Protective Effects of Oleanane and Ursane Type Triterpenoids from Origanum majorana Against the Formation of Advanced Glycation Endproducts

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Abstract
Bioassay guided fractionation of a crude methanol extract of leaves of Origanum majorana led the isolation of six new triterpenes (1-6). The structures of the isolates were established on the basis of extensive 1D and 2D NMR spectroscopic data interpretation and by comparison with those previously reported in the literature as 3β,11α,16β,21β-tetrahydroxy-olean-12-en-28-oic acid (1), 2α,3β,21β,22α,29α-pentahydroxy-olean-12-en-28-oic acid (2), 3β,15α,21α-trihydroxyolean-12-en-28-oic acid (3), 2α,28α-diacetoxy,3β,23α-dihydroxy-olean-5,12-dien-28-oic acid (4), 3β,15α,23α,19α-tetrahydroxyursan-12-ene-28-oic acid (5) and 2α,3β-diacetoxy-ursan-12,19-diene (6). Six other known triterpenes were isolated including oleanolic acid (7), 28-norlup-20(29)-ene-3β,17β-diol (8), 3β,6β,7β-trihydroxy-20(29)lupine-ene (9), ursolic acid (10), 3β,15α,dihydroxy-30-norurs-12-ene (11), and lupeol (12). All of the isolates were subjected in vitro bioassays to evaluate their inhibitory activity on the formation of advanced glycation end products (AGEs) including AGEs-BSA, methylglyoxal, Amadorin activity, carboxymethyl-lysine, protein carbonyl content, α-dicarbonyl compounds formation, cellular oxidative stress inhibition and protein structural changes were evaluated by Maldi-TOF-MS. Triterpenes 1-5 displayed inhibitory effects on these specific AGEs, more effectively than the positive control, aminoguanidine. Among these, compounds 2, and 1 exhibited the most potential inhibitory activity against AGEs formation. This activity is attributed in part to carbonyl scavenging capacities. This edible plant may be used for controlling oxidative stress and inhibiting the AGE formation, which are implicated in the pathogenesis of diabetic complications (Image 1).

Keywords: Origanum majorana; Protein glycation; Triterpenes; Ursene; Oleanane

Introduction
Glycation, a nonenzymatic reaction between reducing sugars and lysine residues is an important source of reactive carbonyl species (RCS) that leads to the formation of protein advanced glycation end products (AGEs) such as Nϵ-carboxymethyllysine (CML), methylglyoxal lysine dimers and glyoxal lysine dimers [1]. These adducts are formed in all stages of the glycation process, by Schiff’s bases in early glycation, or degradation of glucose or Amadori products (fructosamine) in the intermediate stages of glycation. Thus, α-dicarbonyl could be considered very important to understand how glucose can form AGEs by the Maillard reaction [2]. Methylglyoxal and glyoxal are reactive dicarbonyl species (RCS) common intermediates in protein damage. In the presence of protein dicarbonyl compounds, reducing sugars and transition metal ions, can auto-oxidize to form superoxide radical that can subsequently converted to hydroxyl radical which is highly toxic. Lipoxidation, AGEs, reactive oxygen species (ROS) generation can provoke tissue damage y activate inflammation [3]. The hyperglycaemia
en diabetic can increase the production of free radicals and reactive oxygen species [4]. Glycation, is increased in hyperglycemic leading to an acceleration of AGEs [5]. Increased oxidative stress and accumulation of AGEs can induce cellular changes producing diseases, such as diabetic, atherosclerosis, retinopathy, neuropathy and nephropathy [6].

**Origanum majorana** L., is a perennial herb of the mint family (Lamiaceae or Labiatae). Marjoram is used traditionally, as a folk remedy against indigestion, asthma, rheumatism, and headache. Marjoram is used as a spice and its flavour is highly search to consumers worldwide. The spice is valued not only for its flavour but also for its antimicrobial and antioxidant activities [7-9]. Because marjoram has been known to possess medicinal effects, it has been used in pharmaceuticals and the industries of cosmetics [10]. In the present study isolation, structures and anti-AGEs activity of derivatives triterpenes of the oleanane and ursane from the leaves of *Origanum majorana* are described with some comments on the structural requirements for their activity.

**Experimental Section**

**General experimental procedures**

IR spectra were obtained on a Perkin-Elmer 1720 FTIR. A Bruker DRX-300 NMR spectrometer, operating at 599.19 MHz for 1H and 150.86 MHz for 13C, using the UXNMR software package, was used for NMR experiments; chemical shifts are expressed in ppm using TMS as an internal standard. DEPT 13C, ID TOCSY, IH-1H DQF-COSY, and HMBC/NMR experiments were carried out using the conventional pulse sequences as described in the literature [11]. HREIMS were measured on a JEOL JX 110 mass spectrometer (JEOL, Tokyo, Japan). Precoated TLC silica gel 60 F254 aluminium sheets from Sigma-Aldrich (St. Louis, USA) were used. Column chromatography was carried out on Silica gel 60 (230-400 mesh, Merck Co. New Jersey, USA); solvents used as eluents were from Fermont (California, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, USA).

**Plant material**

Fresh leaves of *Origanum majorana* (Lamiaceae) were collected in the county of Amecameca de Juarez (Mexico State). They were identified by the Herbarium of the Metropolitan Autonomous University-Xochimilco. A representative specimen was kept (No. 7419) for further reference.

**Isolated of the active constituents of *Origanum majorana***: Air-dried leaves of *Origanum majorana* (9 kg) were ground and extracted successively three times with methanol under reflux (3 h). The filtered samples were combined, and the solvent was evaporated in vacuum to yield the crude residue (874 g). The methanol extract was initially separated by chromatography over a silica gel column (8 x 8 cm, 500 g) and eluted with chloroform. Each fraction (75 ml) was monitored by (thin layer chromatography) TLC; fractions with similar TLC patterns were combined to yield four major fractions (F1–F9). The fractions rich in triterpenes were identified by TLC using the Liebermann-Burchard spray reagent. Each fraction was monitored for its glycation effect in AGEs-BSA formations. Fractions F2, F3 and F5 were rechromatographed over a silica gel column, (2 kg each), fraction F3 was fractionated using as eluent n-hexane-chloroform-ethyl ether 0.5:3:5.0:5.5, to produce six subfractions (F2-1 to F2-6). However, in the F3 fraction use n-hexane-ethyl ether 1:2 to collect nine subfractions (F3-1 to F3-9). F5 was further purified eluted with n-hexane-ethyl acetate 3:1 to yield five subfractions (F5-1 to F5-5). Fraction F2-4 was then loaded onto a Sephadex LH-20 column eluted with CHCl3, MeOH (2:3); the eluate was then loaded onto a silica gel preparative chromatographic plate and eluted with a gradient system of chloroform-methanol (70:30-90:100). These procedures yielded pure 1 (40 mg), 7 (45 mg) and 3 (27 mg). The F3-6 mixture was further purified using CHCl3-acetone (5:1) yielded compounds 6 (38 mg), 9 (20 mg) and 4 (44 mg). Compounds 8 (15 mg), 10 (10 mg) 2 (35 mg) and 12 (28 mg) were purified from fraction F5-5 using a Sephadex LH-20 column chromatography (2.5 x 40 cm, 15 g) using chloroform-methanol gradient system (70:30-90:100). F2-5 yielded 10 (15 mg), 11 (9 mg) and 5 (30 mg) using identical chromatographic conditions. All compounds identified by NMR spectra and comparison of their physical and spectroscopic data to those reported in literature.

3β,11α,16β,21β-tetrahydroxy-olean-12-en-28-oic acid (1): Amorphous powder, IR (KBr) νmax (cm\(^{-1}\)) : 3491 (OH), 1645 (C=C), 1710 (C=O), 1462, 1371, 1250, 1085, 805; HRMS m/z 504.3492 (M\(^+\)) calcd for C30H40O5, 504.3451; 1H- and 13C NMR (CDCl3) see Table 1.

2α,3β,21α,29α-pentahydroxy-olean-12-en-28-oic acid (2): Amorphous powder, substance, IR (KBr) νmax (cm\(^{-1}\)): 3456 (OH), 1632 (C=C), 1732 (C=O), 1463, 1134, 1014; HRMS m/z 520.3472 (M\(^+\)) calcd for C29H40O5, 520.3440; 1H- and 13C NMR (CDCl3) see Table 1.

3β,15α,21α-trihydroxyolean-12-en-28-oic acid (3): Amorphous powder, IR (KBr) νmax (cm\(^{-1}\)): 3457 (OH), 1732 (C=O), 1640 (C=C) 1380; HRMS m/z (M\(^+\)) 488.3541 calcd for C28H36O5, 488.3502; 1H- and 13C NMR (CDCl3) see Table 1.

2α,29α-diacetoxy-3β,23α-dihydroxy-olean-5,12-dien-28-oic acid (4): Amorphous powder, IR (KBr) νmax (cm\(^{-1}\)): 3461 (OH), 1735 (C=O), 1643 (C=C); HRMS m/z 572.3336 (M\(^+\)) calcd for C32H38O9, 572.3349; 1H- and 13C NMR (CDCl3) see Table 1.

3β,15α,23α,19α-tetrahydroxyursan-12-ene-28-oic acid (5): Amorphous powder, IR (KBr) νmax (cm\(^{-1}\)): 3405 (OH), 1732 (C=O), 1668 (C=C); HRMS m/z 504.3430 (M\(^+\)) calcd for C29H38O5, 504.3451; 1H- and 13C NMR (CDCl3) see Table 2.

2α,3β-diacetoxy-ursan-12,19-diene (6): Amorphous powder, IR (KBr) νmax (cm\(^{-1}\)): 1732 (C=O), 1668 (C=C); HRMS m/z 524.3835 (M\(^+\)) calcd for C30H36O9, 524.3866; 1H- and 13C NMR (CDCl3) see Table 2.

**In vitro glycation of proteins**

**Determination of glycation product formations by fluorescence spectroscopy**: According to the method of Vinson and Howard [12] the reaction mixture, 10 mg/ml of bovine serum albumin (BSA; Sigma, St Louis, MO, USA) in 50 mM phosphate buffer (PH 7.4) with 0.02% sodium azide to prevent bacterial growth, was added to 0.2 M of glucose. The reaction mixture was then mixed with compounds or aminoguanidine (Sigma, St. Louis, MO, USA). After incubating at 37°C for 15 day the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350 nm, Em: 450 nm).

**BSA-methylglyoxal assay**

The assay evaluates the middle stage of protein glycation [13]. BSA and methylglyoxal were dissolved in phosphate buffer (100 mM, pH 7.4) to a concentration of 20 mg/ml and 60 mM, respectively. Compounds were dissolved in the same phosphate buffer. 1 ml of the BSA solution was mixed with 1 ml of methylglyoxal solution and 1 ml of the samples. The mixture was incubated at 37°C. Sodium azide (0.2 g/l) was used as an aseptic agent. Phosphate buffer was used as a blank. Aminoguanidine and philorogluconol were used as positive controls. After seven days of incubation, fluorescence of the samples was measured using an excitation of 340 nm and an emission of 420 nm, respectively.
Amadorin activity

Amadorin activity was determined using a post-Amadori screening assay [14]. Lysozyme (10 mg/ml) was incubated with 0.5 M ribose in 0.1 M sodium phosphate buffer containing 3 mM sodium azide, pH 7.4 at 37°C for 24 h. Unbound ribose was removed by dialysis against 0.1 M sodium phosphate buffer, pH 7.4 at 4°C for 48 h with 5-6 changes. Following dialysis, the protein concentration was determined using the Bio-Rad standard protein assay kit based on the Bradford dye-binding method [13].

Determination of protein carbonyl content

Protein carbonyl content, a common marker for protein oxidative damage, was measured according to a previous method with minor modifications [17]. Glycated BSA was incubated with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl at room temperature for 1 h. Afterwards, it was precipitated by 20% (w/v) trichloroacetic acid (TCA), left on ice for 5 min, and centrifuged at 10,000 g at 4°C for 10 min. The pellet was washed three times using 1: 1 (v/v) ethanol-ethyl acetate mixture. The final pellet was dissolved in 6 M guanidine hydrochloride. The absorbance was recorded at 370 nm. The level of protein carbonyl content was calculated by using an absorption coefficient of 22,000 M⁻¹cm⁻¹. The results were expressed as nmol carbonyls/mg protein.

Cellular oxidative stress inhibition

Cell-culture and treatment: C2C12 cell purchased from ATCC (Manassas, VA, USA) was cultured in Dulbecco’s modified eagle’s medium (DMEM) medium supplemented with 10% Fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in 5% CO₂ incubator. When the cells were about to cover 80% of the flask area, they were disrupted and seeded on 24 well plates. After attaining ~70–80% confluency, the cells were rinsed twice with phosphate buffer saline (PBS) and changed with...
medium containing compounds at different concentrations. After 24 h incubation, the cells were washed twice with PBS and 50 μM H₂O₂ was maintained in individual well for 1 h at 37°C. These cells were detached by trypsin to assay in flow cytometry.

Cytoprotective effect against the oxidative stress induced by H₂O₂ was measured by determining intracellular content of ROS. Intracellular ROS levels were measured employing 2',7'-dichlorofluorescin-diacetate (DCFH-DA). DCFH-DA is cleaved intracellularly by non-specific esterase and turn to high fluorescent (DCF) upon oxidation by ROS, which were analyzed with FACS Aria II Flow Cytometer (BD Bioscience, San Jose, USA). C2C12 cells pretreated with triterpenes were incubated with DCFH-DA at 37°C for 1 h and then read in FACS Aria II.

**Table 2:** 1H and 13C NMR spectra data for compounds 5-6 (δ in ppm, J in Hz).

| 5      | 6      |
|--------|--------|
| δ_4   | δ_5   |
| 1.54 m, 1.57 m | 1.07 m, 2.03 m |
| 1.92 m, 1.99 m | 1.09 m |
| 3.59, dd (11.5, 5.3) | 4.34, d (10.3) |
| -     | -     |
| 1.30, m | 1.55 m, 1.40 m |
| 1.57 m, 1.66, m | 1.52 m, 1.33 m |
| -     | -     |
| 1.88, m | 1.83, m |
| -     | -     |
| 1.27, m, 2.0, t (12.2) | 2.09 td (13.5, 4.3) |
| -     | -     |
| 1.39, m | 1.24, m |
| -     | -     |
| 0.87, m | 1.62 m, 1.33 m |
| 3.49, d (11.2), 3.70 d (11.2) | 0.92, s |
| -     | -     |
| 1.04, s | 1.02, s |
| 0.92, s | 1.07, m |
| 1.16, s | 0.89, s |
| 1.07, s | 1.0, s |
| -     | 0.82, s |
| 1.01, d (6.1) | 0.89, d (6.4) |
| 0.95, d (6.1) | 1.65, s |
| -     | -     |
| -     | 2.08, s |
| -     | 172.31, 21.32 |
| -     | 174.19, 21.59 |

Assignments were confirmed by coupling constant, 1H-H COSY, NOESY, HMQC, and HMBC analysis in CDCl₃.

**Results and Discussion**

The methanol extract of stems of *Origanum majorana*, was fractionated by column chromatography over silica gel and purified by column with sephadex LH-20 and preparative chromatography. In the present study we report the isolation of twelve triterpenes. The compounds were identified by comparison of their physical and spectroscopic data to those reported in literature. The known triterpenes including 28-norlup-20(29)-ene-3β,17β-diol (8, CAS: 202651-66-9) and lupeol (12, CAS: 545-47-1) that were previously isolated from extract of *Origanum majorana* [18]. Lupeol treatment caused decreases in serum glucose, nitric oxide, and glycated haemoglobin, also increase antioxidant levels and serum insulin level [19]. While ursolic acid (10,
Due to the lack of UV chromophore group in the structure of pentacyclic triterpenes, this study was not conducted.

Compound 1 showed strong absorption bands at 3491, 1710 and 1645 cm⁻¹ in the IR spectrum indicative of the presence of hydroxyl, carbonyl and double bond groups respectively. The mass spectrum of 1 exhibited a weak molecular ion at m/z 504, which was in agreement with the formula C₇₆H₈O₁₀ and seven degrees of unsaturation. This was also supported with analysis of ¹H- and ¹³C-NMR and the HMBC spectrum, data which were assigned to 1 as shown in Table 1. This compound displayed in DEPT experiments signals for thirty carbons which were distinguished as seven methyls, seven methylenes, eight methines (four oxygenated, three aliphatic, and one vinyl) and eight quaternary carbons (one oxygenated, six aliphatic, and one vinyl). The ¹³C NMR spectrum showed the presence of a trisubstituted double bond at δc 123.56 and δc 141.56 (Table 1) together with the signals of ¹H NMR spectrum for seven singlet methyl groups (δH 0.82, 0.83, 0.81, 0.89, 1.07, 0.98, 1.19, 1.03) consistent with an 12-oleanene-28 oic acid skeleton [25]. The presence of four oxygen bearing carbon atom in the molecule were indicated for signals at δc 79.10, 76.59, 75.52 and 72.67 in the ¹³C NMR.

A cross peaks of two methyl protons, H-3-23 (δH 0.84) and H-3-24 (δH 0.82), correlated with C-3 (δc 79.10) carbon showed a hydroxyl group located at C-3 (δc 79.10). In the ¹H NMR spectrum of 1 the doublet of doublet at δH 3.54 and doublet at δH 4.40 were indicative of the deshielded protons attached to the two of the oxygen bearing methine carbons C-3 (δc 79.10) and C-11 (δc 72.67), respectively. The oxygenated doublet of doublets at δH 3.54, which correlated to the methine carbon at δc 72.67 in the HETCOR spectrum, was assigned to H-11. The trans diaxial coupling constants J = 10.7 and 9.0 Hz for a doublet (δc 3.54) and doublet (δc 4.40) respectively, suggested that both the 3-OH and 11-OH groups should be equatorial. This was also supported on the observed COSY coupling with the olefinic proton doublet resonating at δH 5.32 (H-12) and the proton doublet absorbing at δH 1.71 (H-9).

The presence of the hydroxyl group at C-21 was suggested by the ¹H-NMR signal at δ 3.47 (1H, m), the ¹³C-NMR signal at δc 75.52 and HMBC correlations of the methyl protons H-29 (δc 1.26) and H-30 (δc 1.03) with C-18 (δc 55.17), C-19 (δc 34.37), C-21 (δc 75.52) and C-22 (δc 45.37) carbons; H-22α (δc 2.42) and H-22β (δc 1.26) with C-21 (δc 75.52) carbon. The β configuration of the hydroxyl group at H-21 in the NOESY spectrum, was identified from the correlations H-21 (δc 3.47) with H-29 (δc 1.26).

In the HMBC spectrum, proton H-22α (δc 2.47) correlated with C-16 (δc 76.59) carbon, which revealed a hydroxyl group located at C-16 (δc 76.59). This suggestion could be confirmed by the correlations as H-15 (δc 3.50 and 2.22) correlated with C-16 (δc 76.59) and C-27 (δc 27.41) carbons, respectively. β configuration of the hydroxyl group at C-16 was identified from the correlation between H-16 (δc 4.34) and H-27 (δc 0.98). The other oxygenated carbon, which appeared at δc 181.34 as a quaternary carbon in the DEPT spectrum, was attributed to C-28 as a carboxylic acid group. The structure of compound 1 being, 3β,11α,16β,21β-tetrahydroxy- olean-12-en-28 oic acid

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Figure 1: Triterpenoids isolated from Origanum majorana.
eight quaternary carbon including one carboxyl group, with a degree of unsaturation of seven. 

The H NMR spectrum of 2 (Table 1) showed signals for six tertiary methyl groups at δ 1.23 (3H, s), δ 1.08 (3H, s), δ 1.33 (3H, s), δ 1.09 (3H, s), δ 1.24 (3H, s), 1.25 (3H, s). Thus, 2 was deduced to be a oleanane derivative. HMBC experiment showed the correlation of H-23 (δ 1.23) and H-24 (δ 1.08), with C-3 (δ 83.25) which have a hydroxyl group. The carbonyl group of the C-3 (δ 83.25) is 7 ppm downfield shift compared to other analogues [26], indicated that C-2 have an oxygenated carbon. In the HMBC spectrum, correlation signals from δ 3.69 (H-3) to δ 69.64 (C-2), δ 40.98 (C-4), δ 28.65 (C-23) and δ 16.59 (C-24) were observed, supported the linked of the two hydroxyl groups at C-2 and C-3. In NOESY spectrum showed correlations of H-3 (δ 3.69) with Me-23 (δ 1.23) of H-2 (δ 4.19) with Me-24 (δ 1.08) and Me-25 (δ 1.13) together with the large proton–proton coupling constant of H-3 (J 9.4 Hz) indicated the α- and β-orientation of the hydroxyl groups at C-2 and C-3, respectively [27]. AB system signal, for one hydroxymethyl group in the H NMR spectrum at δ 3.62 (2H, s) together with HMBC spectrum showed correlations from δ 3.62 (H-29) to C-19 (δ 46.72), C-20 (δ 36.49) and C-30 (δ 19.73), supported the linked of the hydroxy hydroxyl group at C-29 but not at C-30. The α-orientation of hydroxyl group and the β-axial configuration of H-18 was suggested by NOE correlations of H-18J (δ 2.38) with H-12 (δ 5.50) and H-30 (δ 1.25) and the coupling constant of H-18 (δ 11.5 Hz) [31]. HMBC correlations from δ 5.50 (H-12) to C-9 (δ 48.01), C-14 (δ 43.49) and C-18 (δ 43.49) indicated the double bond at C-12(13). The signal H-16 (δ 1.93) and H-18 (δ 2.38), correlated with carbonyl signal at δ 179.22 suggested that the carbonyl group was connected to C-17.

The location of the other two hydroxy groups was carried out by 1H-1H COSY spectrum indicated correlations of H-18 (δ 2.38) to H-19 (δ 2.90, 1.32) and H-21 (δ 4.81) to H-22 (δ 4.64) [28]. In the NOESY spectrum NOE correlations between H-18 (δ 2.38) to H-30 (δ 1.25) and H-18 (δ 2.38) to H-22 (δ 4.64) supported the orientation of the hydroxyl groups linked to C-22 (δ 76.31) and C-29 (δ 73.73). Moreover, the β-orientation of the hydroxyl groups linked to C-21 was confirm by large values of the proton coupling constant between H-21 and H-22 (J 10 Hz) [29]. However, HMBC spectrum showed correlations of H-18 (δ 2.38) with C-20 (δ 36.49), H-19 (δ 2.90, 1.32) with C-21 (δ 73.68), C-29 (δ 73.73), and C-30 (δ 19.73); H-21 (δ 4.81) with C-29 (δ 73.73), C-30 (δ 19.73); H-22 (δ 4.64), with C-20 (δ 36.49) confirm the location of the three hydroxy groups in E ring. On the basis of these results the structure of 2 was determined as 2a,3β,12β,23α-trihydroxyolean-12-en-28-0ic acid (Figure 1).

Compound 3 was isolated as amoraphous powder. Its molecular formula was established as C19H19O3 on the basis of HRESI-MS. The IR spectrum showed the presence of hydroxyl (3461 cm−1), carbonyl (1735 cm−1), and double bond (1643 cm−1) groups absorption bands. The 13C NMR and DEPT spectra revealed thirty-three signals assigned to seven methyl groups, nine methylenes, six methynes and eleven quaternary carbon, with a degree of unsaturation of ten. The H NMR spectrum of 3 showed typical absorptions bands for a double bond (1643 cm−1) and C-20 (δ 50.19). C-20 (δ 32.45) and C-21 (δ 73.32); H-29 (δ 91.94/1.88) to C-21 (δ 73.32). The relative configuration of hydroxyl group was determined as 1α-axial orientation according on the coupling constant of H-21 (J 4.0, 10 Hz) [31]. Therefore compound 3 was concluded to be 3β,15α,21α-trihydroxyolean-12-en-28-0ic acid (Figure 1).

Compound 4 obtained as amoraphous powder. The molecular formula was C19H19O4 on the basis of HRESI-MS. The IR spectrum showed the presence of hydroxyl (3461 cm−1), carbonyl (1735 cm−1), and double bond (1643 cm−1) groups absorption bands. The 13C NMR and DEPT spectra revealed thirty-three signals assigned to seven methyl groups, nine methylenes, six methynes and eleven quaternary carbon, with a degree of unsaturation of ten. The H NMR spectrum of 4 showed signal attibutable to two downfield protons at δ 5.29 and δ 5.15 assigned to H-6 and H-12 respectively, the sharp of three proton singlets at δ 2.03 and δ 1.98 revealed the presence of two acetyl groups and five tertiary methyl group at 0.85, 0.93, 1.03, 1.17 and 1.24. The H-30 methyl appeared as a three proton singlet at 1.24, corresponding of the oleanane-type triterpenic skeleton. Also was observed an acetoxyl group at δ 2.03 (3H, s), a methyne proton attached an acetoxyl group at δ 4.7. HMBC correlations of the proton signals at H-3 (δ 3.41) with δ 47.80 (C-1), δ 43.89 (C-4), δ 71.23 (C-23), δ 22.78 (C-24), H-23 (δ 3.45/3.68) with δ 81.31 (C-3), δ 43.89 (C-4), and δ 145.36 (C-5) supported the location of hydroxyl groups at C-3 and C-24 respectively. Based on NOE correlations between H-3a and H-23 and coupling constant of H-3 (J 9.5 Hz) hydroxyl group was assigned β-equatorial and the hydroxyethyl group at C-23 as α-orientation. The signal H-16 (δ 1.93) and H-18 (δ 3.38), correlated with carbonyl signal at δ 179.22 suggested that the carbonyl group were connected to C-17. The position of acyl groups at C-2 was clarified by HMBC experiment. Long range correlations were observed between the proton of C-2 and the carbonyl carbon of the acetyl group. In HMBC spectrum the C-30 methyl protons displayed correlations with carbonyl of the C-29 and the methylene carbons 19 and 21. The other acetyl group was assigned at C-20 by NOE correlations with H-30 to H-18; H-18 to H-22 which suggested the α-orientation of the 29-COOMe at C-20. The double bonds were located at C-5/C6 and C-12/C13 by HMBC correlations of H-23 and H-24 to C-5; H-23 to C-6; H-24 to C-6; H-25 to C-5; H-27 to C-13. Consequently compound 4 was established as 2a,29α-diacetoxyl, 3β,23α-dihydroxy-olean-12-en-28-oic acid (Figure 1).

Compound 5 was isolated as amoraphous powder and showed positive Molish and Liebermann-Burchard reaction tests. The molecular formula was established as C19H18O4 according to HRESI-MS, m/z 504.3630 [M]+ (calcld. for C19H18O4 504.3451) indicating seven degrees of unsaturation. The IR spectrum contained typical absorptions of hydroxyl (3450 cm−1) and carbonyl (1732 cm−1) groups, and a double bond (1668 cm−1) function. The 13C and DEPT spectrum data indicated the presence of thirty carbons assigned to six methyl groups, eight methylenes including one hydroxymethyl, nine methylenes including one olefinic and three hydroxyl groups, seven quaternary carbon including one carbonyl. These data are in agreement with the molecular formula.
indicated that 5 was ascribed to be derivate of ursane triterpenoids. The location of the C-3 hydroxy group, C-23 hydroxy group, C-28 acid group and C-12 double bond function were determined via an HMBC and NOESY experiments indicated the similarity with those of 4 (Table 1) showed a common 3β,23α-diol-urs-12-en-28 oic acid nucleus for the triterpene. The HMBC correlations between H-15 (δH 4.19) with C-8 (δC 41.26), C-17 (δH 4.27) and C-27 (δC 13.14); H-16 (δH 1.57/20) with H-27 (δC 1.07) and C-15 (δC 68.41) indicated that the second hydroxy group was located at C-15. The configurations of the hydroxy groups at C-3 and C-15 were assigned as β and α-orientations respectively, due to the trans-diaxial coupling constants of H-3 (J = 11.5 Hz) and H-15 (J = 10.6 Hz). Furthermore, in the NOESY experiments correlations were observed between δH 3.59 (H-3), δC 1.30 (H-5) and those at δH 4.19 (H-15) and δC 1.16 (H-26) confirmed the α- and β-orientations of H-3 and H-15 respectively (Table 2). Therefore compound 5 was concluded to be 3β,15α,23α,19α-tetrahydroxyursan-12-ene-28-oic acid (Figure 1).

Triterpene 6 was isolated as a colorless solid. The molecular formula C29H42O8 was established by its 13C NMR and MS data, contained double bond, and acetoxyl groups attributable to the IR absorption bands at 1606, and 1732 cm−1 respectively. The 1H NMR spectrum confirmed that a triterpene skeleton with an ursolic acid type with a double bond at C-12-C-13, and two carboxyl group at δH 172.31, and δC 174.19. The large coupling constant (J0.J) of 10.3 Hz is typical to an antiperiplanar (axial-axial) relationship between H-2 and H-3 (Table 2) suggested the α- and β-orientation of acetyl groups at C-2 and C-3 respectively. The NOE correlations of H-2 (δH 4.56) with H-24 (δH 1.02) and H-25 (δH 1.07); H-3 (δH 4.34) with H-23 (δH 0.92) supported these positions. The 1H NMR spectrum of 6 exhibited signals for a methine proton at δH 2.21 (1H, d, J = 6.7 Hz, H-18a), and the presence of one olefinic double bond at δH 5.23/128.54 (H-12/C-12) and δH 139.27 (C-13) an additional olefinic double bond at δH 5.27/16.43 (H-21/C-21) and δC 134.65 (C-20) and an olefinic methyl group at δH 1.65/23.56 (H-30/C-30). The position of the double bond was established to be at C-20/C-21 based on the HMBC correlations of H-29/C-20, H-21/C-19, H-21/C-20, H-30/C-20, H-21/C-30 and H-30/C-21. Thus, the structure of compound 6 was elucidated to be 2α,3β-diacetoxy-ursan-12,19-diene (Figure 1).

In this study triterpenes 1-6 were evaluated for their ability to retard glycation reaction between glucose and albumin (Table 1). Among them, triterpenes 2 and 1 exhibited the most potential inhibitory activity against AGEs formation, with IC50 values of 39 µM, 26 µM and 82 µM respectively. Compounds 3, 4 and 5 also showed stronger inhibitory activities (IC50 values ranging from 128 to 200 µM) than a well known positive control, aminoguanidine (IC50 value of 959 µM). However 6 showed very little activity (IC50 value of 1301) than that produced by the AG.

The inhibitory effects of triterpenes on methylglyoxal-mediated protein glycation were evaluated. Compounds 2, 1, and 3 exhibited significant inhibition, and their IC50 values were 201, 188, and 224 µM respectively, compared to AG (IC50 335 µM). compounds 4 and 5 showed strong inhibition with IC50 values of 300 and 242 µM respectively (Table 3). Methylglyoxal is a dicarbonyl intermediates as mediators of advanced glycation endproduct formation and are known to react with arginine, cysteine and lysine residues in proteins to form glycosylamine protein crosslinks [32,33].

A G. K. peptide containing a lysine residue was incubated with D-ribose for 24 h. This model system was used to evaluate the inhibitory effects of triterpenes on protein cross-linking. As shown in Table 3, compound 2 exhibited substantial anti-cross-linking activities. At a concentration of 10 mM, the inhibitory effect of compound 2 and pyridoxamine was 78% and 64%, respectively (Table 3).

For a period of 24 h exposure of lysozyme to ribose produced glycated protein rich in Amadori but not advanced glycation adducts [32]. Lysozyme is widely used for investigation of glycation-induced crosslinking. Triterpenes inhibit cross-linked advanced glycation endproducts and also have Amadorin activity to concentration of 50 µg/ml (Table 3). Pyridoxamine, used as positive control may inhibit at multiple stages of advanced glycation endproduct formation. This contrasts with other inhibitors, such as aminoguanidine which has no Amadorin activity [34].

These results indicated that for each stage of protein glycation compound 2 had the most potent inhibitory effect of all the compounds isolated from O. majorana. These observations suggested that the compounds can potentially inhibit the glycosylative modification of proteins.

Nε-CML, is a glyco-oxidation product which is not fluorescent and not reactive, is produced from the oxidative degradation of Amadori products [32]. CML is an indicator of the advanced stages of the Maillard reaction. Table 4 shows the effect of 1-6 on Nε-CML level in glycated BSA after 4 weeks of incubation. Triterpenes 1-5 significantly (p<0.05) reduced the concentration of Nε-CML level in BSA + Glucose (39.53%, 51.16%, 34.88, 25.58%, and 30.23%), Fructose-glycated BSA (44.44%, 50.61%, 38.27%, 22.22% and 28.39%) and BSA + Ribose (43.44%,

| Inducer | Treatment | Inhibitory effects IC50 value µM |
|---------|-----------|--------------------------------|
| Glucose | Methanol extract | 656 ± 12.56 |
| Aminoguanidine | 959 ± 32.85 |
| Phloroglucinol | 710 ± 7.59 |
| Pyridoxamine | 82 ± 17.64 |
| 2 | | 39 ± 11.53 |
| 3 | | 128 ± 22.17 |
| 4 | | 200 ± 21.06 |
| 5 | | 161 ± 29.41 |
| 6 | | 1301 ± 19.78 |
| Methyglyoxal | Methanol extract | 196 ± 28.21 |
| Aminoguanidine | 335 ± 15.34 |
| Phloroglucinol | 205 ± 17.63 |
| 1 | | 201 ± 28.31 |
| 2 | | 188 ± 18.76 |
| 3 | | 224 ± 15.83 |
| 4 | | 300 ± 30.21 |
| 5 | | 242 ± 21.65 |
| 6 | | 523 ± 18.76 |
| Lysozyme/ribose | Methanol extract | 70.5% |
| Aminoguanidine | 0% |
| Pyridoxamine | 64% |
| 1 | | 73.2% |
| 2 | | 77.9% |
| 3 | | 66.5% |
| 4 | | 50.3% |
| 5 | | 60.5% |
| 6 | | 11.4% |

Data are mean ± standard deviation of triplicate tests. The concentration of triterpenoids used in the AGEs formation methods were of 20, 30, 50, 100, 200, 300, 500, 750, 1000, 1200, and 1300 µM. However, in lysozyme/ribose method was used 10 mM.
The effect of triterpenes 1-5 at concentration of 1 mM on protein carbonyl content (PCO) in BSA incubated with ribose, fructose and glucose is shown in Table 4. The results indicated a significant reduction of PCO content level in BSA+Glu (47.78%-64.60%), BSA+Rib (38.08%-53.49%) and BSA+Fruc (47.01%-65.57%). In addition, BSA in the presence of AG also to concentration of 1 mM significantly inhibited PCO (47.34% in BSA+Glu, in 31.52% BSA+Rib and 40.39% in BSA+Fruc). Comparing with the percent reduction in PCO of 1-5 were more effective than AG at the same concentration.

The ability of triterpenes 1-5 to inhibit α-dicarbonyl compounds formation is showed in Table 5. The effect of 1-5 on glyoxal content showed all isolated have less inhibitory effect than that produced by the quercetin used as positive standard. Among the isolated compounds 2 had stronger inhibition activity.

The ability of the triterpenes 1-5 to decrease the oxidative stress in cells was evaluated by intracellular oxidative stress induced by H$_2$O$_2$ in C2C12 cells. The oxidative stress reduction is showed in Table 5. The capacity of triterpenes (50 μg/ml) to reduce the oxidative stress was compared with that of ascorbic acid (25 μg/ml). The triterpenes 1-5 were less potent than ascorbic acid.

Glycation of triterpenes was monitored by MALDI-MS through the increase in the molecular weight of protein as a result of glucose adducts formation with the honnone. Figure 2 shows the typical mass spectra of positive (a) and mass spectra of BSA. In Figure 3, we can observe a typical spectrum of glycated protein where glycation of the protein was performed by incubation with 220 mM glucose for 30 d at 37°C in the absence of reducing conditions and glycated protein with 1 (a). The major peak at m/z 67925.163 corresponds to native BSA whereas the second peak at m/z 3999.656 corresponds to a diglycated form of BSA. A third minor peak at m/z 5397.765 is also observable and should correspond to a monoglycylated form. Figure 3b shows a different mass profile, especially in terms of relative abundance of each specie. There are two additional peaks in the spectrum besides the peak corresponding to native BSA. The major peak, at m/z of 5397.765, is the protonated

| Concentration (50 μg/ml) | Inhibition of CML (ng/ml) | Protein carbonyl content (nmol/mg/protein) |
|--------------------------|---------------------------|------------------------------------------|
| BSA                      | 1.9 ± 0.4                 | 0.29 ± 0.008                             |
| BSA + Glucose (Glu)      | 4.3 ± 1.2$^a$             | 2.26 ± 0.034$^a$                        |
| BSA + Glucose + 1        | 2.6 ± 0.8$^a$             | 0.88 ± 0.011$^a$                        |
| BSA + Glucose + 2        | 2.1 ± 0.7$^a$             | 0.80 ± 0.037$^a$                        |
| BSA + Glucose + 3        | 2.8 ± 1.0$^a$             | 0.95 ± 0.043$^a$                        |
| BSA + Glucose + 4        | 3.2 ± 1.3$^a$             | 1.18 ± 0.016$^a$                        |
| BSA + Glucose + 5        | 3.0 ± 0.9$^a$             | 1.07 ± 0.039$^a$                        |
| BSA + Glucose + 6        | 4.0 ± 1.5                 | 2.03 ± 0.039                            |
| BSA + Glucose + AG       | 2.3 ± 0.7$^a$             | 1.19 ± 0.079$^a$                        |
| BSA + Fructose (Fruc)    | 8.1 ± 2.1$^a$             | 3.02 ± 0.082$^a$                        |
| BSA + Fructose + 1       | 4.5 ± 1.2$^a$             | 1.18 ± 0.021$^a$                        |
| BSA + Fructose + 2       | 4.0 ± 1.9$^a$             | 1.10 ± 0.017$^a$                        |
| BSA + Fructose + 3       | 5.0 ± 0.8$^a$             | 1.31 ± 0.045$^a$                        |
| BSA + Fructose + 4       | 6.3 ± 0.6$^a$             | 1.60 ± 0.048$^a$                        |
| BSA + Fructose + 5       | 5.8 ± 0.7$^a$             | 1.49 ± 0.050$^a$                        |
| BSA + Fructose + 6       | 7.8 ± 0.7                 | 2.81 ± 0.093                            |
| BSA+ Fructose + AG       | 4.1 ± 1.8$^a$             | 1.80 ± 0.045$^a$                        |
| BSA + Ribose (Rib)       | 53.4 ± 3.17$^a$           | 7.01 ± 0.034$^a$                        |
| BSA + Ribose + 1         | 30.2 ± 6.19$^b$           | 3.88 ± 0.059$^b$                        |
| BSA + Ribose + 2         | 27.89 ± 5.12$^b$          | 3.75 ± 0.087$^b$                        |
| BSA + Ribose + 3         | 33.8 ± 4.19$^b$           | 4.05 ± 0.073$^b$                        |
| BSA + Ribose + 4         | 35.4 ± 6.11$^b$           | 4.34 ± 0.037$^b$                        |
| BSA + Ribose + 5         | 34.7 ± 3.93$^b$           | 4.21 ± 0.026$^b$                        |
| BSA + Ribose + 6         | 50.9 ± 5.17               | 6.77 ± 0.062$^b$                        |
| BSA+ Ribose + AG         | 34.5 ± 3.76$^b$           | 4.80 ± 0.078$^b$                        |

Data are mean ± standard deviation (n = 3) *p<0.05 when compared to BSA ; $p<0.05 when compared to BSA + Glu, BSA + Fruc and BSA + Rib.

Table 4: Effect of triterpenes 1-6 on non-fluorescence N-CML level and protein carbonyl content.

| Groups             | % DCF fluorescence | α-dicarbonyl compounds (IC$_{50}$ μg/ml) |
|--------------------|-------------------|------------------------------------------|
| Blank              | 7.12              | -                                        |
| Control            | 68.34             | -                                        |
| Ascorbic acid      | 16.38             | -                                        |
| Quercetin          |                   | 62.5 ± 13.19                            |
| 1                  | 40.23             | 124.8 ± 8.56                            |
| 2                  | 34.27             | 105.7 ± 9.28                            |
| 3                  | 45.67             | 140.2 ± 10.38                           |
| 4                  | 51.30             | 173.0 ± 13.78                           |
| 5                  | 48.47             | 160.3 ± 12.26                           |

Values represents mean ± SD (n = 3).

Table 5: Reduction activity of triterpenes 1-5 on cellular oxidation stress and α-dicarbonyl compounds.
Figure 2: Positive ion MALDI linear TOF MS, with the standard (sinapic acid) SEM detector (a) and BSA (b).

Figure 3: Chromatogram (a) show mono-, di- and tri- adducts of BSA+ glucose after incubation 15 days at 37°C. MALDI TOF MS spectra (b) of monomero-glucose, dimero-glucose and trimero-glucose adducts of 2α,3β,21β,22α,29α-pentahydroxy-olean-12-en-28-oic acid.
monoglycated form. The other peaks at m/z 3999.656 correspond to the protonated diglycated and triglycated forms, respectively.

In all screening compound 2 displayed the most potent activity whereas compounds 1, 3, 5 and 4 gave significantly activity, instead compound 6 has much weaker activity considered inactive. This results suggested that the loss of the hydroxyl groups caused a significant loss of activity, revealing also the importance of the carboxyl group since no activity was observed in compound 6 which lacks this group. In general, the most active derivatives were oleanane followed by ursene. Interestingly, acetyl groups greatly diminished activity. Structural variations involving the hydroxyl groups substituted rings resulted in considerable improvement in inhibited AGEs.

In conclusion, according to the data obtained in this study *Origanum majorana* leaves extract contain various triterpenoids which showed excellent effect anti-glycation and they are effective inhibiting cellular oxidations. In particular, 2α,3β,21β,22α,29α-pentahydroxy-olean-12-en-28-oic acid (2) displaying potent activity when compared to aminoguanidine a known glycation agent.

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