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ORIGINAL ARTICLE

Anticancer Activity of Abietane Diterpenoids from *Salvia libanoticum* Grown in Lebanon

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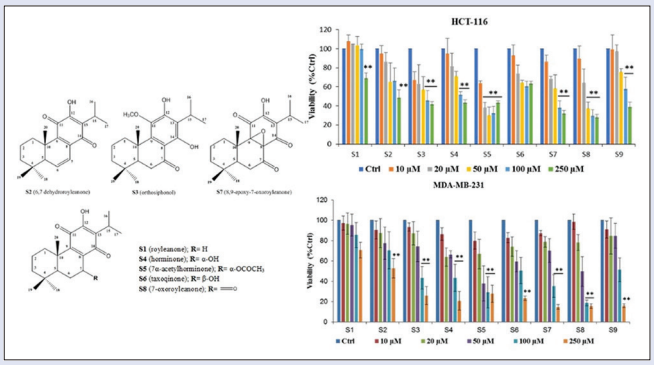
ABSTRACT

Background: The Salvia plant and their metabolites are well reported for their valuable therapeutic effects and as potential remedies for treatment of many diseases. *Salvia libanoticum* is an endemic species to Lebanon where its metabolites have never been investigated. **Objectives:** The objectives were to evaluate the potential of abietane diterpenes from the roots of *Salvia libanoticum* as anticancer agents and explore some essential chemical features. **Materials and Methods:** Crude extract from the roots of *Salvia libanoticum* was separated using chromatographic techniques and spectroscopic analysis. The anticancer activities of the isolated compounds along with the crude extract were evaluated against MDA-MB-231 breast cancer cells and HCT116 human colon cancer cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **Results:** Eight abietane diterpenes were isolated to be royleanone (1), 6,7-dehydroroyleanone (2) orthosiphonol (3), horminone (4), 7α-acetylhorminone (5), taxoquinone (6), 8,9-epoxy-7-oxoroyleanone (7), and 7-oxoroyleanone (8). All compounds including the extract revealed dose-dependent inhibitory effects that varied between the two cell lines, indicating cell-type specificity and suggesting different cell-compound interactions. **Discussion:** The most effective compound was found to be 7α-acetylhorminone, with IC_{50} of 18 and 44 μM on HCT116 and MDA-MB-132 cells, respectively. The results suggested that oxygenated C7 is essential for the cytotoxic activity. Moreover, the carbonyl group at position C7 leads to higher activity than the hydroxyl group. **Conclusion:** This study reported the potential of abietane diterpinoids as anticancer agents. It also suggested *Salvia libanoticum* and its diterpinoids as promising remedies in colon and/or breast cancer therapy. Further studies are needed to explore the exact interaction of these compounds with cancer cells at molecular level.

Key words: *Cytotoxicity,* diterpinoids, salvia, spectroscopic analysis, structure activity

SUMMARY

- This article reported the phytochemical investigation of quinoidal compounds from *Salvia libanoticum* using chromatographic techniques and spectroscopic analysis. Eight abietane diterpenes were identified as royleanone, 6,7-dehydroroyleanone, orthosiphonol, horminone, 7α-acetylhorminone, taxoquinone, 8,9-epoxy-7-oxoroyleanone and 7-oxoroyleanone. All compounds and the total extract exhibited a dose-dependent anticancer activity against HCT116 human colon cancer cells and MDA-MB-231 breast cancer cells, with 7α-acetylhorminone being the most active. Comparing the difference in activity among the eight compounds, together with literatures, it was noticed that oxygenated C7 is essential for the cytotoxic activity and carbonyl group at position C7 that leads to higher activity than the hydroxyl group. Further studies are recommended to determine the activity of these compounds at molecular level.



Abbreviation used: Anticancer activity of Salvia compounds against HCT-116 and MDA-MB cells

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INTRODUCTION

Salvia constitutes the largest genus in the Family of Lamiaceae with about 1000 species around the world.[1] Salvia plants are small woody herbaceous perennial shrubs, ranging from 17 to 100 cm high, with characteristic flowers.[2] Salvia species, commonly known as sages, have long been used as ornamental plants, in cosmetics flavoring agents and in perfumery.[1] They have been used also in folk medicine for treatment of most kinds of ailments and as food additives and preservatives.[3] In Mediterranean countries, infusions of several Salvia species mainly *Salvia officinalis* and/or *Salvia fruticosa* are commonly used.[4]

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Phytochemical investigations of Salvia species resulted in the isolation of a large number of secondary metabolites, of which many are biologically active. The major secondary metabolites present in the aerial parts of Salvia plants are flavonoids, terpenoids, and volatile oils.[5] A large number of diterpenoids, belonging mainly to abietane, labdane, pimarane, kaurane, and clerodane types, have been isolated from the roots of different Salvia species[6] that possess a broad spectrum of interesting medicinal activities and ecological roles.[7] Extracts and many of the isolated compounds from Salvia plants are well known for their antioxidant, antiseptic, anti-inflammatory, and antibacterial properties.[2] They also possess promising antifungal, antiviral, cytotoxic, diuretic, hypoglycemic, hemostatic, wound healing, spasmolytic, and sedative activities. Recent studies have demonstrated the potential use of Salvia plants in treating colds, diabetes, bronchitis, dementia, tuberculosis, obesity, depression, and menstrual disorders.[8]

In Lebanon, more than 19 Salvia species were reported to grow widely, of which *Salvia libanotica*, an endemic species, is the most common species with maximum density and distinct chemotype.[9] Besides the fact that natural products are major sources of valuable chemical entities effective against human tumors, many promising secondary metabolites have not been characterized yet.[10] Moreover, it was reported that about 75% of compounds with cytotoxicity effects were directly or indirectly obtained from natural products.[7] On the other hand, Most of the studies on *Salvia libanotica* were only carried out either on the crude extracts or its volatile oil,[11-13] and there are no available data that describing the diterpene contents of this species and their possible biological activities. Accordingly and in continuation to our previous work on Salvia,[14-19] we decided to carry out a phytochemical study on the roots of *Salvia libanotica* to explore its diterpenoides contents and evaluate their anticancer activities against human colon and breast cancer cell lines.

MATERIALS AND METHODS

General

All NMR data were measured on a Bruker Ascend™ 500 spectrometer fitted with Avance III HD, using deuterated solvents $CDCl_3$.

Plant material

The roots of *Salvia libanotica* were collected during the early flowering period in March and April of the year 2018 from Mount Lebanon (300 m above sea level). The plant was authenticated by Dr. George Tohme (taxonomist) from the National Council for Scientific Research, Beirut, Lebanon. A voucher specimen (SL-18-12) was kept at Faculty of Pharmacy Herbarium, Beirut Arab University, Lebanon. The plant roots were dried at normal conditions and then grounded into a fine powder.

Preparation of plant extract

The air-dried powdered roots (4 kg) were extracted with hot acetone using Soxhlet apparatus until exhaustion. The extracts were separated and the solvent was evaporated under reduced pressure and then lyophilized to get 40 g crude extract.

Chromatographic isolation of compounds

Crude acetone extract was subjected to open column chromatography (CC, 120 cm × 7.5 cm) over silica gel (2000 g). Elution was carried out with gradient mixtures of petroleum ether and dichloromethane (PE: DCM, 20%–100% DCM) then continued by dichloromethane and methanol (DCM: MeOH, 5%–100% MeOH) to give 100 fractions (F1–F100) each 250 ml. Each fraction was subjected to TLC analysis and screening using a suitable solvent system, ultraviolet lamp detection, exposure to conc. Ammonia and different spraying reagents (ferric chloride and anisaldehyde/sulfuric). Similar fractions were combined for further purification either through CC, preparative TLC (PTLC, Silica-gel GF_{254}, 20 cm × 20 cm, 0.5 mm thickness), or crystallization to get single pure crystalline compounds. Fractions F15 and F16 showed two major spots that gave intense violet color upon exposure to ammonia. These two fractions were gathered (0.5 g) and subjected to another CC using silica gel (50 g, column diameter 2 cm) eluted by gradient mixture petroleum ether and dichloromethane (PE: DCM, 5%–30% DCM to give the compounds S1 and S2. S1 and S2 were further purified using Preparative TLC (PTLC) then recrystallized from $CHCl_3$: MeOH solution. Fractions F18–F20 revealed one major and two minor spots. They were pooled again and separated as mentioned above to give the compound S3. S4 precipitated freely as yellow needles after gathering fractions F21–F27. It was further purified by recrystallization from PE: $CHCl_3$ solution. Separation of F21–F27 (1.1 g) was performed using CC (40 cm × 2.5 cm) over silica gel and eluted with gradient mixtures of petroleum ether and dichloromethane (PE: DCM, 5%–60% DCM) to give 20 fractions (A1–A20) where S5 and S6 precipitated freely from A10 and A11, respectively. Fractions F35–F36 were combined and subjected to PTLC to afford S7. S8 precipitated freely as a major component from fraction F37 as shiny orange crystals.

Chemicals

All organic reagents (PE, DCM, and MeOH) were obtained from Sigma-Aldrich® (Germany, analytical grade). The used silica gel for CC and PTLC was purchased from Fluka® (Switzerland). TLC plates (silica gel 60 F_{254}) were purchased from ALUGRAM® SIL G (Germany). MeOH, PE, and DCM used for recrystallization and analysis were HPLC-grade from Sigma-Aldrich®.

Cell culture and treatment

MDA-MB 231 and HCT 116 cells were cultured in DMEM (Lonza) and RPMI-1640 (Sigma Aldrich) medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin streptomycin. Cells were kept in a humidified incubator (95% air, 5% CO_2). They were seeded in 96-well plates at 6×10^5 and 5×10^5 cells/mL, respectively. After 24 h, cells were treated with Salvia total extract (S9) and isolated compounds (S1, S2, S3, S4, S5, S6, S7, and S8) at different concentrations (0, 10, 20, 50, 100, and 250 μM).

Cell viability assays

Compound effect on cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is based on the ability of metabolically active cells to reduce the MTT dye to purple formazan. After treatment, MDA-MB 231 and HCT 116 cells were incubated with 5 mg/mL MTT solution (Abcam) for 3 h then with isopropanol for 45 min. The absorbance of purple formazan was measured at 595 nm using an enzyme-linked immunosorbent assay microplate reader. The results were represented as a percentage of viable cells relative to untreated control. The percentage of viable cells was calculated using this formula:

% viability = (mean O.D. treatment/mean O.D. untreated control) × 100.

Statistical analysis

Each experiment was repeated at least three times. Data were presented as the mean ± standard deviation and two-tailed Student's *t*-test was used to determine the statistical significance between different groups. Statistical significance was defined as a $^*P < 0.05$ and $^{**}P < 0.01$.

RESULTS AND DISCUSSION

Structure elucidation

Compounds S1–S8 were isolated from the roots of *Salvia libanotica*. Physical, chemical, and spectral data of all compounds indicated that they are homologous components related to the abietane diterpene group. The chemical structures of the isolated compounds are presented in Figure 1.

All compounds (except S3) responded to the common tests for hydroxyl-*p*-quinone producing purple-to-dark violet colors upon exposure to NH_3 vapor, reddish-orange or reddish-brown with sulfuric acid and greenish-black with $FeCl_3$ solutions.[20]

^{1}H-NMR and ^{13}C-NMR spectra of all compounds (S1–S8) displayed the presence of two doublets at δ 1.1–1.2 (J = 7 Hz) assigned for the methyl groups at positions 16 and 17 (δ 19.5–20) [Tables 1 and 2]. The appearance of one proton signal (*hept*, J = 7 Hz) assigned for C15 (at δ 3.1) is an indicative of the isopropyl group. Gem dimethyl group at δ 0.86–0.98 for C18 and C19, in addition to one angular methyl group at δ 1–1.3 for C20, was evident in all compounds. In addition, the singlet proton signal at δ 7–7.3 assigned for hydroxyl group at position C12, with two carbonyl signals in the ^{13}C-NMR at δ 183 and 187 elaborated the presence of hydroxylated para-benzoquinone chromophore in all compounds (except S3).[21] All the above-mentioned spectral data were reminiscent of normal abietane diterpene skeleton for all of the isolated compounds.[22]

Compound S1 was crystallized from PE: $CHCl_3$ in the form of yellowish plates, m. p. 181°C –182°C. Mass spectra of S1 exhibited a parent molecular ion peak at m/z 316 in agreement with the molecular formula $C_{20}H_{28}O_3$. ^{13}C-NMR spectrum showed the presence of twenty resolved carbon signals. DEPT experiment indicated them to be as follows: five methyls, five methylenes, two methines, and eight quaternary carbons. Full structure elucidation was achieved by analysis of C-H shift correlations (HCCOSW), ^{1}H-^{13}C shift COSY correlation, ^{1}H-^{13}C long-range correlation (HMBC) spectrum, and comparison with the literature data to be royleanone.[23]

Compound S2 was obtained in the form of dark orange needles, m. p. 170°C–171°C. MS exhibited the molecular ion peak at *m/z* 314, i.e., two mass units less than S1, in agreement with the molecular formula $C_{20}H_{26}O_3$. ^{1}H-NMR spectrum showed the appearance of two double doublet at δ 6.8 (1H, dd, J = 10, 3.5 Hz) and 6.45 (1H, dd, J = 10, 3 Hz) for the two vinylic protons at C6 and C7, respectively. All the obtained data were found to be similar to those reported for 6,7-dehydroroyleanone.[24]

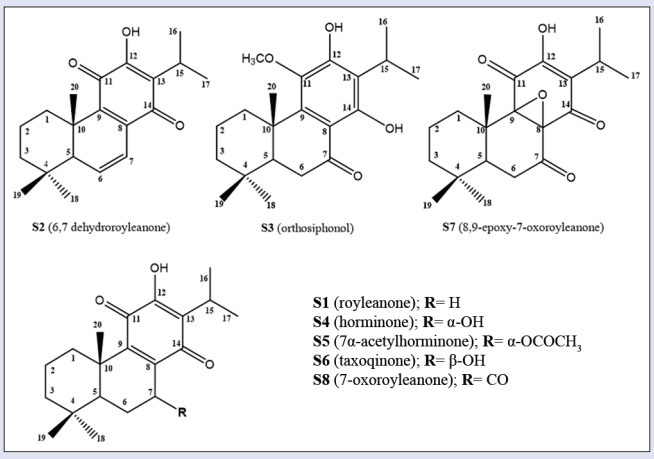

Figure 1: Chemical structures of compounds S1-S8

Compound S4 was isolated in the form of orange to yellowish deposits, m.p. 173°C–174°C. Combined MS (*m/z* 332), ^{1}H, and ^{13}C-NMR spectra indicated the molecular formula to be $C_{20}H_{28}O_4$, i.e., one more oxygen atom than in S1. In ^{1}H-NMR spectrum, the appearance of a one broad proton signal at δ 3.07, exchangeable with D_2O, suggested the presence of hydroxyl group at position C7. This hydroxyl group was further confirmed by the appearance of double doublet signal at δ 4.74 (1H, dd, J = 7, 1.5 Hz) assigned for H-7. α-configuration of 7-OH group was also confirmed by the appearance of a carbon signal resonating at δ 63.3 in ^{13}C-NMR assigned for C7.[25] All spectral data were in agreement with those previously reported for horminone.[26]

Compound S5 was crystallized in the form of yellow crystals, m. p. 211°C–212°C. The molecular formula ($C_{22}H_{30}O_5$) was deduced from the combined analysis of MS (*m/z* 347) and ^{1}H and ^{13}C NMR spectra [Tables 1 and 2]. In comparison to S4, ^{13}C-NMR spectrum showed an additional carbon signal at δ 169.9 suggesting the presence of an additional carbonyl group. ^{1}H and ^{13}C-NMR spectra indicated that C7 is oxygenated, showing carbon signal at δ 64 and proton signal at δ 5.92 (1H, d, J = 1.5 Hz) which was assigned for H-7. Thus, the acetylation site was confirmed to be at C7. J value (1.5 Hz) together with ^{13}C data for C7 and NOSY correlations indicated 7 β-H and α-configuration for the acetyl group.[27] HMBC, NOSY, and HMQC correlations were also interpreted to confirm the chemical structure of compound S5 to be 7α-acetylhorminone.[28]

Compound S6 was obtained as orange small needles, m. p. 212°C–214°C. Mass spectrum showed a molecular peak ion at *m/z* 332 which was in good agreement with the deduced molecular formula $C_{20}H_{28}O_4$. All spectral data were closely related to that of S4 with slight differences in the shifts of carbons and protons at position 6 and 7. ^{1}H-NMR spectrum of S6 showed a hydroxyl methine signal at δ 4.75 (1H, dd, J = 7.5, 10 Hz) assigned for H-7 and an oxygenated proton at δ 3.8 (1H, br). The presence of carbon signal in the ^{13}C spectrum at δ 68 confirmed the position of hydroxyl group at C7. By comparison of the multiplicity of H-7 and the chemical shifts both in the ^{1}H and ^{13}C-NMR data, the structure of this compound was confirmed to have 7 β-OH at C7.[29] Accordingly, the structure of compound S6 was determined to be taxoquinone. All its data were identical to those previously reported in literature.[30]

Compound S8 was obtained in the form of orange–red needles, m. p. 196°C–197°C. The molecular formula was deduced from combined analysis of mass spectrum (*m/z* 331) and ^{1}H and ^{13}C-NMR spectra to be $C_{20}H_{26}O_4$. All spectra data, physical and chemical properties, confirmed the abietane diterpinoid structure as discussed for the compounds before. ^{13}C-NMR spectrum revealed a carbon signal at δ 197 indicating the presence of an additional carbonyl group, which was assigned to carbon C7. The appearance of a double doublet at δ 2.5 (1Hβ, dd, J = 14.5, 18 Hz) and δ 2.67 (1Hα, dd, J = 18, 4 Hz) assigned for H-6 and the absence of any proton signal correlated to C7 (C-H correlation spectrum) further confirmed the presence of 7-oxo moiety at position C7. All proton and carbon values were fully interpreted and compared with literature[31] to confirm the structure of S8 to be 7-ketoroyleanone or 7-oxoroyleanone.

Compound S7 was isolated in the form of faint yellowish needles. ^{1}H and ^{13}C-NMR spectra were similar to that of compound S8 (shift, multiplicity, correlations, J value) with only a difference in the shift of C8 and C9 in the ^{13}C spectrum. The lower values of carbon signals C8 and C9 at δ 66.8 and 61.3, respectively, indicated the absence of conjugated double bonds and confirmed that both carbons are oxygenated. The mass spectrum showed a molecular peak ion at *m/z* 346 in agreement with the molecular formula $C_{20}H_{26}O_5$, thus the same structure of S8 with additional oxygen between C8 and C9. All spectral data were interpreted and compared to that in literature to reveal the structure of compound S7 as 8,9-epoxy-7-oxoroyleanone.[32,33]

Table 1: ^{1}H-NMR (500 MHZ) data of isolated compounds ($CDCl_3$, δ in ppm, *J* in Hz)

	S1	S2	S3	S4	S5	S6	S7	S8
H_1	2.7 ($1H_\beta$, m) 1.1 ($1H_\alpha$, m)	2.87 ($1H_\beta$, dt, J=13.5 Hz) 1.37 ($1H_\alpha$, m)	3.2 ($1H_\beta$, br.d) 1.22 ($1H_\alpha$, m)	2.7 ($1H_\beta$, dt., J=13 Hz) 1.18 1.06 ($1H_\alpha$, m)	2.72 ($1H_\beta$, d, J=13 Hz) 1.2 ($1H_\alpha$, m)	2.64 ($1H_\beta$, dbr., J=13 Hz) 1.06 ($1H_\alpha$, m)	2.9 ($1H_\beta$, br, d) 1.55 ($1H_\alpha$, dd, J=13.5, 4 Hz)	2.8 ($1H_\beta$, dt, J=12.9,3.2) 1.4-1.7 ($1H_\alpha$, m)
H_2	1.67 ($1H_\beta$, m) 1.5 ($1H_\alpha$, m)	1.52 ($1H_\beta$, m) 1.6 ($1H_\alpha$, m)	1.47 (1H, m) 1.65 (1H, tt, J=14, 3.5 Hz)	1.74 (1H, qt, J=13.5, 3.5 Hz) 1.6 1.5 (1H, m)	1.59 (2H, m)	1.69 (1H, dt, J=14, 3.5 Hz) 1.5 (1H, m)	1.7 (1H, m) 1.65 (1H, m)	1.4-1.7 (2H, m)
H_3	1.42 (2H, m)	1.45 ($1H_\beta$, m)	1.42 (1H, br.d) 1.19 (1H, m)	1.45 (1H, dm, J=12.5Hz) 1.27 (1H, m)	1.48 (1H, m) 1.2 (1H, m)	1.1 (1H, dd, J=4.5 Hz) 1.44 (1H, m)	1.49 (1H, br.d) 1.26 (1H, m)	1.4-1.7 (2H, m)
H_5	1.05 (1H, m)	2.13 ($1H_\alpha$, t, J=3 Hz)	1.72 (1H, dd, J=11.5,6 Hz)	1.5 (1H, br.)	1.46 (1H, m)	1.09 (1H, m)	1.94 (1H, dd, J=12.5,6 Hz)	1.8 (1H, dd, J=24.5, 4 Hz)
H_6	1.83 ($1H_\beta$, dd, J=13.5, 6 Hz) 1.35 ($1H_\alpha$, m)	6.4 (1H, dd, J=10, 3 Hz)	2.56 (2H, dd)	1.97 ($1H_\beta$, d, J=14 Hz) 1.54 (1H, m)	1.93 ($1H_\beta$, d, J=14.5 Hz) 1.64 ($1H_\alpha$, m)	2.16 ($1H_\beta$, dd, J=7.5, 5.5 Hz) 1.48 ($1H_\alpha$, m)	2.58 (1H, dd, J=17, 6 Hz) 2.3 (1H, dd, J=12.5, 8.5 Hz)	2.5 ($1H_\beta$, dd, J=14.5, 18 Hz) 2.67 ($1H_\alpha$, dd, J=18, 4 Hz)
H_7	2.66 ($1H_\beta$, dd, J=4.5, 1 Hz) 2.3 (1Hα, m)	6.8 (1H, dd, J=10, 3.5 Hz)		4.74 ($1H_\alpha$, dd, J=7, 1.5 Hz)	5.92 ($1H_\alpha$, d, J=1.5 Hz)	4.75 ($1H_\beta$, dd, J=7.5, 10 Hz)		
7-OH				3.07 (7-OH, br.)		3.8 (1H, br)		
H_7-O-Ac					2.02 (3H, s)			
11-OCH_3			3.71 (3H, s)					
12-OH	7.15 (1H, s)	7.34 (1H, s)	5.62 (1H, s)	7.4 (1H, br.)	7.14 (1H, br.)	7.2 (1H, br.)	7.04 (1H, s)	7.01 (1H, s)
H_{14}-OH			13.29 (1H, s)					
H_{15}	3.13 (1H, hept, J=7 Hz)	3.15 (1H, hept, J=7 Hz)	3.2 (1H, hept, J=7 Hz)	3.17 (1H, sept, J=7.5 Hz)	3.15 (1H, sept, J=7 Hz)	3.1 (1H, sept, J=7.5 Hz)	3.14 (1H, hept., J=7 Hz)	3.17 (1H, hept, J=7 Hz)
H_{16}	1.17 (3H, d, J=7 Hz)	1.2 (3H, d, J=7 Hz)	1.3 (3H, d, J=7 Hz)	1.21 (3H, d, J=3 Hz)	1.16 (3H, s)	1.17 (3H, d, J=3.5 Hz)	1.19 (3H, d, J=7 Hz)	1.21 (3H, d, J=7)
H_{17}	1.16 (3H, d, J=7 Hz)	1.19 (3H, d, J=7 Hz)	1.32 (3H, d, J=7 Hz)	1.22 (3H, d, J=3 Hz)	1.18 (3H, s)	1.18 (3H, d, J=3.5 Hz)	1.2 (3H, d, J=7 Hz)	1.22 (3H, d, J=7)
H_{18}	0.86 (3H, s)	0.97 (3H, s)	0.86 (3H, s)	0.98 (3H, s)	0.86 (3H, s)	0.9 (3H, s)	0.88 (3H, s)	0.91 (3H, s)
H_{19}	0.9 (3H, s)	1 (3H, s)	0.88 (3H, s)	0.91 (3H, s)	0.87 (3H, s)	0.88 (3H, s)	0.9 (3H, s)	0.95 (3H, s)
H_{20}	1.18 (3H, s)	1.02 (3H, s)	1.31 (3H, s)	1.22 (3H, s)	1.22 (3H, s)	1.31 (3H, s)	1.23 (3H, s)	1.34 (3H, s)

Table 2: ^{13}C-NMR data for compounds S1-S8 (at 500MHz in $CDCl_3$, δ in ppm)

	S1	S2	S3	S4	S5	S6	S7	S8
C_1	36.24	36.24	35.88	35.7	35.77	36.26	33.47	35.73
C_2	18.9	18.9	19	18.3	18.8	18.67	18.2	18.68
C_3	41.29	41.29	41.1	41	40.9	40.9	40.75	40.88
C_4	33.28	33.45	33.1	33.1	32.9	33.4	34	33.41
C_5	51.7	51.7	49.6	45.7	46.1	49.05	42.76	49.12
C_6	26.68	139.7	36.4	25.7	24.6	26.33	38.25	36.86
C_7	29.38	121.1	206.1	63.1	64.5	68.22	198.99	197
C_7-OC					169.5			
C_8	145.9	146	112.5	143.1	139.4	144.47	66.8	132.9
C_9	146.5	146.52	135.8	147.8	149.9	148.1	61.3	155.5
C_{10}	38.41	39.26	40.3	39.1	39.06	39.62	37.7	40.08
C_{11}	187.5	187.57	152.2	183.8	183.7	183.9	186.28	185.13
C_{11}-OCC3			62					
C_{12}	150.5	150.55	158.2	151.1	151.09	151.15	151.5	150.88
C_{13}	123.69	123.69	126.1	124.2	124.66	124.47	127.47	125.55
C_{14}	183.4	183.46	138.6	189.1	185.47	188.8	187.9	184.9
C_{15}	24	24	26	23.9	24.15	24.11	25.01	24.37
C_{16}	19.8	19.84	20.33	19.77	19.69	20.01	19.5	19.87
C_{17}	19.96	19.96	20.38	19.88	19.86	20.4	19.2	19.94
C_{18}	33.4	33.49	33.4	33	32.95	33.32	32.8	32.85
C_{19}	20.08	17.4	21.5	21.7	21.62	21.81	20.7	21.4
C_{20}	21.76	22.83	17.9	18.85	18.48	19.97	16	18.08

Compound S3 was crystallized in the form of yellow to orange crystals, m. p. 143°C–145°C. EI-MS spectrum showed the molecular ion peak at *m/z* 346 in agreement with the molecular formula $C_{21}H_{30}O_4$. ^{1}H and ^{13}C-NMR spectra confirmed the presence of normal abietane skeleton.[22] ^{13}C-NMR revealed the presence of carbonyl group at δ 206, which was assigned for C7. Moreover, the presence of six quaternary aromatic

signals in the ^{13}C-NMR spectrum and the absence of any aromatic protons in ^{1}H-NMR spectrum indicated a fully substituted benzoyl moiety in ring C. ^{1}H-NMR showed the presence of two singlet signals, exchangeable with D_2O, at δ 5.6 and 13.2. The appearance of the latter singlet signal (δ 13.2) indicated the presence of a hydroxyl group *peri* to carbonyl group within a six-membered ring structure. Accordingly, the carbonyl and the hydroxyl groups should be at positions C7 and C14, respectively. The spectrum also indicated the presence of methoxyl group, through the appearance of a singlet signal at δ 3.71 (3H, s). HMBC correlated this signal to carbons C9 (δ 135) and C12 (δ 158) with a strong correlation with C11, and thus, its existence at C11 was confirmed. Extensive analysis and interpretations of 2D-NMR spectra (COSY, NOSY, HMBC, and HMQC) allowed full assignment of all protons and carbons. Interestingly and referring to Scifinder, compound S3 was reported only twice,[31] and all our results were in full agreement with published data for orthosiphonol.

Biological importance of isolated compounds

Although all isolated compounds were reported before, this study showed the distinct chemotype of the Lebanese species of Salvia, whereby some known rare compounds were isolated as major components. According to Scifinder, this is the first report of isolating S3 from the genus Salvia.[31] Furthermore, the study provided evidence for the therapeutic potential of *Salvia libanotica* as it constitutes biologically important components. S1 was reported to have neuroprotective activities via the prolyl endopeptidase inhibitory effect.[34] S4, S5, and S8 were reported to have promising antimicrobial activities, especially against MRSA[34] with remarkable anticandidal effects for S6.[35] S6 also showed a potent inihibitory effect for α-glucosidase and tyrosinase enzymes by 9.24%–51.32% and 11.14%–52.32%, respectively, with a possibility for using it as a natural alternative medicine to prevent diabetes mellitus type-2 related disorders and as a depigmentation agent.[36] S1, S2, S4, and S6 showed gastroprotective activities against HCl/EtOH-induced gastric ulcers in mice (71%, 54%, 53%, and 36%, respectively) and cytotoxicity against human gastric adenocarcinoma AGS cells with IC_{50} of 18, 366, 11, and 27 μM, respectively.[37]

Cytotoxic activity and structure relationships

The anticancer activity of crude Salvia extract and the isolated compounds were tested against human colon HCT116 and breast MDA-MB-231 cancer cell lines at different concentrations ranging from 10 μM to 250 μM for 24 h using MTT assay. The results revealed that almost all compounds (including the total extract) showed dose-dependent inhibitory effects on both cancer cell lines [Figure 2]. The most effective was 7α-acetylhorminone, with IC_{50} of 18 and 44 μM on HCT116 and MDA-MB-231 cells, respectively [Table 3]. However, the least effective compounds were royleanone and dehydroroyleanone with IC_{50} >250 μM. The activity of the extract and all the isolated compounds varied between the cell types indicating cell-type specificity and suggesting different interaction modes between the compounds and the cells. MDA-MB-231 cell line does not display progesterone receptors, estrogenic receptors, or human epidermal growth factor receptor 2 because it is a highly metastatic triple-negative breast cancer cell line. This is the reason behind being clinically difficult to target.[38] Besides, 7α-acetylhorminone and 7-oxoroyleanone showed promising inhibitory effects with IC_{50} of 44 and 55 μM on MDA-MB-231 cells. 7α-acetylhorminone, taxoquinone, 8,9-epoxy-7-oxoroyleanone, and 7-oxoroyleanone were more active against colon cancer cells (HCT 116), while horminone and orthosiphonol were more active against MDA-MB-231 cells. This specificity was also reported previously, where 6,7-dehydroroyleanone (as an example) was very active against prostate and cervical cancer cells with IC_{50} 6.5 and 9.4 μM, respectively, but inactive against colon or breast cancer cells.[34]

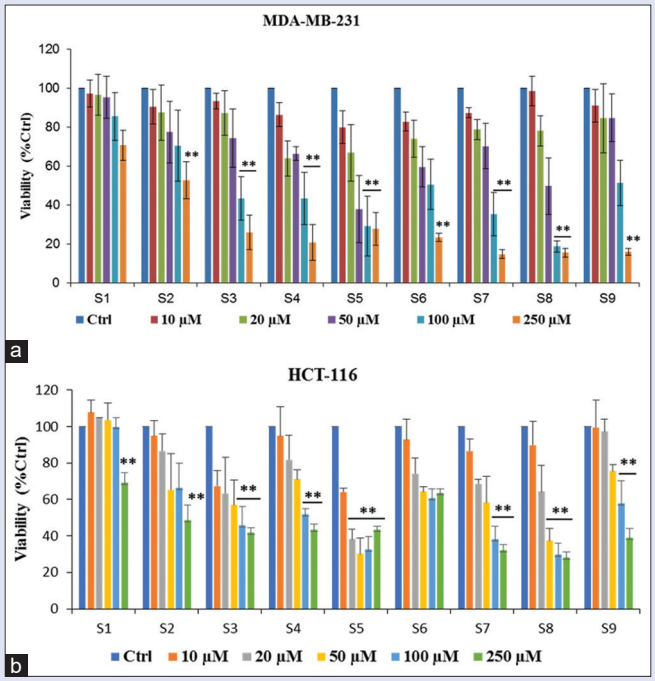

Figure 2: MDA-MB 231 breast cancer cells (a) and HCT 116 human colon cancer cells (b) were treated with Salvia total extract (S9) and eight isolated compounds at different concentrations (0, 10, 2 0, 50, 100 and 250 μM). At 24 h post-treatment, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The values are expressed as percentage of viable cells relative to untreated control. Each value demonstates the mean ± standard deviation of three independent experiments. ** ($P < 0.01$) are significantly different from untreated control using two-tailed Student's *t*-test

Table 3: IC_{50} of tested compounds and of the total extract (S9) (μM)

Compound	IC_{50} (μM)	
	HCT116	MDA-MB-132
S1	>250	>250
S2	157	≥250
S3	105	89
S4	118	100
S5	18	44
S6	69	85
S7	60	78
S8	38	55
S9 (extract)	177	110

IC_{50}: Inhibition concentration 50

Studies on the structure–activity relationships SARs of royleanon derivatives are rare.[39] Thus, the obtained cytotoxicity data [Table 3] when combined with other studies on the cytotoxicity of abietane diterpene compounds allowed the proposal of some structure–activity relationships for royleanone-type abietanes. The results indicated that oxygenated C7 is essential for the cytotoxic activity (the case of 7α-acetylhorminone, taxoquinone, 8,9-epoxy-7-oxoroyleanone and 7-oxoroyleanone). Moreover, the carbonyl group at position C7 appears to lead to higher activity than the hydroxyl group (taxoquinone vs 7-oxoroyleanone). More specifically, β-OH at C7 were more active than α-OH (taxoquinone vs horminone), but both led to less activity than the

carbonyl moiety (8,9-epoxy-7-oxoroyleanone and 7-oxoroyleanone). On the other hand, non-oxygenated C7 (even with unsaturation between C6 and C7) diminishes the inhibitory effect of the compounds (royleanone and 6,7-dehydroroyleanone). These findings are in good agreement with previous literature on abietane diterpene SARs. Studies suggested that the carbonyl and hydroxyl groups, attached at the C7 position of ring B, may play a significant role in the reactivity properties of these compounds and such activity is suppressed when a hydrogen atom replaces them.[40] In addition, a clear potential was observed for increased cytotoxicity with the higher lipophilicity of the 7α substituent corresponding to an improved fitting on the target or favorable log *P* for membrane crossing because the cytotoxicity of several diterpenes has been associated with a mechanism that involves membrane-disrupting properties.[38] For that, 7α-acetylhorminone was also the most active among its derivatives when tested against pancreatic MIAPaCa-2 and melanoma (MV-3) cancerous cell lines with IC_{50} of 4.7 and 7.4 μM, respectively.[31] The relatively lower activity of orthosiphonol with respect to 8,9-epoxy-7-oxoroyleanone and 7-oxoroyleanone suggested the importance of para quinone structure and the carbonyl group at C14 position for the observed cytotoxic activity.[39] Similar findings were reported for orthosiphonol when tested against human pancreatic (MIAPaCa-2) and melanoma (MV-3) tumor cell lines with IC_{50} >100 and >80 μM, respectively.[31] Further extensive investigations at the molecular level are needed to completely explore the relationship between the structural features of the compounds and their subsequent biological activity.

CONCLUSION

This study reported the potential of abietane diterpinoids as anticancer agents. The highest activity was for 7α-acetylhorminone with IC_{50} of 18 and 44 μM on HCT116 and MDA-MB-132 cells. It also suggested *Salvia libanoticum* and its diterpinoids as promising remedies in colon and/or breast cancer therapy with high selectivity and cell specificity.

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Conflicts of interest

There are no conflicts of interest.

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