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Antibacterial, antioxidant and cytotoxic activities of triterpenes and flavonoids from the aerial parts of *Salvia barrelieri* Etl.

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Abstract

From the aerial parts of *Salvia barrelieri* Etl. (Lamiaceae), six flavonoids and six triterpenes including a new compound 3 β -acetoxy-olean-18-ene-2 α -ol, were isolated. Their structures were established by the combination analyses of spectroscopy including 1D-, 2D-NMR, HRESIMS and in comparison with the reported data in the literature. The antibacterial evaluation of isolated compounds by bioautography on *Staphylococcus aureus* followed by the determination of MIC values of actives compounds by serial dilution technique against 5 bacteria revealed that *epi*-germanidiol (**2**), apigenin-7-*O*- β -D-glucuronopyranoside (**10**) and cynaroside (**12**) were the most active against *Enterococcus faecalis* ATCC 1034, *Staphylococcus aureus* CIP 53.154, *Escherichia coli* CIP 54.127, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* ATCC 9027, with minimum inhibitory concentration values of 15.1 to 125 μ g/mL. Apigenin-7-*O*- β -D-glucuronopyranoside (**10**) and cynaroside (**12**) exhibited moderate to good DPPH radical scavenging activity (IC₅₀ 79.1 and 21.2 μ g/mL, respectively). The isolated compounds did not show significant tyrosinase inhibitory activities (IC₅₀ > at 1.5 mg/mL). The cytotoxic activity of isolated compounds was evaluated against promyelocytic leukemia (HL60), human erythromyeloblastoid leukemia (K562) and fibrosarcoma (HT1080) cell lines. Compound **1**, *epi*-germanidiol (**2**), germanicol (**4**) and micromeric acid (**5**) showed a moderate growth inhibitory activity against HL60, K562 and HT1080 cell lines (IC₅₀ 28.75 to 85.0 μ M).

Keywords: *Salvia barrelieri*, Lamiaceae, Triterpenoids, Flavonoids, Antibacterial Activity, Cytotoxic activity, Radical scavenging activity.

1. Introduction

The genus *Salvia* (Lamiaceae) comprises more than 900 species widely distributed, of which 23 species are distributed in Algeria [1]. Some *Salvia* species such as *S. officinalis* and *S. miltiorrhiza* have been applied as medicinal plants and used for the preparation of various

remedies in various countries [2,3]. *S. barrelieri* Etl. has been used as diuretic, emmenagogue and to treat gastric disorders and microbial infections in Algeria [4].

The anticholinesterase, neuroprotective, anticancer, antiviral, anti-inflammatory, antioxidant, antibacterial and cytotoxic activities of *Salvia* plants were summarized in many reviews papers [3,5]. Other reviews were interested on diterpenoids and triterpenoids isolated from *Salvia* [6-8].

Salvia barrelieri is an herbaceous perennial plant found in northern Africa, Algeria, Morocco, Tunisia, and southwestern Spain. It grows 1-2 meters tall, with large, wavy, gray-green leaves. The inflorescence is a verticillaster and can grow nearly one meter tall, with flowers of light lavender or sky blue [9].

In continuation of our studies on the *Salvia* genus [8,10,11,12], we have now investigated the aerial parts of *S. barrelieri*, from the root of which seven abietane diterpenoids have been previously isolated, and evaluated for their antioxidant activity [4,11]. Herein, we report the isolation and structural characterization of one new triterpene together with eleven known compounds from the aerial parts of this plant. Taking into account the use of this plant to treat gastric disorders and microbial infections, the antibacterial activities of isolated compounds were evaluated. In addition, the antioxidant, anti-inflammatory and cytotoxic activities of *Salvia* plants encouraged us to evaluate the DPPH radical scavenging rates and tyrosinase inhibitory activity. The cytotoxic activity of isolated compounds was evaluated against promyelocytic leukemia (HL60), human erythromyeloblastoid leukemia (K562) and fibrosarcoma (HT1080 cell lines).

2. Results and discussion

Chromatographic separations of exudate and the 80% EtOH extract of the aerial parts of *S. barrelieri* led to the isolation of twelve compounds (**1-12**). Six triterpenoids (**1-6**), including a previously undescribed 3β -acetoxy-olean-18-ene-2 α -ol (**1**) were isolated from the exudate

whereas from the EtOH 80% extract, six flavonoid glycosides were isolated (**7-12**). The elucidation of their structures was performed by NMR analysis (^1H and ^{13}C NMR, ^1H - ^1H -COSY, HSQC, and HMBC) and high resolution mass spectrometry (HRESIMS).

The known compounds were identified as *epi*-germanidiol (**2**) [18], olean-18-ene-1 β ,2 α ,3 β -triol (**3**) [19], germanicol (**4**) [20], micromeric acid (**5**) [21], ursolic acid (**6**) [22], salvigenin (**7**) [23], apigenin-7-*O*- β -D-glucuronopyranoside methyl ester (**8**) [24], apigenin-7-*O*- β -D-glucopyranoside (**9**) [25], apigenin-7-*O*- β -D-glucuronopyranoside (**10**), apigenin (**11**) and cynaroside (**12**) [25,26] (Fig. 1). Their spectroscopic data were in perfect agreement with those reported in the literature.

Compound **1** was obtained as white solid. Its molecular formula was determined as $\text{C}_{32}\text{H}_{52}\text{O}_3$ from the pseudo-molecular $[\text{M} + \text{Na}]^+$ ion peak observed at m/z 507.3820 in the positive HRESIMS (calcd $\text{C}_{32}\text{H}_{52}\text{O}_3\text{Na}$, 507.3814). The ^1H NMR spectrum of **1** showed typical signals of eight tertiary methyl groups (each, 3H, s) at δ_{H} 0.76 (H-27), 0.88 (H-24), 0.90 (H-23), 0.96 (H-29), 0.97 (H-30), 0.98 (H-25), 1.04 (H-28) and 1.09 (H-26), one olefinic proton at δ_{H} 4.89 (*brs*, H-19), two oxygenated methines at δ_{H} 3.84 (td, $J = 10.6, 4.1$ Hz, H-2) and 4.52 (d, $J = 10$ Hz, H-3), and one acetoxy group at δ_{H} 2.16 (3H, s) (see experimental part). Analysis of the ^{13}C NMR and HSQC spectra of **1** revealed 32 signals, of which 30 were assigned to an aglycone and 2 belonged to acetoxy group (δ_{C} 172.4 and 21.1). The aglycone of **1** was deduced to be a germanicol-*type* triterpene with two olefinic carbons of tri-substituted double bond (δ_{C} 129.8 and 142.5), two oxygenated methine carbons (δ_{C} 67.8 and 85.0) and eight quaternary methyl carbons (δ_{C} 14.5, 16.0, 17.4, 17.8, 25.2, 28.4, 29.1 and 31.3). All proton and carbon signals of **1** were assigned by analysis of 2D-NMR, including HSQC, HMBC and ^1H - ^1H -COSY. The HMBC correlations of H₃-23 and H₃-24 to C-3 (δ_{C} 85.0), C-4 (δ_{C} 39.3) and C-5 (δ_{C} 55.4) indicated the location of the oxygenated carbon and *gem*-dimethyl groups at C-3

and C-4 in A ring, respectively. The correlation observed in the ^1H - ^1H COSY spectrum between H-3 (δ_{H} 4.52) and the second oxygenated methine (δ_{H} 3.84) indicated that the second hydroxyl was located at C-2 (δ_{H} 67.8) which was confirmed by the long range correlations in HMBC spectrum between H-2 (δ_{H} 3.84) and C-1 (δ_{C} 48.3) and C-3 (δ_{C} 85.0). Similarly, the HMBC correlations between H₃-25/C-1, C-10, C-9 and C-5, H₃-26/C-7, C-8, C-9 and C-14, H₃-27/C-8, C-13, C-14 and C-15, H₃-28/C-16, C-17, C-18, C-22, H₃-29 and H₃-30 /C-19, C-20, and C-21 confirmed some aspects of the structure. The HMBC correlation between the carbon methyl signal of C-27 at δ_{C} 14.5 and the proton at δ_{H} 2.28 (*brd* ($J = 11.4$ Hz) attributed to the angular methine proton H-13 and the HMBC correlations between this proton H-13 and the two ethylenic carbons at δ_{C} 129.8 (C-19) and δ_{C} 142.5 (C-18) suggested a Δ^{18} -unsaturated oleanene skeleton as in germanidiol or *epi*-germanidiol (**2**) [18]. The long range 4J ^1H - ^1H COSY correlation observed between H-19 and H-13 in addition to the HMBC correlations observed between H-19/C-13, C-17, C-20, C-21, C-29, and C-30 confirmed the position of the double bond at C-18/C-19. The acetoxy group was assigned at C-3 as deduced from the HMBC correlation observed between H-3 and H₃-acetyl to the carboxyl carbon at δ_{C} 172.4. Thus, the aglycone of **1** was considered to be 3-acetoxy-olean-18-ene-2-ol. The 2- α and 3- β orientations of hydroxy and acetoxy groups were deduced from the value of the vicinal coupling constant ($J_{2-3} = 10.0$ Hz) and by comparison with spectroscopic data with those reported in the literature for compounds possessing 3 β -acetoxy and 2 α -hydroxyl substitution ($J_{\text{H}3\alpha/\text{H}2\beta} = 10.0$ Hz) [27] and with *epi*-germanidiol (2 α ,3 β -di-OH, $J_{2-3} = 10$ Hz) and germanidiol (2 α ,3 α -di-OH, $J_{2-3} = 3$ Hz) [18]. The β orientation of H-13 was deduced by comparison of the vicinal coupling constant value and by comparison with spectroscopic data with those reported in the literature of germanidiol and *epi*-germanidiol (**2**) [18]. The large vicinal coupling constant value between H-12_{ax} and H-13 ($J_{12\text{ax}-13} = 11.4$ Hz) suggested a quasi *trans*-diaxial relationship, like in 2 α ,3 β ,21 β ,23-tetrahydroxyolean-18-en-28-oic [17].

Consequently, the structure of **1** was elucidated as 3 β -acetoxy-olean-18-ene-2 α -ol, or 3-acetyl *epi*-germanidiol.

The antibacterial activity of the exudate and the 80% EtOH extract obtained from the aerial parts of *S. barrelieri* were initially tested for their antibacterial capacity using TLC bioautography method against *S. aureus* CIP 53.154, a Gram positive cocci bacterium frequently found on the skin and in the respiratory tract and that can be responsible for nosocomial infections [13]. Currently its resistance to antibiotics is an acute problem for the treatment of patients. The results showed good activity for the 80% EtOH extract and a milder activity for the exudate by comparison with the reference gentamicin. Subsequently, the test was repeated for compounds **1-12** isolated from the 80% EtOH extract and the exudate. Compounds **2, 4, 5-8, 10-12** were the only active compounds as revealed by the white inhibition zones observed on the TLC plate around all compound spots. The minimum inhibitory concentration values (MIC) of these compounds were measured by a serial dilution technique using 96-well microliter plates against three Gram-positive (*E. faecalis* ATCC 1034, *S. aureus* CIP 53.154 and *S. epidermidis*) and two Gram negative (*E. coli* CIP 54.127 and *P. aeruginosa* ATCC 9027) bacteria (Table 1). The results showed a high inhibitory effect of compound **10** against *P. aeruginosa* (MIC 15.1 μ g/mL), *E. faecalis*, *S. epidermidis* and *E. coli* (MIC 31.2 μ g/mL). Compound **12** was also active against these four bacteria (MIC 31.2 μ g/mL). The triterpenes **1-6** were less active compared to flavonoids **10** and **12**. Only compound **6** showed a high antibacterial activity against *E. faecalis* (MIC 31.2 μ g/mL). Triterpene **2** showed also good antibacterial activity against the five tested bacteria with MIC values ranging from 62.5 to 125 μ g/mL (Table 1). A moderate antibacterial activity against the five tested bacteria with MIC values ranging from 125 to 250 μ g/ mL was obtained with compounds **5** and **8**.

The DPPH radical scavenging activity of 80% EtOH extract and exudate obtained from the aerial parts of *Salvia barrelieri* was measured. In this assay, antioxidants were able to reduce the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine. Only the 80% EtOH showed a DPPH radical scavenging activity (IC_{50} 50.1 μ g/mL). Subsequently, the test was repeated for compounds **7-12** isolated from this extract. Only compounds **10** and **12** exhibited moderate to good DPPH radical scavenging activity (IC_{50} 79.1 and 21.2 μ g/mL, respectively) compared with the reference ascorbic acid (IC_{50} 11.2 μ g/mL). The five other compounds showed low or no antiradical activity.

The tyrosinase inhibitory activity of the 80% EtOH extract and the exudate obtained from the aerial parts of *S. barrelieri* was tested. The 80% EtOH extract showed a low tyrosinase inhibitory activity at the dose of 1.5 mg/mL (27 % inhibition). The test was repeated for compounds **7-12** isolated from this extract and 50% inhibition was not achieved at the concentration of 1.5 mg/mL. Kojik acid was used as positive control (IC_{50} 20 μ g/mL).

The cytotoxic activity of compounds **1-12** was evaluated *in vitro* using two tumor cell lines, promyelocytic leukemia (HL60), human erythromyeloblastoid leukemia (K562) and fibrosarcoma (HT1080). The resulting IC_{50} values are listed in table 1. Compounds **2**, **4** and **5** showed a moderate cytotoxic activity against HL60 (IC_{50} 54.0, 42.0, 52.0 μ M, respectively) and against K562 cell lines (IC_{50} 85.0, 82.0, 82.0 μ M, respectively). Compound **1** showed good cytotoxic activity against HT1080 (IC_{50} 28.75 μ M) whereas compounds **2**, **4** and **5** showed moderate cytotoxic activity against HT1080 (IC_{50} 50, 37.5 and 41.5 μ M, respectively).

3. Experimental

3.1. General experimental procedures

HRESIMS experiments were performed using a Micromass Q-TOF micro instrument (Manchester, UK). Optical rotations of pure compounds were measured in CHCl₃ using a Perkin-Elmer 341 Polarimeter. NMR spectra were recorded in CD₃OD or CDCl₃ on a Bruker Avance III 500 spectrometer (¹H at 500 MHz and ¹³C at 125 MHz). 2D-NMR experiments were performed using standard Bruker microprograms (TopSpin 3.2 software). Flash chromatography was carried out on a Grace Reveleris system equipped with dual UV and ELSD detection using Grace® cartridges (Silica gel or RP-C₁₈). HPLC separations were performed on a Dionex apparatus equipped with an ASI-100 autosampler, an Ultimate 3000 pump, a STH 585 column oven, a diode array detector UVD 340S and a Chromeleon software. A prepacked RP-C₁₈ column (Phenomenex 250 x 15 mm, Luna 5 µ) was used for semi-preparative HPLC. The eluting mobile phase consisted of H₂O with TFA (0.0025%) and CH₃CN with a flow rate of 5 mL/min and the chromatogram was monitored at 205 and 210 nm. Thin-layer chromatography (TLC) was carried out using silica gel 60 F₂₅₄ pre-coated aluminium plates (0.2 mm, Merck). After developing with solvent systems, spots were visualized under UV light (254 and 366 nm) and sprayed with 50% H₂SO₄ followed by heating. An Armen instrument equipped with an AP 250/500 pump, ACC 250/500 sampler, and a Merck UV-detector K-2501 was used for preparative HPLC. A Lichrospher RP18 prepacked column (Merck 250x50mm, 12µm) was used with binary gradient eluent (H₂O and CH₃CN) and a flow rate of 50 mL/min; the chromatogram was monitored at λ 250 nm.

3.2. Plant material

The aerial parts of *Salvia barrelieri* Etl. (Lamiaceae) were collected from Constantine (North-Eastern of Algeria), in April 2014 and it was authenticated by Mr. Kamel Kabouche. A voucher specimen (LOST.Sb.04.14) was deposited at the University of Constantine, Algeria.

3.3. Extraction and isolation

The fresh aerial parts of *S. barrelieri* (7 kg) were first immersed in CHCl₃ for 20 s. After filtration and evaporation of the solvent under reduced pressure, 48 g of exudate were obtained. Then, the powdered air-dried aerial parts were macerated in EtOH–H₂O (8:2, 7 L); three time at room temperature. The combined extracts were concentrated under reduced pressure and the residue was suspended in H₂O (1 L), to be partitioned successively with CHCl₃, EtOAc and *n*-BuOH (3 × 300 mL each), to yield respectively 1.8, 2.7 and 11 g of each fraction.

A portion of the exudate (10 g) was fractionated by silica gel-*vacuum* liquid chromatography (VLC) using a step-gradient solvent system from 100% *n*-hexane to 100% EtOAc with a 10% increment to obtain 21 fractions [E1-E21]. Fraction E5 (129 mg) was subjected to flash chromatography on RP18 using a gradient 90-100% of MeOH in water in 30 min, affording compound **4** (32 mg). Fraction E11 (380 mg) was further purified by flash chromatography on RP18, eluted with MeOH from 80 to 100% in water, to yield compound **1** (67 mg). Fraction E14 (1.28 g) was also subjected to flash chromatography on RP18 eluted with the same system of the previous fraction (E11), to give compounds **2** (23.3 mg), **5** (10.5mg) and **6** (8 mg). Compound **3** (1.5 mg) was obtained from the flash chromatography over silica gel of the fraction E16 (1.47 g), eluted by gradient system toluene-acetone (100:0-80:20).

A part of the *n*-BuOH fraction (2 g) was submitted to a VLC over RP18 eluted with MeOH:H₂O solvent system (from 0% MeOH to 100% MeOH) to give 6 fractions. The 60% MeOH fraction (310 mg) was subjected to flash chromatography over silica gel, eluted by a gradient system of CHCl₃-MeOH (100:0-90:10), in 25 min, affording compounds **7** (4.6 mg), **8** (6.8 mg), **9** (18.5 mg) and **11** (6.4 mg). The 20% MeOH fraction (200 mg) was further subjected to HPLC preparative eluted by CH₃CN:H₂O (from 0% to 100% CH₃CN), to give compound **10** (13.8 mg). The first sub-fraction (98.7 mg) was purified by semi-prep HPLC eluted with isocratic system (22% CH₃CN), to yield compound **12** (*t_R* 19.0 min, 2mg).

3.4. Antibacterial assay

An immersion bioautography method was adopted to identify the most promising compounds as previously described by Abedini et al. [13,14]. Gentamicin was used as a positive control. A serial dilution technique using 96-well microliter plates was used to determine the MIC values of the active compounds revealed by bioautography. MIC values were determined as the lowest concentrations of samples having an inhibitory effect on bacteria growth. This test was performed in duplicates. Gentamicin and vancomycin were used as positive controls.

2.5. DPPH radical scavenging activity

The scavenging activity of isolated compounds against DPPH was investigated by spectrophotometric methodology [15]. Ascorbic acid was used as positive control.

3.6. Tyrosinase enzyme assay

Tyrosinase activity inhibition was determined spectrophotometrically according to the method previously described by Tadrent et al. [16]. Kojic acid was used as positive control agent.

3.7. Cytotoxicity assay

Compounds **1-12** were tested for their cytotoxicity against promyelocytic leukemia (HL60), human erythromyeloblastoid leukemia (K562) and fibrosarcoma HT1080 cells by mean of the MTT method as previously described by Gossan et al. [17].

3.8. *3 β -acetoxy-olean-18-ene-2 α -ol (compound 1)*: $[\alpha]_D^{20} - 3$ (c 0.1, CHCl₃); ¹H NMR (500 MHz, CDCl₃): δ_H 0.98 (m, H-1), 2.23 (dd, $J = 12.3, 4.2$ Hz, H-2a), 3.84 (td, $J = 12.3, 4.1$ Hz, H-2b), 4.52 (d, $J = 10.0$ Hz, H-3), 0.94 (m, H-5), 1.43, 1.53 (each m, H₂-6), 1.37, 1.52 (each m, H₂-7), 1.36 (m, H-9), 1.37, 1.60 (each m, H₂-11), 1.20, 1.51 (each m, H₂-12), 2.28 (brd, $J = 11.4$ Hz, H-13), 1.21 (m, H-15a), 1.82 (td, $J = 13.6, 4.8$ Hz, H-15b), 1.42, 1.46 (each m, H₂-16), 4.89 (brs, H-19), 1.43, 1.47 (each m, H₂-21), 1.34, 1.45 (each m, H₂-22), 0.90 (s,

H₃-23), 0.88 (s, H₃-24), 0.98 (s, H₃-25), 1.09 (s, H₃-26), 0.76 (s, H₃-27), 1.04 (s, H₃-28), 0.96 (s, H₃-29), 0.97 (s, H₃-30), 2.16 (s, H₃-acetyl); ¹³C NMR (125 MHz, CDCl₃): δ_C 48.3 (C-1), 67.9 (C-2), 85.0 (C-3), 39.3 (C-4), 55.5 (C-5), 18.2 (C-6), 34.4 (C-7), 40.8 (C-8), 51.1 (C-9), 38.4 (C-10), 21.3 (C-11), 26.1 (C-12), 38.3 (C-13), 43.4 (C-14), 27.5 (C-15), 37.7 (C-16), 34.3 (C-17), 142.5 (C-18), 129.9 (C-19), 32.4 (C-20), 33.3 (C-21), 37.4 (C-22), 28.4 (C-23), 17.5 (C-24), 17.9 (C-25), 16.1 (C-26), 14.6 (C-27), 25.2 (C-28), 31.3 (C-29), 29.2 (C-30), 172.4 (acetyl-C-1), 21.2 (acetyl-C-2); HRESIMS *m/z*: 507.3820 [M+Na]⁺ (calcd for C₃₂H₅₂O₃Na, 507.3814).

4. Conclusions

The present paper reported the isolation and structural characterization of a new triterpene 3β-acetoxy-olean-18-ene-2α-ol, along with eleven known compounds from the aerial parts of *S. barrelieri*. The antibacterial potential evaluated initially by using TLC bioautography method followed by a serial dilution technique to determine the minimum inhibitory concentration (MIC) showed a high activity for compounds **2**, **10** and **12**. The DPPH scavenging and tyrosinase inhibitory effects of the exudate, the 80% EtOH extract and flavonoids (**7-12**) isolated from the 80% EtOH extract were evaluated. Only the 80% EtOH extract showed moderate antioxidant activity and low tyrosinase inhibitory activity. Flavonoids **10** and **12** exhibited moderate to good DPPH radical scavenging activity. Flavonoids **7-12** did not show significant tyrosinase inhibitory activity. The cytotoxic activity of isolated compounds, evaluated *in vitro* using two tumor cell lines, the promyelocytic leukemia (HL60), the human erythromyeloblastoid leukemia (K562) and fibrosarcoma (HT1080), showed that compounds **1**, **2**, **4** and **5** had a moderate cytotoxic activity. The overall results may support the use of this plant in traditional medicine for the treatment of gastric disorders and microbial infections

Supporting Information

Supplementary materiel relating to the NMR and HR-ESI-MS spectra of new compound **1** and NMR data of **2-12**.

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Supporting Information

HR-ESI-MS spectra and 1D and 2D NMR of **1** and NMR data of **2-12**.

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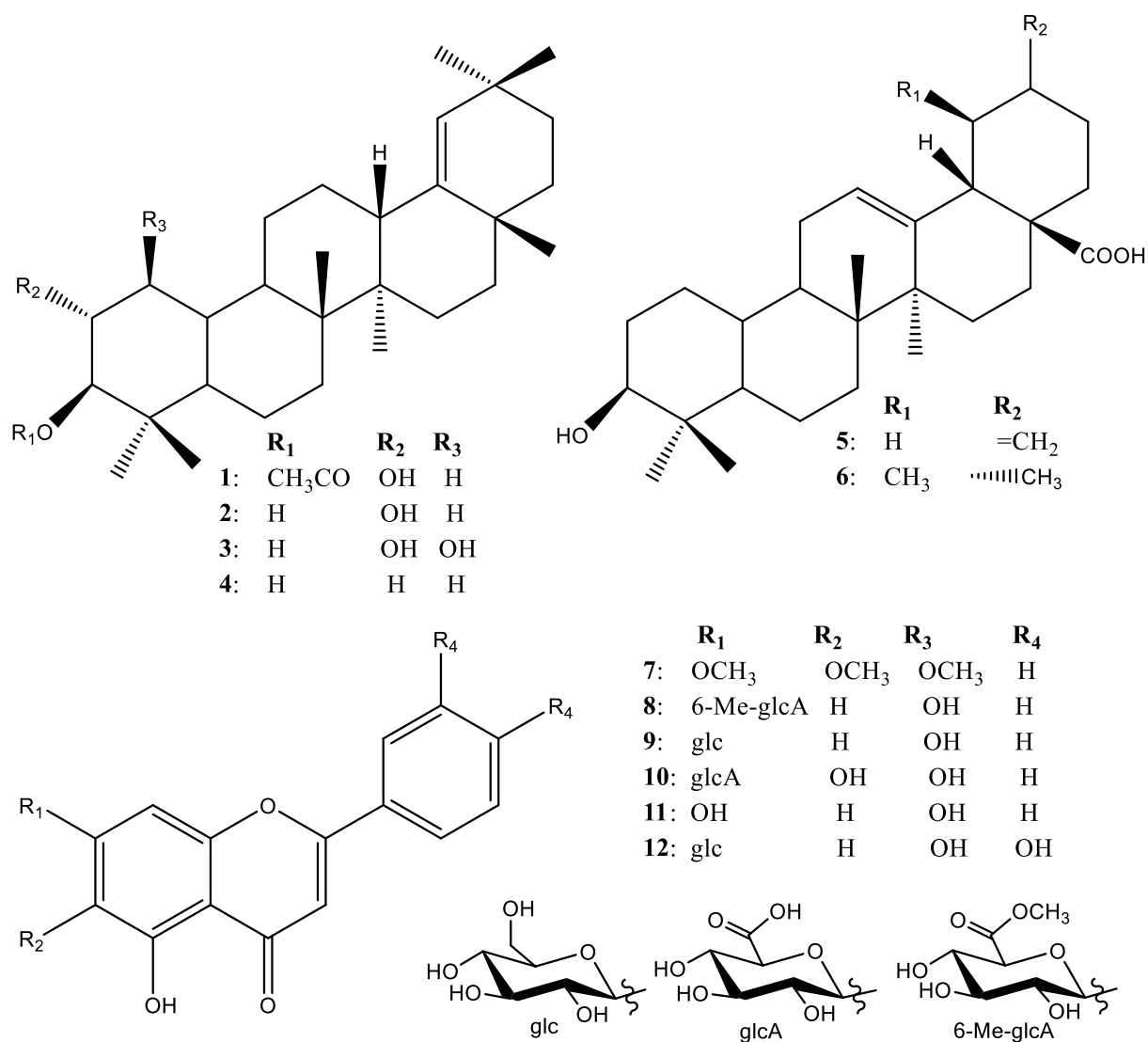


Figure 1. Chemical structures of compounds **1-12** isolated from *Salvia barrelieri*.

Table 1.

Minimum inhibitory concentration (MIC) values in liquid medium and cytotoxic activity against HT1080, HL60 and K562 cell lines of compounds isolated from *S.barrelieri*.

Compounds	Antibacterial activity					Cytotoxic activity	
	<i>E. faecalis</i>	<i>S. aureus</i>	CMI (µg/mL)			IC ₅₀ (µM)	
			<i>E. coli</i>	<i>S. epidermis</i>	<i>P. aeruginosa</i>	HL60	K562
1	> 500	> 500	> 500	> 500	> 500	> 100	> 100
2	125	62.5	125	62.5	62.5	54 ± 0.8	85 ± 1.6
3	> 500	> 500	> 500	> 500	> 500	> 100	> 100
4	> 500	> 500	> 500	> 500	> 500	42 ± 0.6	82 ± 1.4
5	125	250	250	125	250	52 ± 0.9	82 ± 1.0
6	31.2	125	500	250	125	> 100	> 100
7	> 500	> 500	> 500	> 500	> 500	> 100	> 100
8	500	125	500	500	250	> 100	> 100
9	> 500	> 500	> 500	> 500	> 500	> 100	> 100
10	31.2	125	31.2	31.2	15.1	> 100	> 100
11	> 500	> 500	> 500	> 500	> 500	> 100	> 100
12	31.2	250	31.2	31.2	31.2	> 100	> 100
Gentamicin^a	16	4	4	0.25	8	-	-
Vancomycin^a	> 64	> 64	16	4	> 64	-	-
Camptothecin^a	-	-	-	-	-	0.25±0.03	0.30±0.05

^aused as positive control