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Novel biologically active glycosides from the aerial parts of *Cephalaria gazipashensis*

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Two new triterpene glycosides, 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$4)-β-D-xylopyranosyl-(1$\rightarrow$3)-α-L-rhamnopyranosyl-(1$\rightarrow$3)-α-L-arabinopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl-(1$\rightarrow$6)-β-D-glucopyranosyl ester (gazipashoside A, **1**) and 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$4)-β-D-xylopyranosyl-(1$\rightarrow$3)-α-L-rhamnopyranosyl-(1$\rightarrow$2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1$\rightarrow$6)-β-D-glucopyranosyl ester (gazipashoside B, **2**), were isolated together with 11 known compounds including 7 triterpene, 1 flavonoid, and 3 iridoidal metabolites from the aerial parts of *Cephalaria gazipashensis* (Dipsacaceae). The structures of all compounds were elucidated by extensive evaluation of the spectroscopic data (1D- and 2D- NMR, HRESI-MS), as well as chemical examinations. The antimicrobial effects of all pure compounds were tested against gram-positive and gram-negative bacteria. The results indicated that the 2 new triterpene glycosides might be acceptable as antimicrobial active agents.

Key Words: Gazipashoside A, gazipashoside B, Dipsacaceae, *Cephalaria gazipashensis*, triterpene glycoside, antimicrobial activity

Introduction

The genus *Cephalaria* (Dipsacaceae) consists of 94 species distributed mainly in the Mediterranean region and the Middle East. In Turkey, there are 40 *Cephalaria* taxa and 24 of them are endemic.[1–4] *Cephalaria gazipashensis* Sümbül, one of these endemic species, is spread in southwestern Anatolia.[3–5] Many *Cephalaria* species have been used in traditional medicine for their antimicrobial, antifungal,[6,7] antioxidant,[8] and cytotoxic[9,10] properties. The roots of one of these active species, *C. gigantea*, are well known in traditional medicine remedies as a sedative and anti-inflammatory.[10] The last comprehensive activity study on this genus was about

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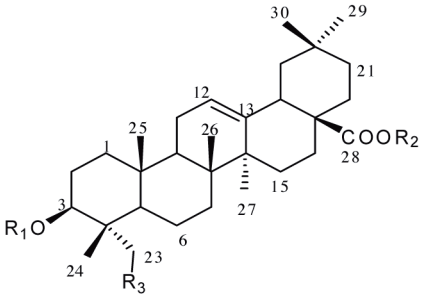
DNA damaging, mutagenic, and clastogenic activities of a secoiridoid glycoside isolated from *C. kotschyi*.[11] *Cephalaria* species in the family Dipsacaceae are characterized by an extremely rich chemical diversity. In particular, triterpene glycosides are widely distributed and they show remarkable structural varieties, as well as notable biological activities. It is known that in plants the triterpene glycosides play an important role as preformed chemical barriers in defense mechanisms against insects and fungi.[12] Several triterpenoid saponins based on an oleanane and hederagenin type aglycon were isolated from some *Cephalaria* species[6,7,9,13–15] in addition to the flavonoid,[8,16] iridoid, and lignan[17–19] glycosides.

In this framework, a study of the bioactive constituents of *C. gazipashensis* was undertaken for the first time and resulted in the isolation of 2 new triterpene glycosides, named gazipashosides A-B (**1** and **2**) (Figure 1). The structures of the new triterpene glycosides were determined as 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-arabinopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (**1**) and 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (**2**), along with 7 known triterpenic compounds: 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (decaisoside E, **3**),[20] 3-*O*-α-L-arabinopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl-(1→6)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl ester (scoposide A, **4**),[6] 3-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (scoposide B, **5**),[6] 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-arabinopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl ester (scoposide F, **6**),[21] 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl olean-12-ene 28-*O*-β-D-galactopyranosyl-(1→6)-β-D-glucopyranosyl ester (scoposide G, **7**),[21] and 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-xylopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl ester. (lycicoside II, **8**).[14] One triterpenic metabolite oleanolic acid (**9**)[22] (Figure 1); 1 flavone glycoside, quercetin 3-*O*-rutinoside (**10**);[23] and 3 iridoid compounds, iridoid aglycon (**11**),[24] loganin (**12**),[18] and laciniatoside I (**13**)[24] (Figure 2) were identified by spectral methods including 1D-, 2D-NMR, and HRESI-MS techniques and some chemical examinations. These compounds were tested for their antimicrobial effects against gram-positive and gram-negative bacteria. The results indicated that 2 new triterpene glycosides (**1** and **2**) can be considered appropriate antimicrobial agents.

Experimental

General: In the present study, several chromatographic techniques, suitable adsorbents, and solvents were used for different purposes in the laboratory. For thin-layer chromatography, silica gel F_{254} (Merck 5554) and RP-18 F_{254S} (Merck 5560) precoated plates were used. Silica gel 60 (0.063-0.200 mm, Merck 7734), Sephadex-LH 20 (25-100 μm, Sigma-Aldrich), and LiChroprep RP-18 (25-40 μm, Merck 9303) were used for column chromatography (CC), vacuum liquid chromatography (VLC), and medium pressure liquid chromatography (MPLC). MPLC was carried out on a Büchi C-605 MPLC instrument and various suitable Büchi glass columns (26-49 mm × 230 mm). In spectroscopic studies, IR spectra were recorded on an ATI Mattson 1000 Genesis Series FT-IR instrument. Samples were measured on KBr disks. Specific optical rotations of samples were run



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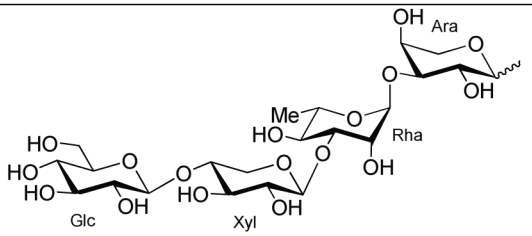
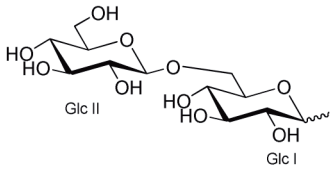
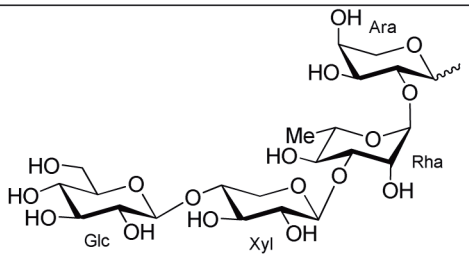
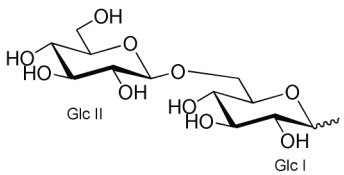
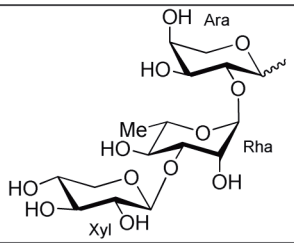
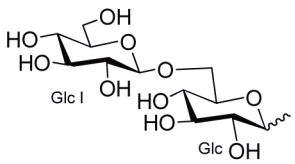
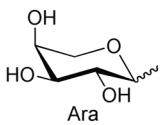
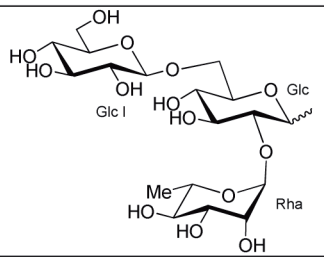
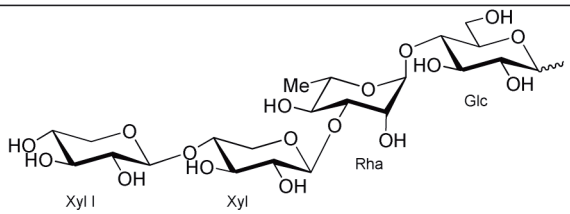
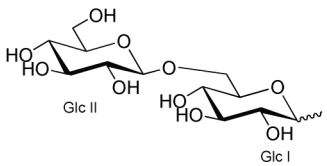
1			H-
2			HO-
3			HO-
4			H-
5			H-

Figure 1. Structures of triterpenic compounds (**1-9**).

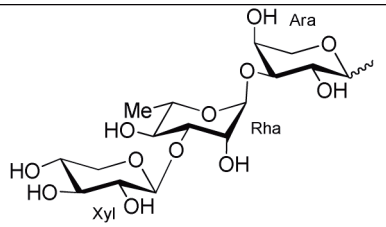
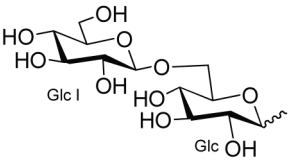
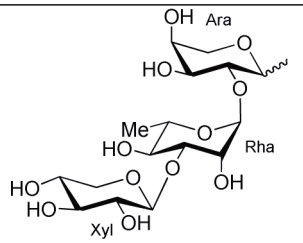
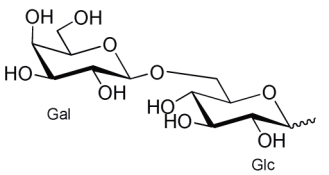
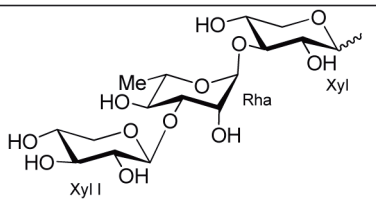
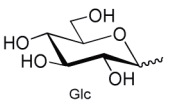
6			H-
7			H-
8			H-
9	H-	H-	H-

Figure 1. Continued.

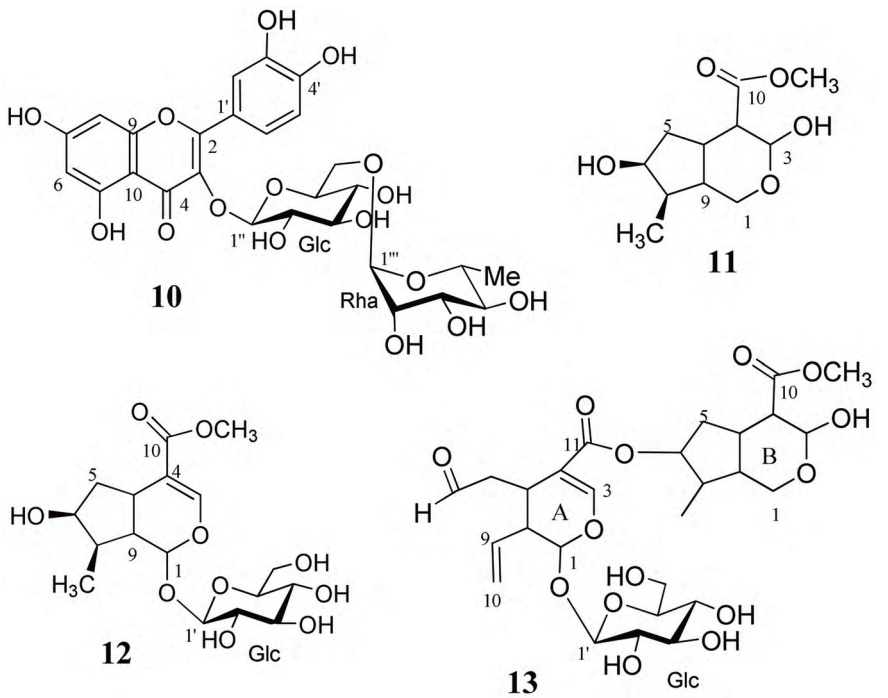


Figure 2. Structures of flavonoid and iridoid compounds (**10-13**).

using a Rudolph Research Analytical Autopol I automatic polarimeter fitted with a sodium lamp and 1 mL or 5 mL cells. 1D- and 2D-NMR measurements were performed on a Varian AS 400 *MHz* in DMSO-d_6. TMS was used as an internal standard and J values are reported in *Hz.* HRESI-MS analyses were carried out using a Bruker LC micro-Q-TOF mass spectrometer.

Plant material: *Cephalaria gazipashensis* Sümbül was collected from Maha, Gazipaşa, Antalya Province, at about 1500 m altitude, in July 2007. It was botanically identified by H. Sümbül and R.S. Göktürk (Department of Biology, Faculty of Arts and Science, Akdeniz University). A voucher specimen (R.S. Göktürk 6099) has been deposited at the Herbarium Research and Application Center of Akdeniz University.

Extraction and isolation: The fresh plant material was dried at room temperature in a shady place for a week. The air-dried and powdered aerial parts of the plant (1.4 kg) were extracted with MeOH (3 × 2L) overnight at room temperature. After filtration the combined extracts were concentrated under vacuum at ∼40 °C to yield crude residue. This residue was extracted with a n-BuOH:H_2O (1:1, 3 × 200 mL) solvent system. After the separation of n-BuOH and H_2O phases, the n-BuOH fraction was defatted with hexane (10 × 50 mL) to remove the chlorophyll and oily substances. Lastly, the re-purified n-BuOH portion, which is the most biologically active fraction, was investigated. The saponin-rich n-BuOH portion (37 g) was subjected to RP-VLC on MPLC with a gradient MeOH:H_2O solvent system (0:100→100:0%) to give 11 fractions. Fractions 8, 9, and 10 (30%, 20%, and 10% H_2O) were combined and chromatographed on a silica gel column with the solvent systems $CHCl_3$:MeOH:H_2O (90:10:1, 1000 mL; 80:20:2, 500 mL; 70:30:3, 500 mL; 61:32:7, 300 mL) to afford 32 fractions. Two new saponins, compounds **1** (333 mg) and **2** (196 mg), were obtained from fractions 25 and 28, respectively. Fractions 21, 12, 23, 18, 15, 7, and 1 gave the compounds **3** (37.3 mg), **4** (101.1 mg), **5** (329 mg), **6** (390 mg), **7** (352 mg), **8** (75.2 mg), and an aglycon **9** (34 mg), respectively. Another isolation process was carried out over fraction 3 of RP-VLC to yield compound **10** (96 mg) by MeOH:acetone (1:3) precipitation. The combined fractions 1 and 2 of VLC were subjected to CC using the solvent system $CHCl_3$:MeOH: H_2O (90:10:1), giving the compounds **11** (155.5 mg) and **12** (96 mg). Lastly, compound **13** (152 mg) was obtained from the combination of fractions 4 and 5 via successive applications of MPLC (silica gel, 36 mm × 460 mm Büchi glass column, solvent systems $CHCl_3$:MeOH:H_2O 90:10:1, 600 mL; 80:20:2, 300 mL; 70:30:3, 400 mL; 61:32:7, 300 mL, flow rate: 30 mL/min, max. pressure: 30 bar) and CC (silica gel, solvent system $CHCl_3$:MeOH:H_2O, 90:10:1).

Gazipashoside A (1): A white, amorphous powder (333.0 mg); $[\alpha]_D^{25}$–63.2 (c1.5, MeOH); IR (KBr): $\nu_{max=}$ 3366, 2942, 1742, 1641, 1387, 1260, 1057 cm^{-1}; ^{13}C-NMR (DMSO-d_6, 100 *MHz*) and ^{1}H-NMR (DMSO-d_6, 400 *MHz*) see Tables 1 and 2, respectively; negative-ion HRESI-MS m/z: 1351.6537 $[M\text{-}H]^-$ (calcd for $C_{64}H_{103}O_{30}$, 1351.6540).

Gazipashoside B (2): A white, amorphous powder (196.0 mg); $[\alpha]_D^{25}$–8.4 (c1.1, MeOH); IR (KBr): $\nu_{max=}$ 3365, 2942, 1744, 1645, 1388, 1261, 1054 cm^{-1}; ^{13}C-NMR (DMSO-d_6, 100 *MHz*) and ^{1}H-NMR (DMSO-d_6, 400 *MHz*) see Tables 1 and 2, respectively; negative-ion HRESI-MS m/z: 1367.6489 $[M\text{-}H]^-$ (calcd for $C_{64}H_{103}O_{31}$, 1367.6483).

Acidic hydrolysis: Acidic hydrolysis was carried out for determination of the carbohydrate units of the glycosides. The sugar analysis of the glycosides was performed by micro-hydrolysis technique and GC-MS.[6,25] In the micro-hydrolysis technique, pure compounds were applied on a TLC layer (silica gel HF 254) and treated with concentrated HCl vapor in a closed vessel and saturated with the acidic vapor for 40 min at 60

°C. After the vessel was cooled and the excess HCl was removed from the plate, the standard monosaccharides were applied on a TLC layer. TLC was eluted using a $CHCl_3$:MeOH:H_2O:*g*AcOH/16:9:2:2 solvent system. For detecting the compounds, it was sprayed with α-naphthol-H_2SO_4 (2%) solution and heated at about 120 °C. While hexoses gave purple spots, 6-deoxy sugars gave orange spots and pentoses gave blue spots on TLC plates.[25] For GC-MS analysis, each compound (5 mg) was hydrolyzed with 1 N HCl (2 mL) for 6 h at 90 °C. After extraction with $CHCl_3$ (3 × 5 mL), the aqueous layer was evaporated to dryness and the residue of the monosaccharides was dissolved in anhydrous pyridine (1 mL), and then 1 mL of HMDS-TMCS (hexamethyldisilazane-trimethylchlorosilane, 1:1) was added, and the mixture was stirred at 70 °C for 1 h. The mixture was concentrated under N_2 stream and solved with *n*-hexane (1 μL), and analyzed by GC-MS.[6] L-Arabinose, L-rhamnose, D-xylose, and D-glucose were detected by co-injection of the hydrolysate with standard silylated sugars. The exact determination of the L-arabinose, L-rhamnose, D-xylose, and D-glucose moieties was confirmed giving the peaks at 14.73, 15.16, 18.26, and 30.64 min for **1** and the peaks at 14.75, 15.15, 18.28, and 30.67 min for **2** by GC-MS, respectively.

Alkaline hydrolysis: The esteric linkages of the sugar moieties in bis-desmosidic triterpene glycosides were hydrolyzed by the following method. All glycosides (10-20 mg each) were refluxed with 5% KOH in water solution (pH 12-13) at 80 °C for 1 h. The reaction mixtures were neutralized with 5% HCl in water solution and then concentrated to dryness.[6] The residues were extracted with *n*-BuOH:H_2O (1:1) and the organic layers were analyzed by ^{1}H-NMR and HRESI-MS spectroscopy to give the pure prosapogenins **1a** and **2a**.

Prosapogenin 1a: White, amorphous powder; 24.7 mg; $[\alpha]_D^{25}$–22.5 (*c* 0.2, MeOH); ^{1}H-NMR (DMSO-d_6, 400 *MHz*) aglycon: δ_H 3.05 (1H, m, H-3), 0.73 (1H, s, H-5), 1.46 (1H, m, H-9), 5.15 (1H, brs, H-12), 0.93 (3H, s, H-23), 0.74 (3H, s, H-24), 0.84 (3H, s, H-25), 0.69 (3H, s, H-26), 1.05 (3H, s, H-27), 0.84 (3H, s, H-29), 0.84 (3H, s, H-30), sugars: δ_H 4.24 (1H, d, J = 7.6 Hz, H-1 of Ara), 5.07 (1H, brs, H-1 of Rha), 4.30 (1H, d, J = 7.2 Hz, H-1 of Xyl), δ_H 4.24 (1H, d, J = 7.6 Hz, H-1 of Glc), named 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-arabinopyranosyl olean-12-ene-28-oic acid (**1a**). Negative-ion HRESI-MS m/z1027.5439 $[M-H]^-$ (calcd for $C_{52}H_{83}O_{20}$, 1027.5478).

Prosapogenin 2a: White, amorphous powder; 17.1 mg; $[\alpha]_D^{25}$–34.6 (*c* 0.1, MeOH); ^{1}H-NMR (DMSO-d_6, 400 *MHz*) aglycon: δ_H 3.41 (1H, m, H-3), 1.12 (1H, s, H-5), 1.46 (1H, m, H-9), 5.15 (1H, brs, H-12), 3.13 (2H, m, H-23), 0.56 (3H, s, H-24), 0.83 (3H, s, H-25), 0.69 (3H, s, H-26), 1.08 (3H, s, H-27), 0.85 (3H, s, H-29), 0.85 (3H, s, H-30), sugars: δ_H 4.23 (1H, d, J = 7.6 Hz, H-1 of Ara), 5.08 (1H, brs, H-1 of Rha), 4.31 (1H, d, J = 7.2 Hz, H-1 of Xyl), δ_H 4.23 (1H, d, J = 7.6 Hz, H-1 of Glc), named 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin (**2a**). Negative-ion HRESI-MS m/z1043.5419 $[M-H]^-$ (calcd for $C_{52}H_{83}O_{21}$, 1043.5427).

Antimicrobial activity: Antibacterial activity tests of all extracts were evaluated using minimum inhibitory concentration (MIC) measurements.[26,27] Four gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella pneumoniae*) and 4 gram-positive (*Staphylococcus aureus, Staphylococcus epidermidis*, *Bacillus cereus*, and *Enterococcus faecalis*) bacterial strains were used in the assays. The bacterial strains were inoculated on Mueller-Hinton broth (Difco) and incubated for 24 h at 37 ± 0.1 °C. The inocula from broth cultures and suspensions were adjusted to 0.5 McFarland standards and diluted 1:100 (v/v) in Mueller-Hinton broth. Dilution series of the extracts were prepared in test tubes and then transferred to the broth in 96-well microtiter plates. Final concentrations were 256 to 0.5 μg/mL in the medium. The last well,

containing 100 μL of nutrient broth without extracts and 10 μL of the inocula on each strip, was used as a negative control. All plates were covered with a sterile plate sealer and incubated at 37 °C for 24 h.

The MIC is defined as the lowest concentration that appeared clear against a black background (no visible growth). Samples from clear wells were sub-cultured by plotting onto Mueller–Hinton agar. Dilutions were prepared from 128 to 0.25 μg/mL concentrations in microtiter plates. Gentamicin (Sigma) was used as the positive control. All of the assays were performed in triplicate.

Results and discussion

Compound **1** gave a pseudo-molecular ion peak at m/z 1351.6537 $[M\text{-}H]^-$ in the negative ion mode HRESI-MS, which indicated that the molecular formula is $C_{64}H_{103}O_{30}$ (Figure 1). The IR spectrum showed the presence of hydroxyl (3366 cm^{-1}) and ester (1742 cm^{-1}) groups, and the glycosidic linkage (1000-1100 cm^{-1}). The structure of the aglycon of **1** was recognized by ^{1}H- and ^{13}C-NMR analysis (Tables 1 and 2) using the correlations observed in COSY, HMQC, and HMBC spectra to be the oleanane-type triterpene, and is in full agreement with the data given in the literature.[15] The ^{1}H-NMR spectrum showed 7 tertiary methyl groups as singlets [δ_H 1.06, 0.93, 0.84 ($\times$3), 0.74, and 0.66] and a series of overlapped signals suggesting an olean-type triterpene glycoside. A careful investigation of the ^{1}H-NMR spectrum revealed a signal at δ_H 5.14 (1H, brs) typical of H-12 of a Δ^{12} oleanane skeleton, which was also confirmed by the signals at δ_C 122.4 and 144.2, due to C-12 and C-13 in the ^{13}C-NMR spectrum (Table 1). In the same spectrum the presence of a carbonyl carbon at δ_C 176.0 suggested the presence of a glycosylated COOH group at C-28. Acidic hydrolysis of **1** with conc. HCl vapor on TLC and GC-MS techniques gave oleanolic acid, L-arabinose, L-rhamnose, D-glucose, and D-xylose moieties, whereas alkaline hydrolysis of **1** with 5% KOH in water yielded 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$4)-β-D-xylopyranosyl-(1$\rightarrow$3)-α-L-rhamnopyranosyl-(1$\rightarrow$3)-α-L-arabinopyranosyl olean-12-ene-28-oic acid (**1a**). The presence of 6 sugar residues were in good agreement with 6 anomeric proton signals resonating at δ_H 5.20 (d, J = 8.0 *Hz*, Glc I), 5.11 (brs, Rha), 4.31 (d, J = 7.6 *Hz*, Xyl), 4.24 (d, J = 7.2 *Hz*, Ara), 4.24 (d, J = 7.2 *Hz*, Glc), and 4.18 (d, J = 8.0 *Hz*, Glc II). The proton resonances of each sugar could be assigned by 2D COSY experiment (Table 2). The HMQC experiment correlated the proton resonances with the relevant carbon signals at δ_C 106.1, 104.8, 103.7, 102.3, 100.4, and 94.8. The interglycosidic linkages were determined by a HMBC experiment. In particular, long-range correlations were observed between the anomeric protons at δ_H 4.24 (Ara) with the carbon at δ_C 88.5 (C-3 of aglycon) and between the anomeric proton at δ_H 5.20 (Glc I) with the carbon at δ_C 176.0 (C-28 of aglycon), which represents the etheric and esteric glycosidations, respectively, by the HMBC experiment. This evidence clarified that 2 sugar chains were located at C-3 and C-28 positions. In addition, the specific correlations between δ_H 4.18 (H-1 of Glc II) and δ_C 68.7 (C-6 of Glc I), δ_H 4.24 (H-1 of Glc) and δ_C 77.0 (C-4 of Xyl), δ_H 4.31 (H-1 of Xyl) and δ_C 81.7 (C-3 of Rha), and δ_H 5.11 (H-1 of Rha) and δ_C 74.5 (C-3 of Ara) were determined by the linking points of the monosaccharide units to each other, clearly. The correlation between rhamnose and arabinose (Rha 1$\rightarrow$3 Ara) was also confirmed using COSY and HMBC spectra of **1**. On the basis of these data, the structure of gazipashoside A (**1**) has been established as 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$4)-β-D-xylopyranosyl-(1$\rightarrow$3)-α-L-rhamnopyranosyl-(1$\rightarrow$3)-α-L-arabinopyranosyl olean-12-ene 28-*O*-β-D-glucopyranosyl-(1$\rightarrow$6)-β-D-glucopyranosyl ester.

Table 1. ^{13}C-NMR data for compounds **1** and **2**[a–c].

	1	2		1	2
Position	Aglycon		Position	Sugars	
				Ara at C-3	**Ara at C-3**
1	39.0	39.4	1	104.8	103.9
2	26.5	26.0	2	73.7	74.4
3	88.5	80.1	3	74.5	74.0
4	39.4	43.0	4	68.7	68.8
5	55.9	46.9	5	65.3	65.5
6	18.5	17.0		**Rha**	**Rha**
7	32.9	32.5	1	100.4	100.4
8	n.d	39.0	2	70.6	70.6
9	47.8	47.8	3	81.7	81.9
10	37.0	36.6	4	71.5	71.6
11	23.7	23.6	5	68.7	68.6
12	122.4	122.4	6	18.4	18.4
13	144.2	144.2		**Xyl**	**Xyl**
14	42.0	42.0	1	106.1	106.1
15	28.0	28.1	2	74.3	74.3
16	23.2	23.2	3	77.4	77.4
17	46.3	46.7	4	77.0	77.0
18	41.4	41.4	5	64.0	64.1
19	46.6	46.3		**Glc**	**Glc**
20	31.0	31.0	1	102.3	102.4
21	33.9	34.0	2	75.0	75.0
22	32.3	32.3	3	77.3	77.5
23	28.1	62.8	4	73.4	73.4
24	17.0	13.8	5	77.6	77.7
25	15.9	16.3	6	61.8	61.8
27	26.2	26.2		**Glc I at C-28**	**Glc I at C-28**
26	17.4	17.4	1	94.8	94.8
28	176.0	176.0	2	72.9	73.0
29	33.5	33.5	3	77.1	77.0
30	24.1	24.1	4	69.9	69.9
			5	77.3	77.2
			6	68.7	68.5
				Glc II	**Glc II**
			1	103.7	103.7
			2	74.1	74.2
			3	77.1	77.0
			4	70.8	70.8
			5	77.4	77.3
			6	61.7	61.7

[a] ^{13}C-NMR data (δ) were measured in DMSO-d_6 at 100 *MHz* .

[b] The assignments are based on DEPT, COSY, NOESY, HMQC, and HMBC experiments.

[c] nd not determined.

Table 2. ^{1}H-NMR data for compounds **1** and **2**$^{a-c}$.

Position	1	2
3	3.00, m	3.44, m
5	0.73, m	1.18, m
9	1.48, m	1.48, m
12	5.14, brs	5.14, brs
23	0.93, s	3.28, m
24	0.74, s	0.55, s
25	0.84, s	0.84, s
26	0.66, s	0.66, s
27	1.06, s	1.08, s
29	0.84, s	0.85, s
30	0.84, s	0.85, s
	Ara at C-3	**Ara at C-3**
1	4.24, d (7.2)	4.31, d (5.2)
2	3.54, m	3.54, m
3	3.52, m	3.50, m
4	3.58, m	3.56, m
5	3.36, 3.64, m	3.34, 3.66, m
	Rha	**Rha**
1	5.11, brs	5.12, brs
2	3.80, m	3.80, m
3	3.59, m	3.60, m
4	3.39, m	3.38, m
5	3.76, m	3.78, m
6	1.06, d (6.4)	1.07, d (6.4)
	Xyl	**Xyl**
1	4.31, d (7.6)	4.32, d (7.2)
2	3.11, m	3.12, m
3	3.04, m	3.12, m
4	3.50, m	3.50, m
5	3.09, 3.86, m	n.d., 3.88, m
	Glc	**Glc**
1	4.24, d (7.2)	4.25, d (7.6)
2	3.26, m	3.26, m
3	3.15, m	3.12, m
4	3.01, m	3.01, m
5	3.11, m	3.02, m
6	3.42, 3.63, m	3.40, 3.60, m

Table 2. Continued.

Position	1	2
	Glc I at C-28	**Glc I at C-28**
1	5.20, d (8.0)	5.20, d (8.0)
2	3.12, m	3.10, m
3	3.49, m	3.28, m
4	3.20, m	3.18, m
5	3.32, m	3.30, m
6	3.58, 3.88, m	3.56, 3.90, m
	Glc II	**Glc II**
1	4.18, d (8.0)	4.18, d (7.6)
2	2.94, m	2.98, m
3	3.18, m	3.20, m
4	3.04, m	3.03, m
5	3.04, m	3.10, m
6	3.42, 3.63, m	3.46, 3.62, m

[a] ^{1}H-NMR data (δ) were measured in DMSO-d_6 at 400 *MHz*.

[b] Coupling constants (J) in *Hz* are given in parentheses.

[c] nd: not determined.

Compound **2** had a molecular formula $C_{64}H_{103}O_{31}$ established by negative-ion HRESI-MS at m/z: 1367.6489 $[M\text{-}H]^-$ (calcd for, 1367.6483) (Figure 1). The ^{1}H-NMR spectrum (Table 2) revealed signals due to 6 tertiary methyl singlets at δ_H 1.08, 0.85 ($\times 2$), 0.84, 0.66, and 0.55; an olefinic proton at δ_H 5.14 (1H, brs); a hydroxymethyl proton at δ_H 3.44 (2H, m); and 6 anomeric protons at δ_H 5.20 (d, $J = 8.0$ *Hz*, Glc I), 5.12 (brs, Rha), 4.32 (d, $J = 7.2$ *Hz*, Xyl), 4.31 (d, $J = 5.2$ *Hz*, Ara), 4.25 (d, $J = 7.6$ *Hz*, Glc), and 4.18 (d, $J = 7.6$ *Hz*, Glc II). The ^{13}C-NMR spectrum (Table 1) displayed signals due to 6 quaternary carbons at δ_C 33.5, 26.2, 24.1, 17.4, 16.3, and 13.8; an oxygen-bearing methine carbon at δ_C 80.1; a set of olefinic carbons at δ_C 144.2 and 122.4; an ester carbonyl carbon at δ_C 176.0; and 6 anomeric carbons at δ_C 106.1, 103.9, 103.7, 102.4, 100.4, and 94.8. A detailed analysis of these spectral data indicated that **2** was a 3,28-bisdesmoside of hederagenin, having 6 monosaccharide units. The acidic hydrolysis results of **2** afforded L-arabinose, L-rhamnose, D-glucose, and D-xylose using micro-hydrolysis and GC-MS techniques comparing with standard monosaccharide moieties. In the HMBC experiments the anomeric proton signals at δ_H 5.20 (Glc I), 4.18 (Glc II), 4.31 (Ara), 5.12 (Rha), 4.32 (Xyl), and 4.25 (Glc) showed long-range correlations with the carbon signals at δ_C 176.0 (C-28), 68.5 (C-6 of Glc I), 80.1 (C-3 of aglycon), 74.4 (C-2 of Ara), 81.9 (C-3 of Rha), and 77.0 (C-4 of Xyl), respectively. These results confirmed that compound **2** differed from **1** mainly in the linking point of rhamnose to arabinose as Rha 1 $\rightarrow$ 2 Ara instead of Rha 1$\rightarrow$3 Ara using COSY and HMBC spectra. Therefore, the structure of **2** was formulated as 3-*O*-β-D-glucopyranosyl-(1$\rightarrow$4)-β-D-xylopyranosyl-(1$\rightarrow$3)-α-L-rhamnopyranosyl-(1$\rightarrow$2)-α-L-arabinopyranosyl hederagenin 28-*O*-β-D-glucopyranosyl-(1$\rightarrow$6)-β-D-glucopyranosyl ester and was named gazipashoside B.

The antimicrobial effects of the gazipashosides A-B (**1** and **2**) were examined using the MIC method (Table 3). As a result, compound **9** was found to be the most active metabolite against both gram-positive and gram-negative bacteria, while **1** and **2** were moderately active. The most active metabolite, oleanolic acid, showed activity equal to that of gentamicin against *E. faecalis*.

Table 3. The MIC results (μg/mL) of compounds **1-13**.

Microorganisms	Compounds													Genta-
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	micin
S. aureus	64	64	32	128	64	32	128	64	32	32	128	64	64	1.0
S. epidermidis	64	64	64	64	64	32	32	32	16	32	64	32	32	1.0
S. typhimurium	32	64	64	128	32	16	128	16	16	32	128	32	64	1.0
E. coli	32	64	32	8	32	16	16	64	16	32	4	32	16	1.0
B. cereus	64	64	64	16	32	16	16	16	16	32	32	32	16	4.0
K. pneumoniae	64	128	64	64	32	32	32	64	16	32	64	32	32	4.0
E. faecalis	64	128	32	32	64	32	32	16	16	32	32	32	16	16.0
P. aeruginosa	32	64	32	64	32	16	128	64	16	32	128	32	16	2.0

The present investigation of the aerial part of *C. gazipashensis* (Dipsacaeae), from the viewpoint of its triterpene glycoside constituents, resulted in the isolation of a total of 9 triterpenic metabolites (**1-9**) including 2 new ones (Figure 1). The new compounds, **1** and **2**, are triterpene glycosides with 2 different aglycons, oleanane and hederagenin, comprising the same monosaccharide moieties in their structures. Among the 13 compounds, 1 flavone glycoside **(10**) and 3 iridoid compounds (**11-13**) were also structurally identified (Figure 2). Among the isolated compounds, although oleanolic acid showed the highest antimicrobial activity, compounds **1** and **2** were moderately active metabolites. Consequently, these new glycosides can be considered appropriate antimicrobial active agents.

Acknowledgements

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Supporting information

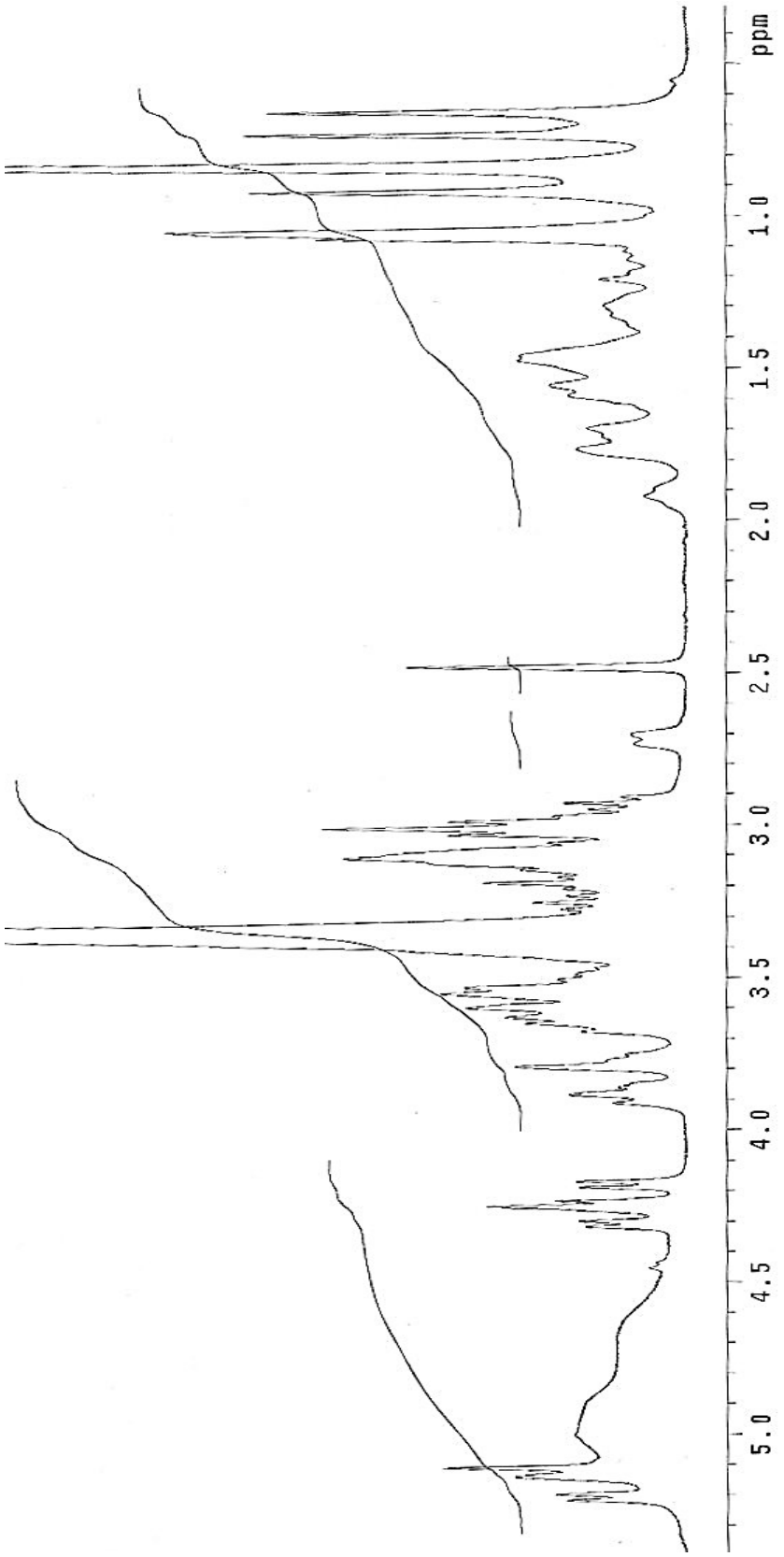

Figure 1. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of Gazipashoside A.

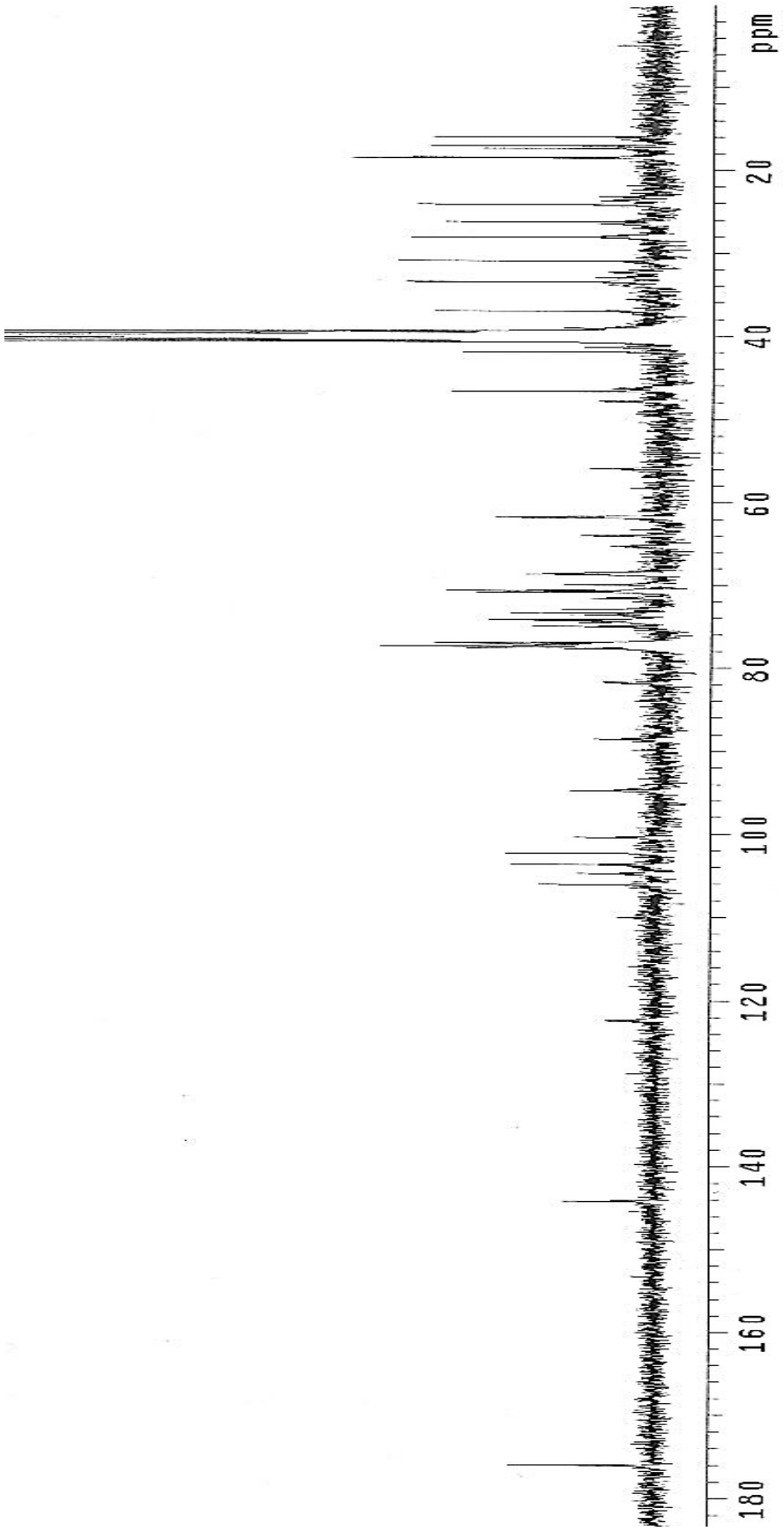

Figure 2. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of Gazipashoside A.

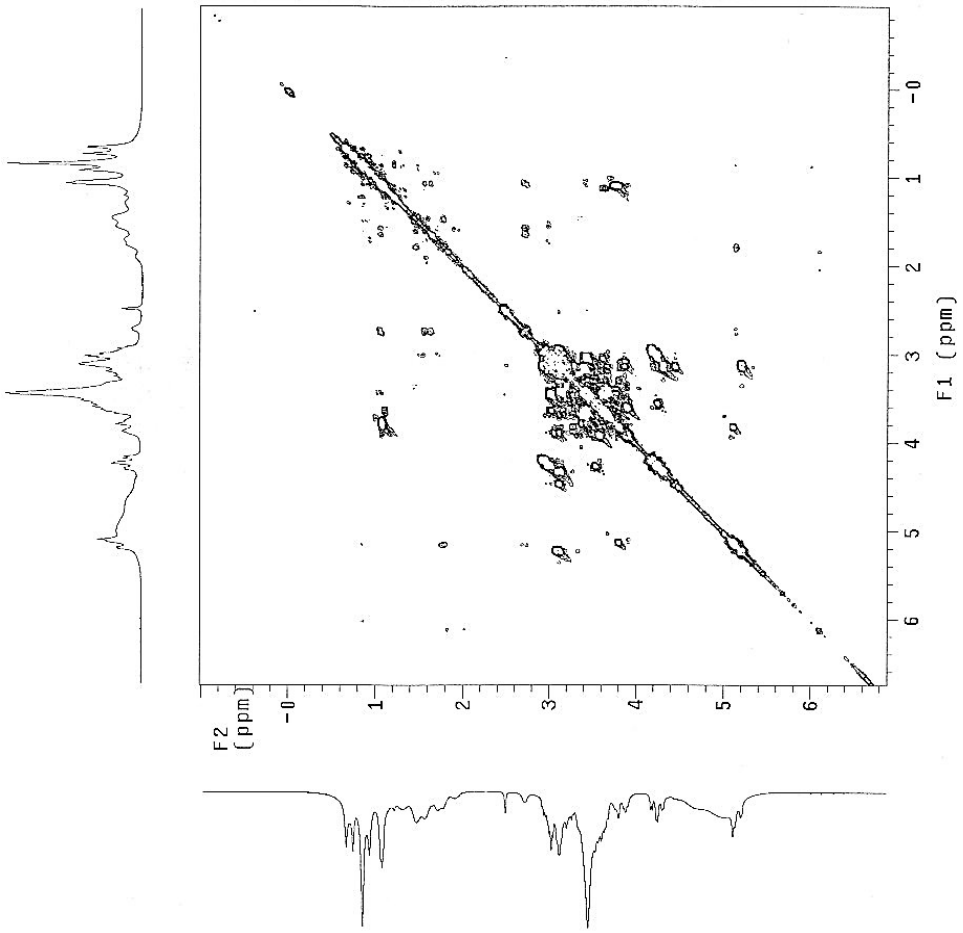

Figure 3. COSY spectrum of Gazipashoside A.

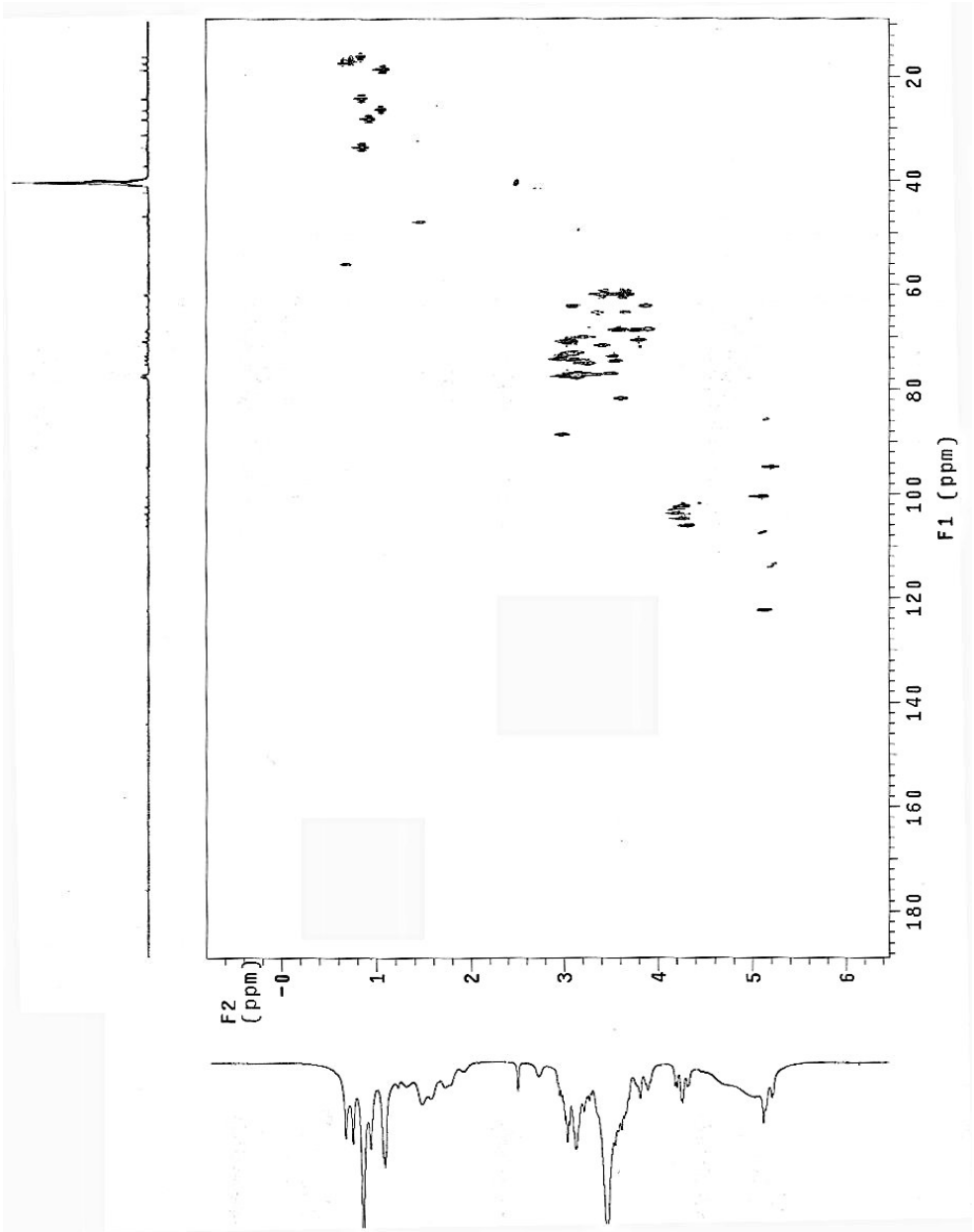

Figure 4. HMQC spectrum of Gazipashoside A.

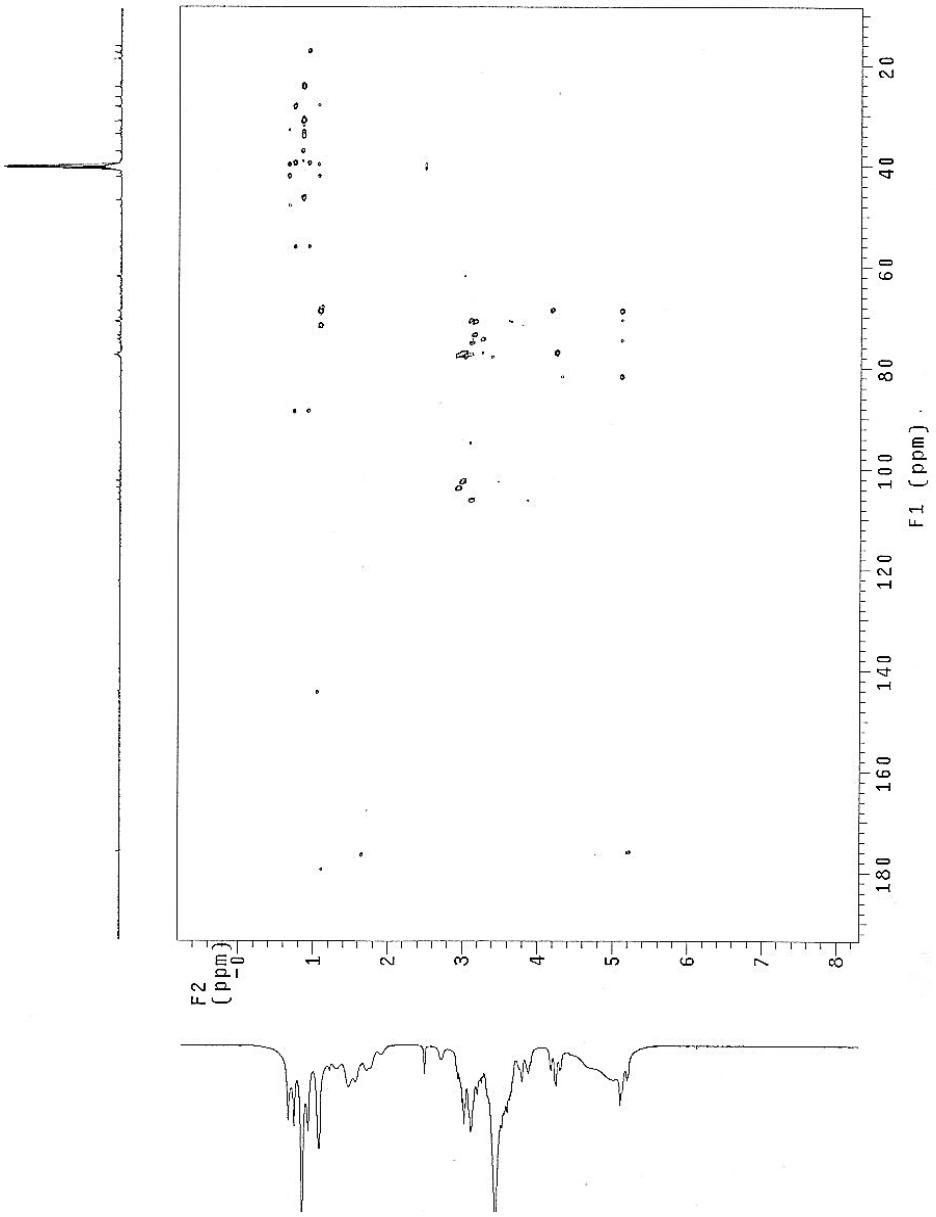

Figure 5. HMBC spectrum of Gazipashoside A.

Acquisition Parameter

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Focus	Active	Set Capillary	4000 V	Set Dry Heater	180 °C
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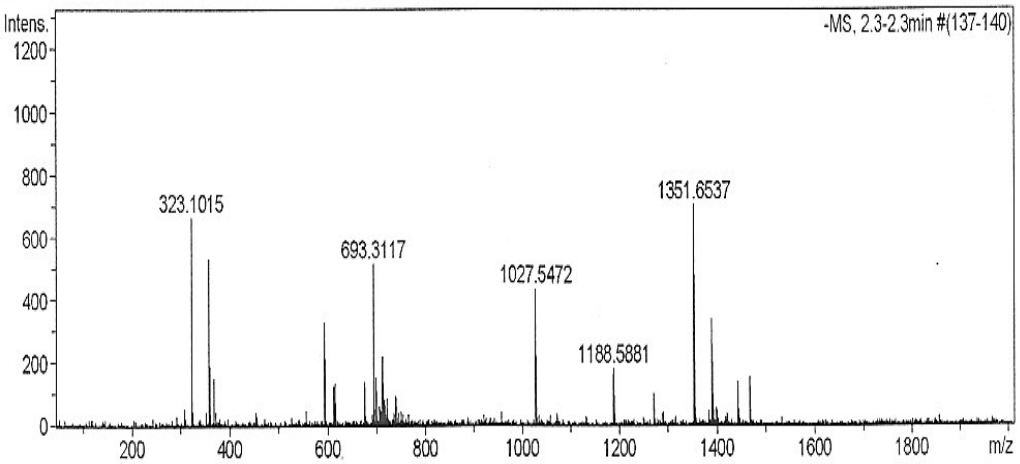
Scan End	2000 m/z	Set Collision Cell RF	600.0 Vpp	Set Divert Valve	Waste

Generate Molecular Formula Parameter

Formula, min.	C53H103O30				
Formula, max.	C64H104O30				
Measured m/z	1351.65	Tolerance	10 ppm	Charge	-1
Check Valence	no	Minimum	0	Maximum	0
Nirogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	yes				



Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 64 H 103 O 30	0.051	1351.6540	0.17	0.41	0.24	13.50	ok	even

Figure 6. HR-ESI-MS spectrum of Gazipashoside A.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Active	Set Capillary	4000 V	Set Dry Heater	180 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	2000 m/z	Set Collision Cell RF	500.0 Vpp	Set Divert Valve	Waste

Generate Molecular Formula Parameter

Formula, min.	C52H83O20				
Formula, max.	C52H83O20				
Measured m/z	1027.54	Tolerance	500 ppm	Charge	0
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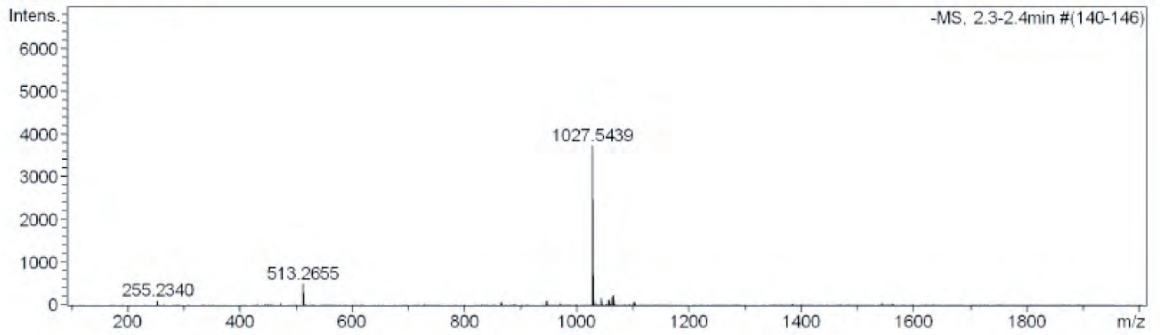
Nirogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	yes				



Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 52 H 83 O 20	0.045	1027.5478	3.80	4.33	3.91	11.50	ok	even

Figure 7. HR-ESI-MS spectrum of prosapogenin (**1a**) of Gazipashoside A.

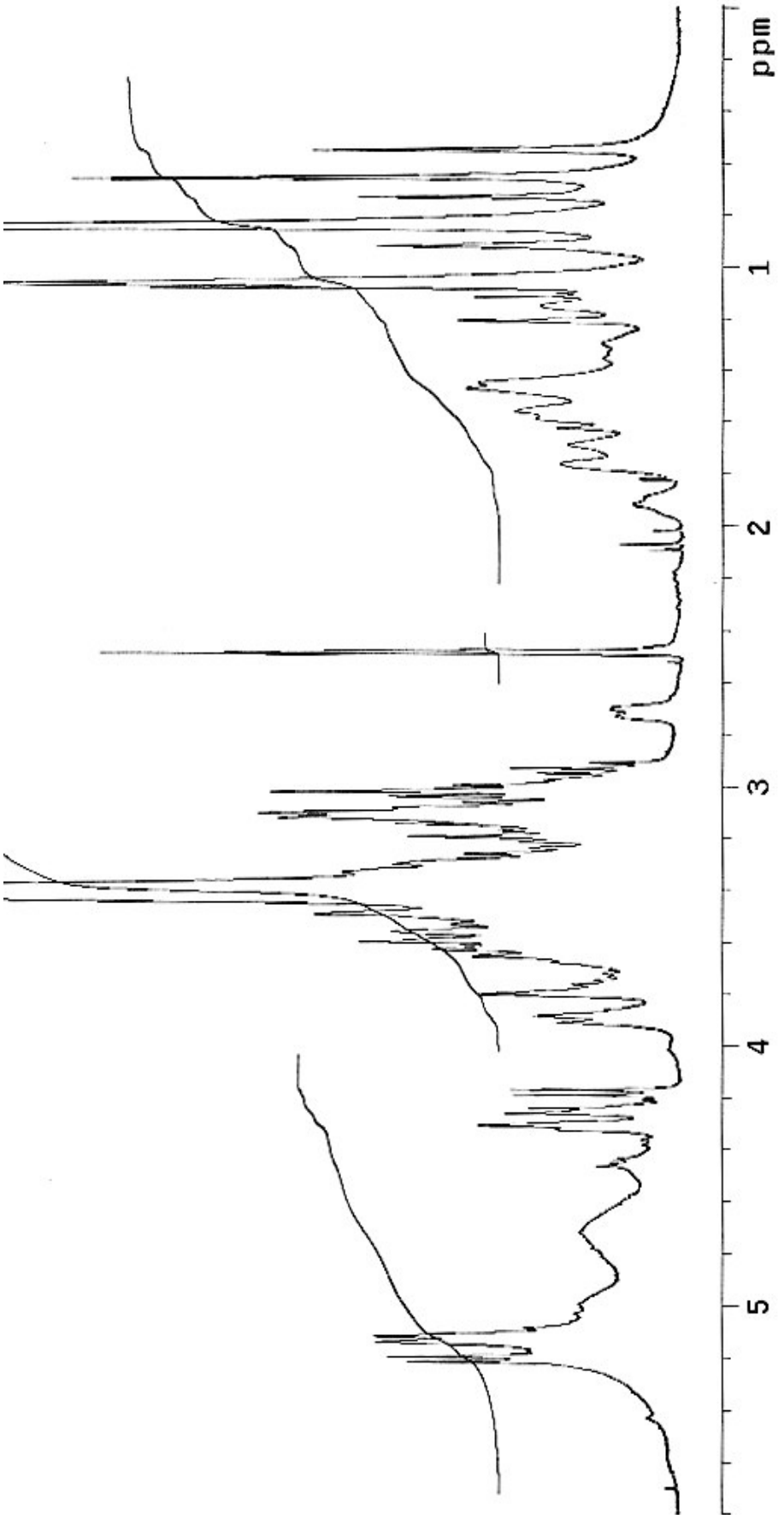


Figure 8. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of Gazipashoside B.

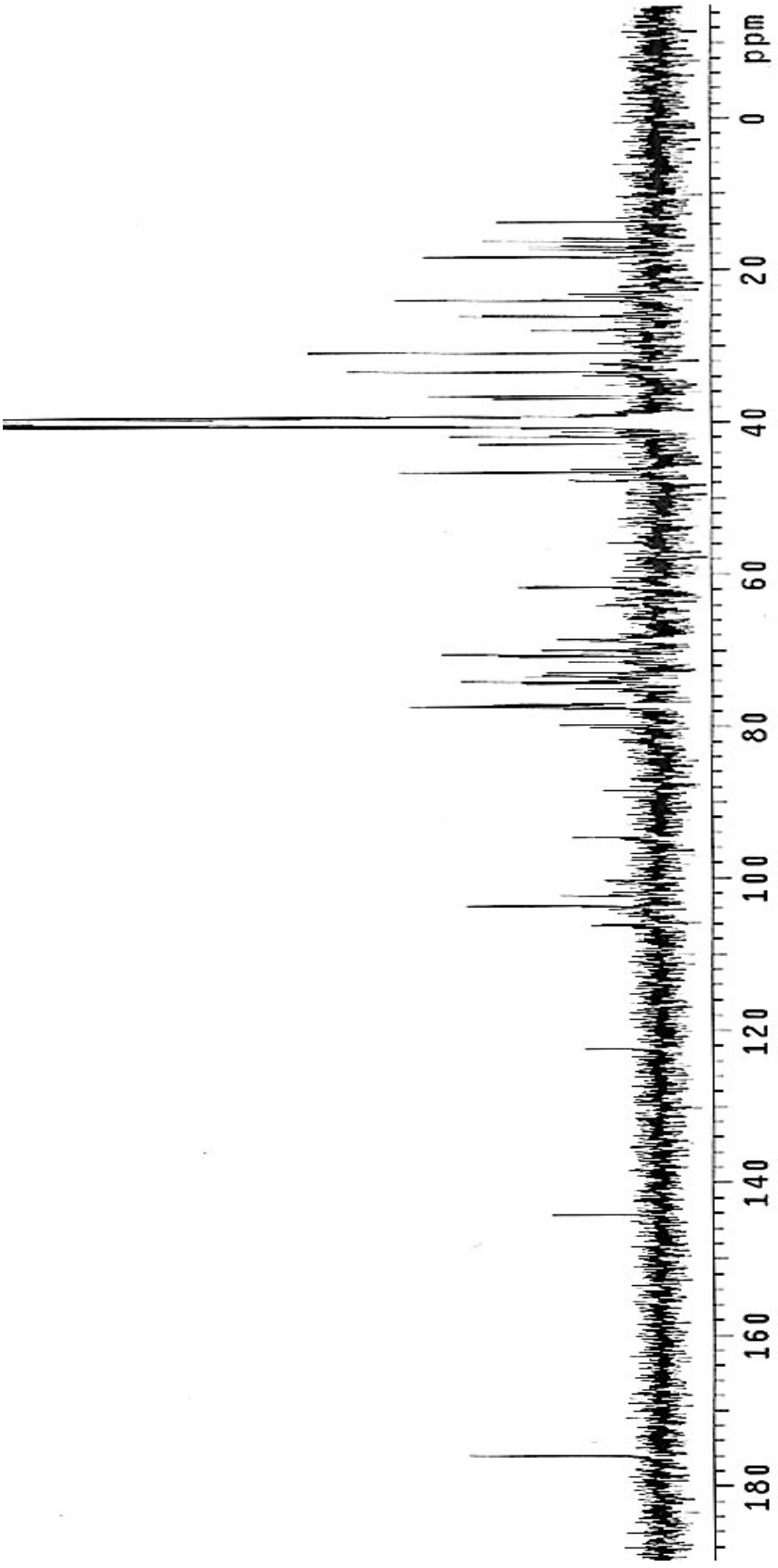


Figure 9. ^{13}C NMR (100 MHz, DMSO-d_6) spectrum of Gazipashoside B.

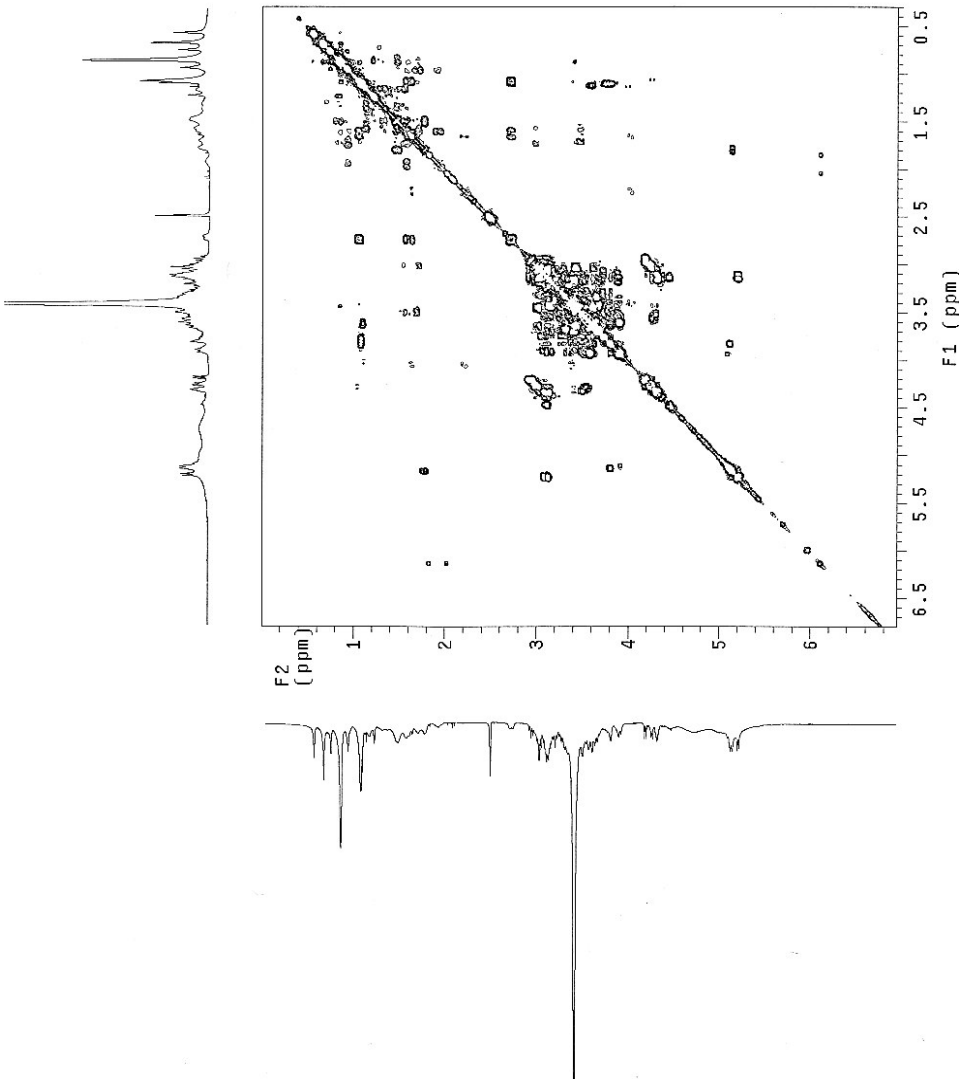


Figure 10. COSY spectrum of Gazipashoside B.

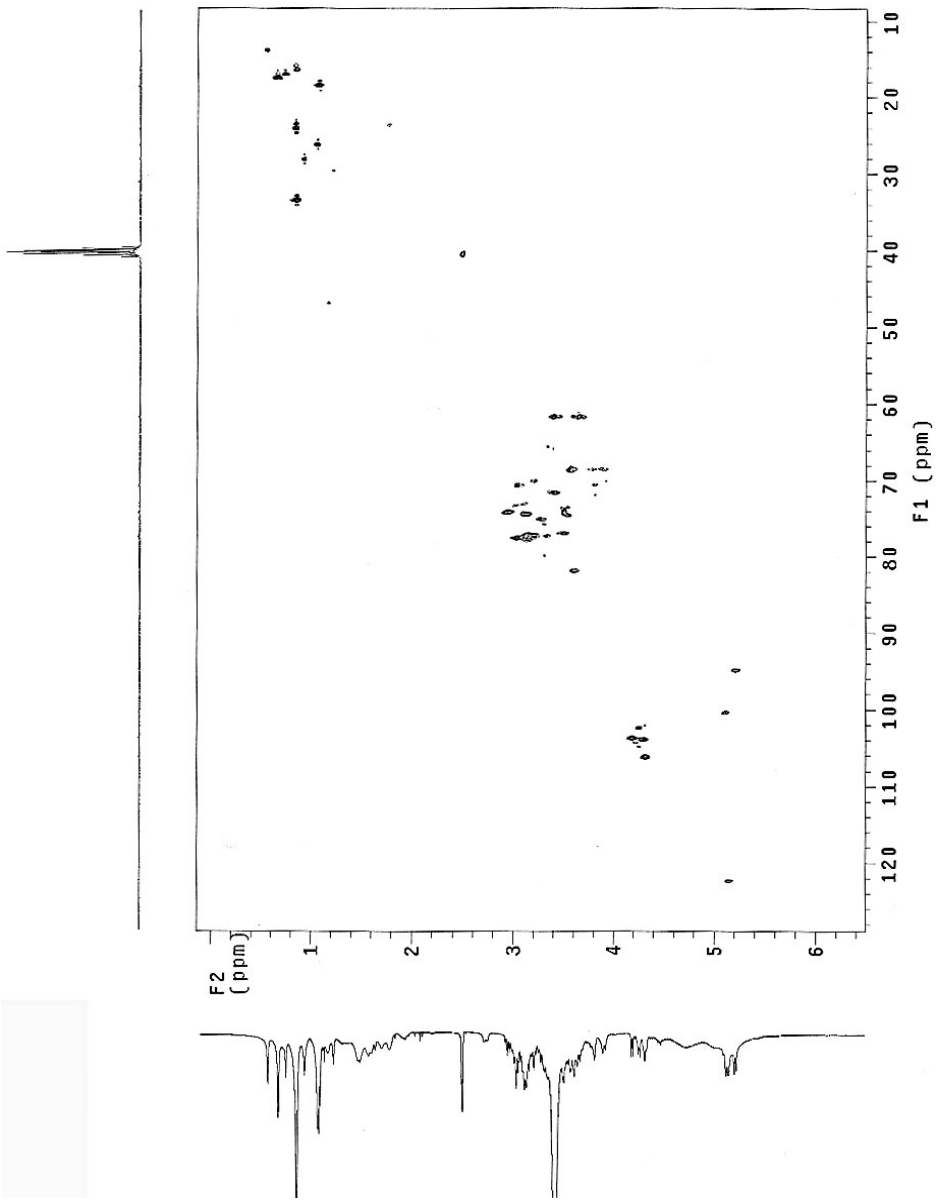


Figure 11. HMQC spectrum of Gazipashoside B.

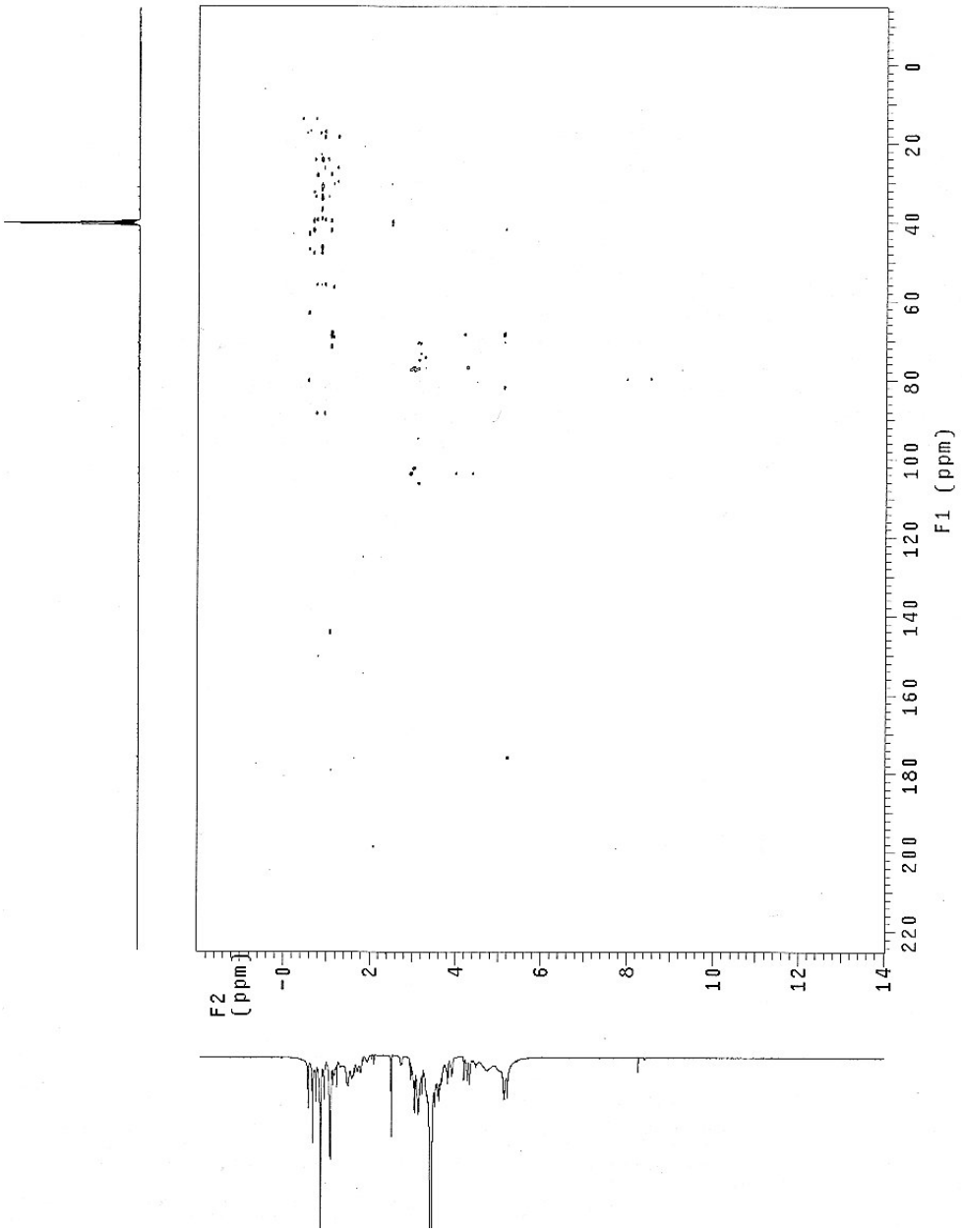


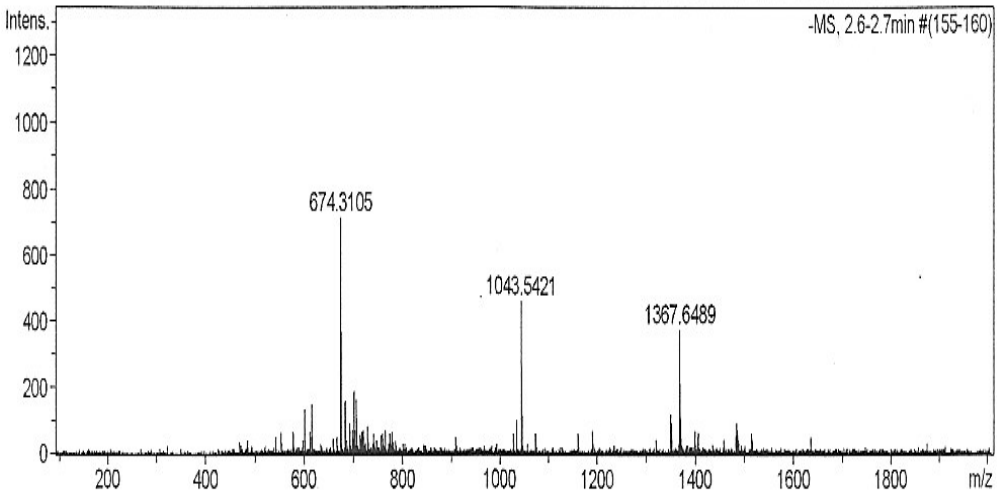
Figure 12. HMBC spectrum of Gazipashoside B.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Active	Set Capillary	4000 V	Set Dry Heater	180 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	1000.0 Vpp	Set Divert Valve	Waste

Generate Molecular Formula Parameter

Formula, min.	C44H103O31				
Formula, max.	C64H103O31				
Measured m/z	1367.65	Tolerance	10 ppm	Charge	0
Check Valence	no	Minimum	0	Maximum	0
Nirogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	yes				



Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 64 H 103 O 31	0.164	1367.6483	-0.43	0.74	-0.59	13.50	ok	even

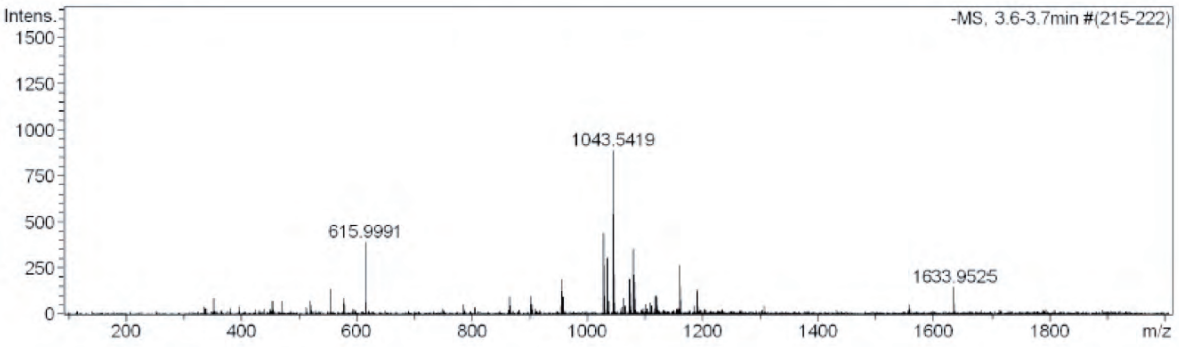
Figure 13. HR-ESI-MS spectrum of Gazipashoside B.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Active	Set Capillary	4000 V	Set Dry Heater	180 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	2000 m/z	Set Collision Cell RF	700.0 Vpp	Set Divert Valve	Waste

Generate Molecular Formula Parameter

Formula, min.	C47H83O21				
Formula, max.	C64H103O31				
Measured m/z	1043.54	Tolerance	1 ppm	Charge	0
Check Valence	no	Minimum	0	Maximum	0
Nirogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	yes				



Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 52 H 83 O 21	0.034	1043.5427	0.80	1.02	0.83	11.50	ok	even

Figure 14. HR-ESI-MS spectrum of prosapogenin (**2a**) of Gazipashoside B.