be described. Analyzing the chemical structure of the main constituents of the essential oil suggests an unlikely role of oxidative stress – and its subsequent formation of reactive oxygen species (ROS) - in such antiproliferative activities. In addition, the weak antioxidant properties of the essential oil rules out a major role for a potential oxidative-mediated cell injury. Further studies should investigate what molecular mechanisms are involved in the growth inhibitory effects of the essential oil of *C. sericeum* on cancer cells.

## 4. Conclusion

A double approach GC and GC-MS was used to identify the chemical component of the essential oil from *C. sericeum*. The oil was shown to contain sesquiterpene hydrocarbons, monoterpene hydrocarbons,

oxygenated monoterpenes and oxygenated sesquiterpenes. After its isolation, the antimicrobial, antioxidant, and antiproliferative properties of the oil were investigated. A weak antibacterial, antioxidant and antitumor potential was found.

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## Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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