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ORIGINAL ARTICLE

A Pentacyclic Triterpene from *Lippia origanoides* H.B.K and its Cytotoxic Activity

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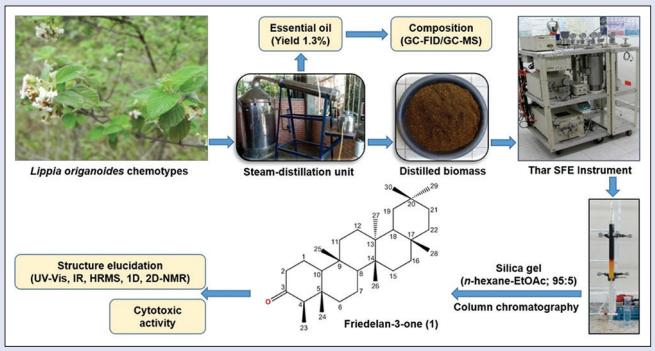
ABSTRACT

Background: *Lippia origanoides* H. B. K. (*Verbenaceae*) is an aromatic small shrub appreciated in the traditional systems of medicine. *L. origanoides* essential oil is an ingredient of commercial poultry feed products and its postdistillation residual biomass is an interesting source of bioactive compounds. During our search for the valorization of this residual biomass, supercritical-CO_2 (SC-CO_2) extraction afforded a mixture that was subjected to an investigation of phytochemicals and of cytotoxicity, which was not reported previously. **Objectives:** The current study was designed to investigate the phytochemicals from the steam-distilled residual biomass of thymol- and carvacrol-rich *L. origanoides* chemotypes and to evaluate their *in vitro* cytotoxic activity. **Materials and Methods:** Steam-distilled aerial parts of *L. origanoides* chemotypes were extracted with SC-CO_2 to obtain a greenish-yellow extract with a strong aromatic odor. The SC-CO_2 extract was subjected to column chromatography, and the isolate obtained was screened *in vitro* for cytotoxicity against human normal embryonic kidney 293, MRC-5, THP-1, and XP4PA cell lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. **Results:** A pentacyclic triterpene, friedelan-3-one (1) was isolated for the first time from *L. origanoides* chemotypes. The structure of the isolate was elucidated with spectroscopic (ultraviolet, infrared, mass spectra, and nuclear magnetic resonance) techniques. The *in vitro* cytotoxic activity of the isolated compound was determined. The results showed no significant results against the selected cell lines using the MTT assay. **Conclusion:** The main significance of the present study was to develop promising routes to utilize the residual biomass for value-addition. This is the first report of friedelan-3-one isolation from the genus *Lippia*.

Key words: Cytotoxicity, friedelan-3-one, *Lippia origanoides*, residual biomass, supercritical-CO_2

SUMMARY

- Valorization of plant residual biomass
- Pentacyclic triterpene, friedelan-3-one was isolated and characterized from the steam distilled supercritical-CO_2 extract of *Lippia origanoides* for the first time
- Friedelan-3-one showed no significant cytotoxicity against the two normal (human normal embryonic kidney 293 and MRC-5) and abnormal (THP-1 and XP4PA) human cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay.



Abbreviations used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; DMSO: Dimethyl sulfoxide; SC-CO_2: Supercritical-CO_2; TLC: Thin-layer chromatography; CC: Column chromatography; NMR: Nuclear magnetic resonance; TMS: Tetramethylsilane; ppm: Parts per million; ESI: Electrospray ionization; m/z: Mass-to-charge ratio; HEK293: Human normal embryonic kidney; MRC-5: Human normal fibroblasts from embryonic lung; THP-1: Human monocytic leukemia; XP4PA: Human dermal fibroblasts from *Xeroderma pigmentosum*; DMEM: Dulbecco's minimal essential medium; CC_{50}: 50% Cytotoxic concentration.

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INTRODUCTION

Lippia origanoides H. B. K. (*Verbenaceae* family) is an aromatic shrub up to 3 m tall, widely distributed from Central America (Mexico, Guatemala, and Cuba) to northern South America, with prominent occurrence in the Amazonian region of Brazil, the Guianas, Venezuela, and Colombia.[1] In Colombia, *L. origanoides* is commonly called "Oregano del monte" (mountain oregano) and distributed in the Andean States and in the Northern peninsula of Guajira at altitudes from 400 m to 2500 m.[1,2] Conventionally, *L. origanoides* is used in the treatment of stomach ache, indigestion, nausea, flatus, and as a general antiseptic for

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the mouth, throat, and wounds.[3] Previous phytochemical investigations of this essential oil have characterized its chemical composition and determined its biological activity.[2,4-9] Several phenolic compounds were detected in the *L. origanoides* extracts by gas chromatography-mass spectrometry (GC-MS) and high-pressure liquid chromatography (HPLC) methods.[10,11] In the course of our search for the valorization of residual biomass from thymol- and carvacrol-rich *L. origanoides* chemotypes by means of a green extraction process, supercritical-CO_2 (SC-CO_2) extraction was used to obtain a compound mixture. An isolate was obtained using column chromatography (CC) and was subjected to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to examine cytotoxic activity against two normal human cell lines (human normal embryonic kidney 293 [HEK293] and MRC-5) and two abnormal human cell lines (THP-1 and XP4PA).

MATERIALS AND METHODS

General experimental procedures

Nuclear magnetic resonance (NMR) experiments were recorded on a Bruker Avance III-400 MHz spectrometer (^{1}H: 400 MHz; ^{13}C: 101 MHz; $CDCl_3$) using TMS as the internal standard. Mass spectra (MS) spectra were obtained on an Ultra HPLC (UHPLC)-electrospray (ESI)-high resolution mass spectrometer Exactive Plus orbitrap system (Thermo Scientific, Bremen, Germany) in positive mode. Melting point (uncorrected) was determined on a capillary point apparatus equipped with a digital thermometer. Ultraviolet (UV) spectrum was recorded on a Flame Ocean Optics spectrophotometer (Ocean Optics, Ostfildern, Germany), and Infrared (IR) spectrum was acquired with a Cary 630 Fourier-transform IR spectrometer (Agilent Technologies, USA). Silica gel (SiO_2) (0.063–0.200 mm, Merck, Darmstadt, Germany) was used for CC and precoated silica gel 60 F_{254} plates (0.2 mm, Merck) were used for thin-layer chromatography (TLC) analysis.

Plant material

Aerial parts (including flowers, leaves, and stems) of thymol- and carvacrol-rich *L. origanoides* were collected from the experimental plot of CENIVAM, Universidad Industrial de Santander, Bucaramanga, Colombia in June 2018 and authenticated by Prof. José Luis Fernández Alonso (National University, Bogota, Colombia). A voucher specimen (COL519799) was deposited at the Colombian National Herbarium (Bogota).

Extraction and isolation

Fresh plant material (16 kg) was chopped and steam-distilled to obtain essential oil (yield 1.3%, w/w). Chromatographic (GC-flame-ionization detection, GC-MS) analysis of essential oil exhibited thymol (32.4%) and carvacrol (12.1%) as major constituents [data depicted in Figure S1 and Table S1; see Supplementary Material]. Distilled vegetal material was further air-dried at room temperature and coarsely ground (particle size <1 mm, 460 g), extracted with CO_2 at 333 K and 50 MPa in a 2 L extraction chamber (Thar SFE 2000-2-FMC50, Thar Instruments, Pittsburgh, Pennsylvania, USA). The initial static period (20 min) was followed by a continuous flow of CO_2 (40 g/min, 120 min), which connected to a vortex collection chamber, where pressure was reduced to 0.1 MPa. The extract (yield 1.4%; w/w) obtained after 2 h of CO_2 recirculation followed by depressurization was dissolved in ethanol (3–5 mL, Merck), centrifuged to remove the fatty layer, and evaporated with a rotary evaporator. The SC-CO_2 extract (3.5 g) was subjected to CC over silica gel (45 g, 50 cm × 1.5 cm) eluted with $CHCl_3$ to yield fractions 1–35. All fractions were monitored by TLC in solvent systems of *n*-hexane-EtOAc (98:2–85:15) and detected with UV light and visualized by spraying with 5% aq. H_2SO_4, followed by heating at 110°C until the characteristic color. Fractions (7–9) were further column chromatographed on silica gel (20 g, 25 cm × 1 cm) eluted with $CHCl_3$ to give 1 (15 mg; 0.43%, w/w).

Cytotoxicity assay

The *in vitro* cytotoxicity of compound 1 against human normal embryonic kidney (HEK293, ATCC® CRL-1573™), human normal fibroblasts from embryonic lung (MRC-5, ATCC® CCL-171™), human monocytic leukemia cells (THP-1, ATCC® TIB-202™), and human dermal fibroblasts from Xeroderma pigmentosum (XP4PA, kindly provided by Dr. Carlos F. Martins, from Institute of Biomedical Sciences, University of São Paulo, Brazil). HEK293 was cultured in Dulbecco's minimal essential medium (DMEM-F12), MRC-5 and XP4PA cells were cultivated in DMEM/high glucose, and THP-1 was cultured in RPMI 1640. The cells were supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotics penicillin (100 U/mL)/streptomycin (100 mg/mL) under a humidified incubator at 37°C in 5% CO_2 for 24 h.

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (tetrazolium) assay

The cell viability of isolated compound 1 was investigated by MTT colorimetric assay.[12] Cells were seeded onto 96-well micro-titer plastic plates (1 × 10^4 cells/well) treated with varying concentrations of the compound (6.25, 12.5, 25, 50, 75, and 150 μM) maintained in a humidified atmosphere at 37°C in 5% CO_2 and incubated for 72 h. The culture medium was removed and 5 mg/mL MTT (Sigma-Aldrich, USA) solution was added to each well, and incubation was continued for 3 h. After incubation, the formed formazan crystals were dissolved in 1 mL of dimethyl sulfoxide and absorbance of wells was measured at a wavelength of 550 nm with an automated spectrophotometric plate reader (Multiskan Go, Thermo Scientific, USA). All experiments were carried out in triplicate. Data analysis was performed using the R Project for statistical computing software (R Development Core Team [2013]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

RESULTS AND DISCUSSION

The SC-CO_2 extract of steam-distilled residual biomass of thymol- and carvacrol-rich *L. origanoides* was selected for phytochemical investigation. The obtained SC-CO_2 extract subjected to CC afforded compound 1 [Figure 1] and structure was elucidated by IR, MS and NMR spectroscopic techniques. The NMR key correlations of ^{1}H-^{1}H COSY and HMBC are shown in Figure 1. The *in vitro* cytotoxicity of 1 showed no significant results against HEK293, MRC-5, THP-1, and XP4PA cell lines using the MTT assay, and results are depicted in Figure 2.

Structural elucidation of compound 1

The UHPLC-ESI(+)-orbitrap-MS data for compound 1 showed the protonated molecule ion (M + H) + *m/z* 427.38940 (calcd. *m/z* 427.39344 for $C_{30}H_{51}O$), indicated six degrees of unsaturation. IR spectrum showed the strong absorption of a ketone group at 1713 cm^{-1}. The compound gave purple color on TLC with 5% aq. H_2SO_4, and positive Liebermann-Burchard reaction indicated that the compound possessed a triterpene skeleton. The ^{1}H and ^{13}C-NMR spectra of 1 showed 50 proton signals corresponding to 30 carbons. ^{1}H NMR spectrum showed eleven methylene groups at H-1α, β (2H, m), H-2α, β (2H, m), H-6α, β(2H, m), H-7α, β(2H, m), H-11α, β(2H, m), H-12α, β(2H, m), H-15α,

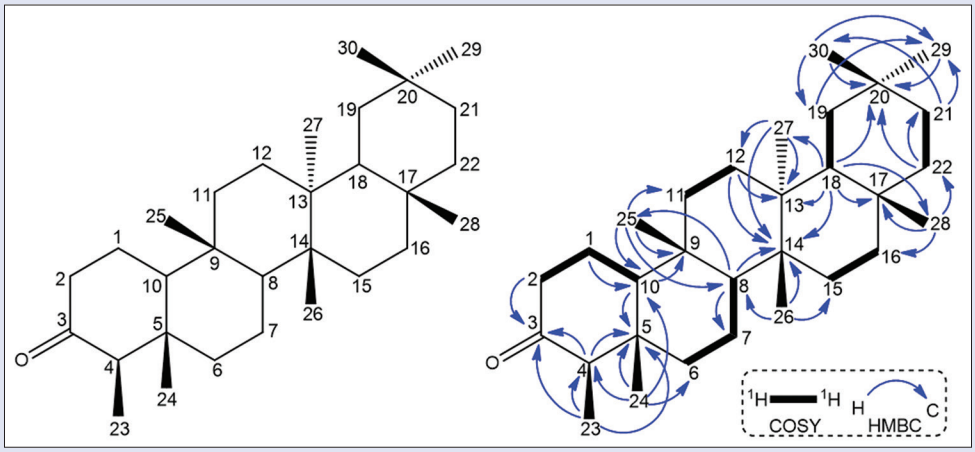

Figure 1: Key^{1}H-^{1}H COSY and HMBC correlations of compound 1

β (2H, m), H-16α, β (2H, m), H-19α, β (2H, m), H-21α, β (2H, m) and H-22α, β (2H, m), eight methyl groups at δ_H 0.89 (3H, d, J = 6.4), 0.75 (3H, s), 0.91 (3H, s), 1.03 (3H, s), 1.07 (3H, s), 1.20 (3H, s), 1.02 (3H, s) and 0.96 (3H, s) and four methine protons at δ_H 2.26 (1H, q), 1.41 (1H, dd), 1.56 (1H, m) and 1.57 (1H, m), indicating that compound was probably a pentacyclic triterpene. ^{13}C NMR spectrum showed a high downfield carbonyl signal at δ_C 213.9 (C-3) correlation with secondary methyl at δ_H 0.89 (J = 6.4 Hz, H-23).[13] This spectrum displayed 30 carbons including eleven sp^3 methylene signals (δ_C 22.9, 42.2, 41.9, 18.9, 36.3, 31.2, 33.1, 36.7, 36.0, 33.5, and 39.9) confirmed with DEPT-135° experiment [Figure S2], eight methyls (δ_C 7.5, 15.4, 18.6, 20.9, 19.4, 32.8, 32.5, and 35.7), six sp^2 quaternary (δ_C 42.8, 38.1, 40.4, 38.9, 30.4, and 28.9) and four sp^2 methine signals (δ_C 58.9, 53.8, 60.2, and 43.5). The COSY (^{1}H-^{1}H) correlations showed between H-1 to H-2 and H-10; H-7 to H-6 and H-8; H-11 with H-12; H-15 with H-16; H-18 with H-19, and H-21 with H-22, and HMBC correlations [Figure 1] from H-1α to C-10, H-2 β to C-3; H-4 to C-3 and C-5, H-8 to C-7, C-9, C-14 and C-25, H-10 to C-5 and C-9, H-11 to C-13, H-12 to C-13 and C-14, H-18 to C-13, C-14, C-17, C-20 and C-28, H-19 to C-29, H-21 to C-29 and C-30, H-22 to C-20, H-23 to C-3, C-4 and C-5, H-24 to C-4, C-5, C-6 and C-10, H-25 to C-8, C-9, C-10 and C-11, H-26 to C-8, C-14 and C-15, H-27 to C-12, C-13 and C-14, H-28 to C-16, C-17 and C-22, H-29 to C-20, H-30 to C-19, C-20 and C-29. The HSQC spectrum revealed the direct-CH signals at δ_H 2.26 (H-4) with δ_C 58.9 (C-4), δ_H 1.41 (H-8) with δ_C 53.8 (C-8), δ_H 1.56 (H-10) with δ_C 60.2 (C-10), and δ_H 1.57 (H-18) with δ_C 43.5 (C-18) [Figure S3]. Based on the above discussion and comparison with literature data,[14-16] the structure of compound 1 was assigned as 4, 4a, 6b, 8a, 11, 11, 12b, 14a-octamethylicosahydropicen-3 (2 h)-one, named friedelan-3-one (friedelin).

Friedelan-3-one (1): White needle crystals dissolved in chloroform. Melting point 260°C –262°C. TLC R_f 0.38 (*n*-hexane: EtOAc, 90:10). UV ($CHCl_3$) λ_{max} (log ε): 245 (0.48), 287 (0.37) nm. IR (neat, ν_{max}, cm^{-1}): 2925, 2867, 1713 (C = O), 1459, 1387. GC-MS: T_R 87.914 min. MS (EI, 70 eV), *m/z* ($I_{rel.}$ %): 426 ([M] +, 14), 273 (24), 205 (26), 163 (31), 125 (53), 123 (64), 109 (72), 107 (42), 95 (87), 81 (72), 69 (100), 55 (75). UHPLC-ESI$^+$-orbitrap-MS *m/z* 427.38940 (M + H) + (calcd. *m/z* 427.39344). ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 1.98 (m, H-1α), 1.73 (m, H-1 β), 2.39 (m, H-2α), 2.28 (m, H-2 β), 2.26 (q, H-4), 1.76 (d, H-6α), 1.29 (d, H-6 β), 1.49 (m, H-7α), 1.38 (m, H-7 β), 1.41 (dd, H-8), 1.56 (m, H-10), 1.47 (m, H-11α), 1.24 (m, H-11 β), 1.31 (m, H-12α), 1.30 (m, H-12 β), 1.48 (m, H-15α), 1.28 (m, H-15 β), 1.59 (m, H-16α), 1.36 (m, H-16 β), 1.57 (m, H-18), 1.40 (m, H-19α), 1.25 (m, H-19 β), 1.51 (m, H-21α), 1.32 (m, H-21 β), 1.54 (m, H-22α), 0.98 (m, H-22 β), 0.89 (d, J = 6.4 Hz, H-23), 0.75 (3H, s, H-24), 0.91 (3H, s, H-25), 1.03 (3H, s, H-26), 1.07 (3H, s, H-27), 1.20 (3H, s, H-28), 1.02 (3H, s, H-29), 0.96 (3H, s, H-30). ^{13}C-NMR (101 MHz, $CDCl_3$) δ_C 22.9 (C-1), 42.2 (C-2), 213.9 (C-3), 58.9 (C-4), 42.9 (C-5), 41.9 (C-6), 18.9 (C-7), 53.8 (C-8), 38.1 (C-9), 60.2 (C-10), 36.3 (C-11), 31.2 (C-12), 40.4 (C-13), 38.9 (C-14), 33.1 (C-15), 36.7 (C-16), 30.4 (C-17), 43.5 (C-18), 36.0 (C-19), 28.9 (C-20), 33.5 (C-21), 39.9 (C-22), 7.5 (C-23,-CH_3), 15.4 (C-24,-CH_3), 18.6 (C-25,-CH_3), 20.9 (C-26,-CH_3), 19.4 (C-27,-CH_3), 32.8 (C-28,-CH_3), 32.5 (C-29,-CH_3), 35.7 (C-30,-CH_3) [Detailed spectral information is provided in Supplementary Material].

Measurement of cytotoxic activity

The cytotoxic activity of compound 1 was studied using the MTT assay against HEK293, MRC-5, XP4PA, and THP-1 cell lines. Significant cytotoxic effect was not observed; the viability of all four cells was reduced between 14% and 37% after treatment with the highest concentration of the compound [Figure 2]. Hence, the 50% cytotoxic concentrations (CC_{50}) were above of or higher than 150 µM. XP4PA cells were deficient against the nucleotide excision repair pathway, which involved the recognition and removal of the DNA lesions.[17] Results suggested that the test compound did not block pathways related to the survival of human cells derived from the embryonic kidney (HEK293), fibroblast from embryonic lung (MRC-5), monocyte from a leukemia patient (THP-1), and fibroblasts from *Xeroderma pigmentosum* patients (XP4PA). Previous report[18] on compound 1 showed low toxicity against the Vero cells using MTT assay at LC_{50} >200 µg/mL, while not toxic against the formaldehyde-fixed red blood cells using the hemagglutination assay. However, compound 1 possesses several biological activities such as anti-inflammatory, analgesic, antipyretic,[19] antioxidant, and liver protective.[20]

CONCLUSION

The present work described the phytochemical investigation on steam-distilled plant residual biomass of *L. origanoides* used for the isolation of a pentacyclic triterpene ketone, friedelan-3-one (1) for the first time from the genus *Lippia*. The structure of the isolate was verified based on various spectroscopic techniques and comparison with literature. Results of cytotoxicity of naturally occurring compound 1 showed no significant effects against four (HEK293, MRC-5, XP4PA, and THP-1) cell lines. A valuable and sustainable way for the management of biomass residues has been proposed.

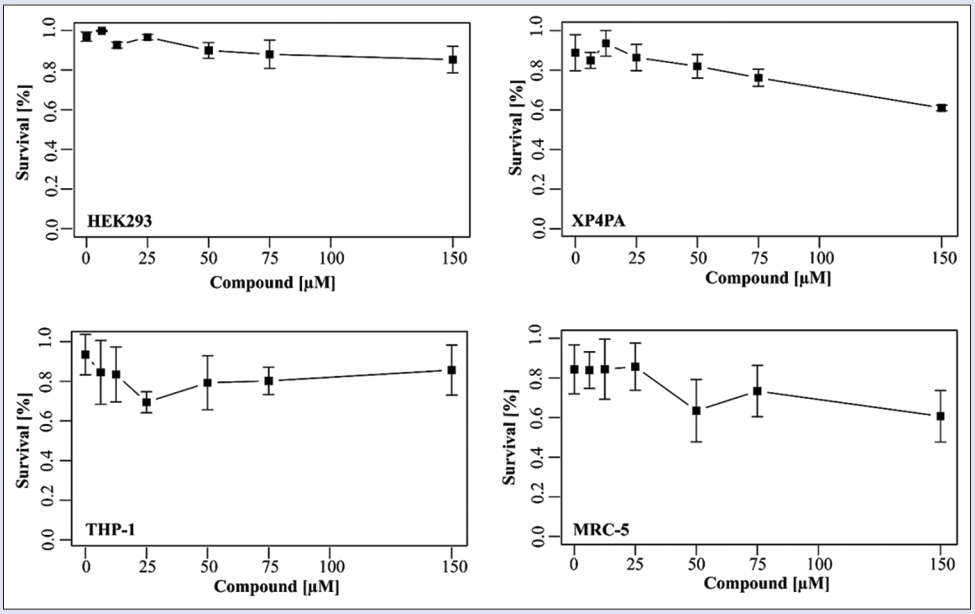

Figure 2: Cytotoxicity of compound 1 against human normal embryonic kidney 293, normal embryonic kidney cells; XP4PA, fibroblast from *Xeroderma pigmentosum* patients; THP-1, monocyte from a leukemia patient, and MRC-5, fibroblast from normal embryonic lung. Data are shown as means ± standard deviation of three-independent experiments and presented relative to untreated control cells

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Conflicts of interest

There are no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Characterization data Ultraviolet/Vis, Infrared, HR-mass spectrometry, and nuclear magnetic resonance (1D and 2D) are available in supplementary material, alongside, Table S1 and Figures S1-S3.

Table S1: Relative amount (%) of the main compounds of *Lippia origanoides* essential oil obtained by steam distillation (total 63 constituents >0.05%)

Peak number [a]	Compound	Type	RI[b]	RI[c]	Relative %[d]	Identification mode
4	β-Myrcene	MH	990	1157	2.1	e) f) g)
6	*p*-Cymene	MH	1029	1264	6.7	e) f) g)
8	1,8-Cineol	MO	1037	1240	2.6	e) f) g)
9	γ-Terpinene	MH	1062	1244	2.4	e) f)
16	Thymol methyl ether	OC	1234	1587	4.2	e) f) g)
19	Thymol	OC	1301	2164	32.4	e) f) g)
20	Carvacrol	OC	1309	2172	12.1	e) f)
21	Thymol acetate	OC	1349	1858	2.8	e) f) g)
28	*Trans*-β-Caryophyllene	SH	1434	1601	7.2	e) f)
36	*m/z*, I% =M^+ 180 (46), 165 (100), 150 (36), 91 (21), 77 (17)	OC	1492	1745	5.9	f)
48	*trans*-Nerolidol	SO	1567	1983	2.9	e) f)
MH					13.4	
MO					5.2	
OC					57.8	
SH					16.9	
SO					6.2	
NI					2.5	
Total					97.5	

a) Peak number in Figure S1, b) Linear retention indices determined on the DB-5MS (60 m) column, c) Linear retention indices determined on the DB-WAX (60 m) column, d) Relative amount determined by GC-FID on the DB-5MS (60 m) column, e) Tentative identification based on retention indices, f) Tentative identification based on mass spectra (EI, 70 eV), g) Identification based on the use of standard substances. NI: Not identified; SO: Oxygenated sesquiterpenes; MH: Monoterpene hydrocarbons; SH: Sesquiterpene hydrocarbons; MO: Oxygenated monoterpenes; OC: Oxygenated compounds; RI: Retention indices

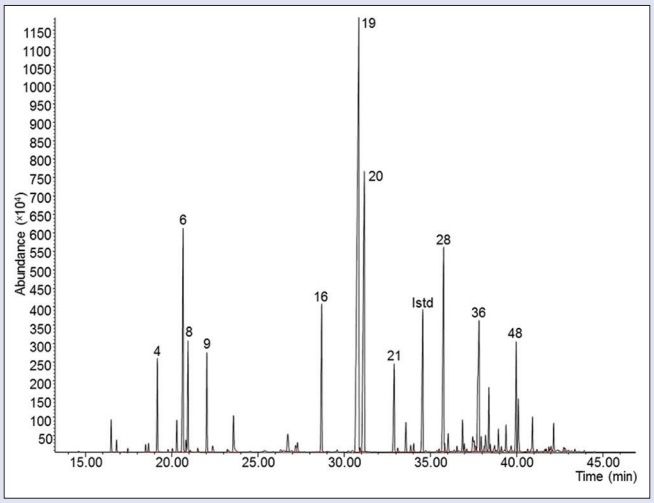

Figure S1: Typical chromatogram (gas chromatography/flame-ionization detection, DB-5MS column, 60 m) of essential oil isolated from *Lippia origanoides*. Peak assignments and identification of main compounds are reported in Table S1

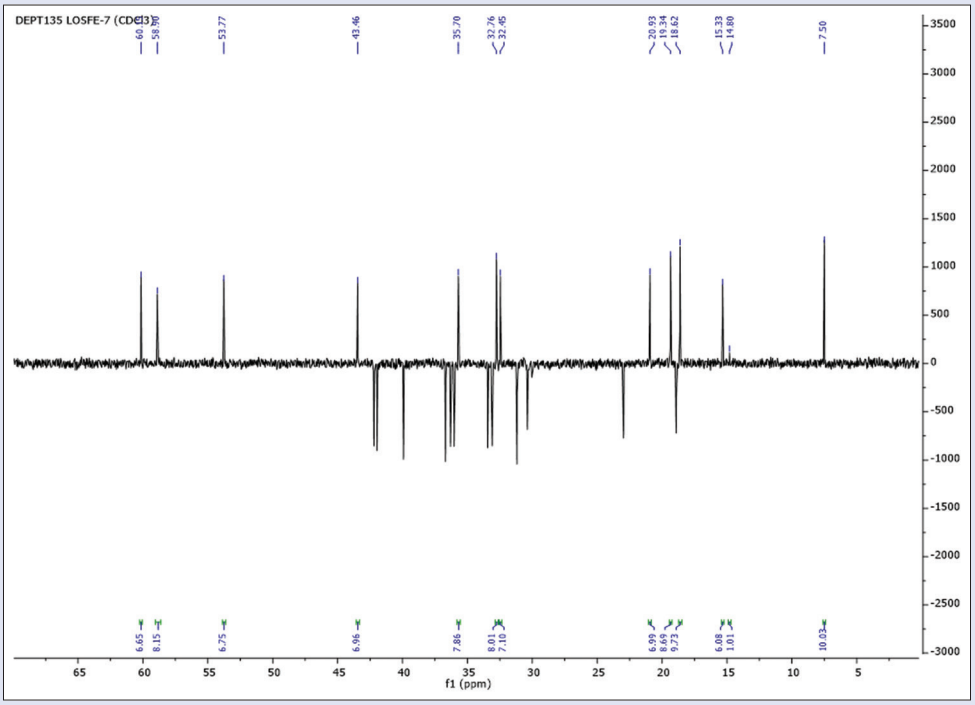

Figure S2: DEPT-135° spectrum ($CDCl_3$) of compound 1

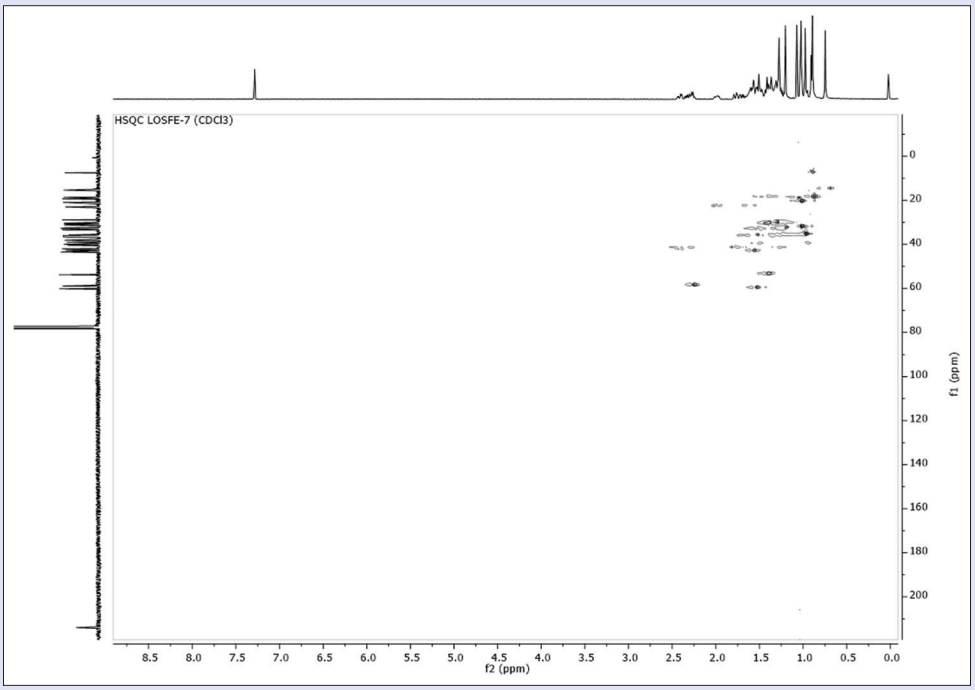

Figure S3: HSQC spectrum (400 and 101 MHz, $CDCl_3$) of compound 1