# Medicinal chemistry

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# Inhibitory Activities of Guaianolides from the Seeds of *Byrsonima Crassifolia* against Protein Glycation *In Vitro*

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## Abstract

In-depth chromatographic investigation on the hexane extract of *Byrsonima crassifolia* led to the identification of eight new guaianolides. Structural elucidation was established on the basis of spectral data as; byrsonina C (1) to byrsonina J (8). *In vitro* inhibitory activity of the 1-8 on advanced glycation end products (AGEs), analysis of protein gels (SDS-PAGE) profile and by matrix assisted laser desorption ionization (MALDI) coupled to time of flight (TOF) analyzers mass spectra (MALDI lineal TOF MS) were evaluated. Guaianolides exhibited glycation inhibitory activity similar to that of aminoguanidine. The major mechanism implied in the inhibition of protein glycation by compounds 1-8 was attributed to their ability to react with carbonyls. SDS-PAGE profiles displayed inhibition of AGE generation, through inhibition of the crosslinked formation of AGEs, which was detectable for MALDI linear TOF MS as an intensity reduction of the dimerized band. We conclude that guaianolides from *Byrsonima crassifolia* can efficiently inhibit AGEs formation and oxidative damage elicited by monosaccharides, suggesting that may prevent AGEs-mediated interaction with multiple targets involved in the pathogenesis of diabetes.

**Keywords:** *Byrsonima crassifol*; Guaianolides; Anti-ages

## Introduction

Advanced glycation end-products (AGEs) are the final products of the nonenzymatic reaction between reducing sugars and amino groups in proteins. They are a group of complex and heterogeneous compounds that are known as brown and fluorescent cross-linking substances [1]. Reactive carbonyl compounds are intermediates in the formation of AGEs and advanced lipoxidation end products in tissue proteins in chronic disease as diabetes and its complications [2].

*Byrsonima crassifolia* fruit is edible and bright yellow when ripened, it has sweet taste and slightly bitter aftertaste. In México, nanche fruit is consumed as liquor, candy and jelly and is wide accepted as nutraceutical too [3]. Since prehispanic times it has been used on ethnobotanical uses include: as wound healing, anti-inflammatory, dysentery infections, and antidiabetic [3]. In the literature there are many researches on the properties and composition of *B. crassifolia* [4-12]. For these reasons, in this study the anti-AGEs activity of guaianolides was tested *in vitro* that may contribute to prevent diabetes or other pathogenic complications.

## Experimental Section

### General experimental procedures

IR spectra were recorded on a Perkin-Elmer FTRI 1720X. NMR experiments were obtained on a Bruker DRX-400. The NMR data were processed using UXNMR software. HREIMS were measured on a JEOL HX 110 mass spectrometer. For Column chromatography was carried on Silica gel 60 (230-400 mesh, Merck Co. New Jersey (USA) and Sephadex LH-20 from Sigma-Aldrich (St. Louis, USA). Precoated TLC silica gel 60 F254 aluminum sheets were used, solvents used as eluents from Fermont (California, USA).

### Plant material

*Byrsonima crassifolia* L. belong to the Malpighiaceae family, fruits were collected at Morelos state and were taxonomically authenticated in the Herbarium of Escuela Nacional de Ciencias Biologicas, Instituto Politécnico Nacional. A voucher specimen of the plant is stored for reference (No. 8976).

### Extraction, isolation and characterization of the sesquiterpene lactones

Seeds from fruit of *B. crassifolia* was air dried and the ground (10 kg) was extracted twice with hexane each for 3 h. The seeds extracts were combined and the solvent was removed by rotatory evaporation to give 572 g residue. The resulting extract was loaded onto a silica gel column chromatography and eluted with petroleum ether-acetone-hexane 2:1:0.5 and 6 fractions (F1-F-6) have been obtained. These fractions were then tested for anti-AGEs activity. Active fractions were pooled together according to their similarities provides by thin layer chromatography analysis (Figure 1a). The fraction F1 was the fraction that showed anti-AGEs properties. F1 further fractionated to silica gel column chromatography eluted with ethyl ether-chloroform (1:5) to produce seven fractions (F1-1 to F1-7). The active fraction F1-1 was subjected to chromatographed over silica gel column using chloroform-EtOAc 5:1 to yield six subfractions (F11-1 to F11-6). The F11-1 fraction was further purified by preparative plate using chloroform-EtOAc 11:2 to produce five fractions (F111-1 to F111-6) and visualized with UV at 254 nm. Fractions F111-1, F111-3 and F111-5 were separated by Sephadex LH-20 using a gradient of CHCl3–MeOH (from 10:1 to 5:1) to yield 1 (87 mg), 2 (79 mg), 3 (88 mg), 4 (58 mg), 5 (36 mg), 6 (91 mg), 7 (80 mg), and 8 (59 mg).

*Byrsonina C* (1): is a colorless gel-like substance. IR (KBr) vmax 1743 (y-lactone), 1724 (benzoyl), 1666 (C=C), 1459, 1376, 1239 (acetate), 1161, 1098, 723 cm$^{-1}$; HR-ESIMS: *m/z* 398. 4508 (calcd. 398.5461, for $C_{23}H_{26}O_6$); $^1$H NMR (500 MHZ, CDCl3) δ: 1.36 (1H, m,
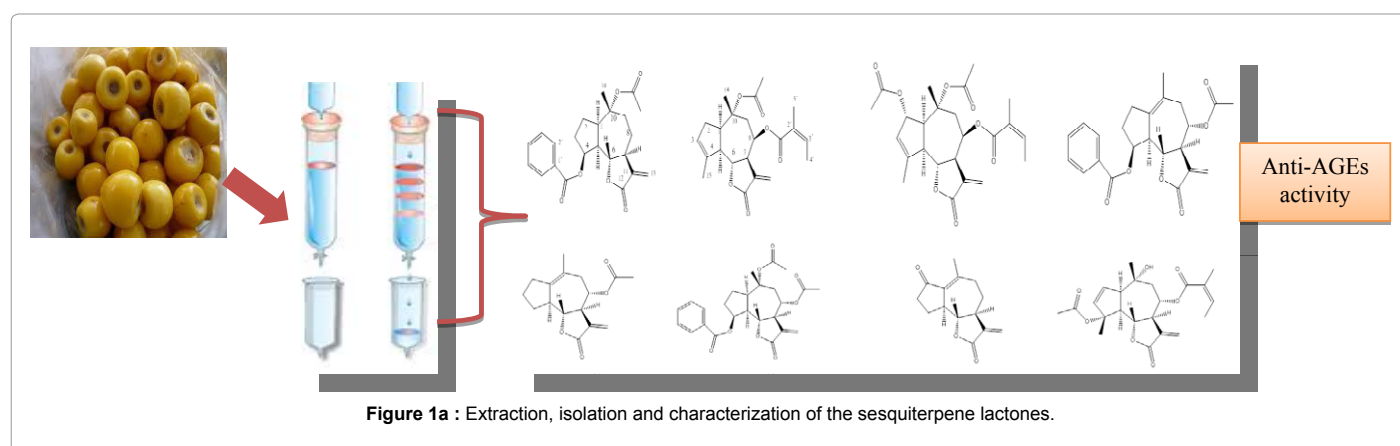
**Figure 1a :** Extraction, isolation and characterization of the sesquiterpene lactones.

H-1), 1.74 (2H, m, H-2), 1.48 (2H, m, H-3), 5.30 (1H, ddd, $J$=11.2, 7.9, 3.5 Hz, H-4), 2.70 (1H, m, H-5), 4.22 (1H, dd, $J$=10.5, 10.2 Hz, H-6), 2.19 1H, ddd, $J$=12.0, 10.5, 10.2 Hz, H-7), 1.46 (2H, m, H-8), 2.90 (2H, m, H-9), 6.79 ( 1H, d, $J$=3.4 Hz, H-13a), 5. 23 (1H, d, $J$=3.0 Hz, H-13b), 1.59 (3H, s, H-14), 7.70 (2H, d, $J$=7.6 Hz, H-2',6'), 7.49 (2H, d, $J$=7.6 Hz, H-3',5'), 7.61 (1H, t, $J$=7.6 Hz, H-4'), 2.20 (3H, s, COOMe) ; $^{13}$C NMR (100 MHZ, CDCl3) δ: 58.21 (C-1), 22.88 (C-2), 32.12 (C-3), 68.17 (C-4), 50.65 (C-5), 68.39 (C-6), 57.51 (C-7), 68.18 (C-8), 30.41 (C-9), 86.51 C-10), 137.65 (C-11), 173.56 (C-12), 121.03 (C-13), 16.06 (C-14), 168.78 (COO), 129.21 (C-1'), 130.98 (C-2',6'), 128.87 (C-3',5'), 132.47 (C-4'), 21.10 (COOMe), 173.45 (COOMe).

*Byrsonina D* (2): IR (KBr) vmax 1762 (ɣ-lactone), 1732 (benzoyl), 1654 (C=C), 1376, 1239 (acetate), 1165, 1091, 722 cm⁻¹; HRESIMS: *m/z* 388. 4508 (calcd. 388.4219, for C22H28O6); ¹H NMR (500 MHZ, CDCl3) δ:2.75 (1H, m, H-1), 2.51 (2H, m H-2), 5.70 (1H, dq, $J$=1.1 Hz, H-3), 2.81 (1H, m, H-5), 4.43 (1H, dd, $J$=10, 10.3 Hz, H-6), 2.61 (1H, ddd, $J$ 3,10,13 Hz, H-7), 4.55 (1H, ddd, $J$=4.2, 10.9, 1.5 Hz, H-8), 2.50, 2.68 (2H, dd, $J$=15.8, 4.5 Hz, H-9), 6.23 (1H, br d, $J$=3.5 Hz,H-13a), 5.56 (1H, br d, $J$=3.5 Hz, H-13b), 1.30 (3H, s, H-14), 1.69 (3H, s, H-15), 2.28, (3H, s, COOMe) OAng: 6.06 (1H, brq, $J$=7.51 Hz, 3'), 1.98 (3H, br d, $J$=7.5 Hz, H-4'), 1.85 (3H, br, s, H-5'); $^{13}$C NMR (100 MHZ, CDCl3) δ: 57.19 (C-1), 46.68 (C-2), 129.02 (C-3), 137.80 (C-4), 52.44 (C-5), 68.94 (C-6), 58.96 (C-7), 68.18 (C-8), 30.75 (C-9), 86.43 (C-10), 137.26 (C-11), 172.94 (C-12), 120.81 (C-13), 16.03 (C-14), 22.76 (COOMe), 173.38 (COOMe), OAng: 167.83 (C-1'), 129.01 (C-2'), 139.64 (C-3'), 15.53 (C-4'), 20.52 (C-5').

*Byrsonina E* (3): IR (KBr) vmax 1754 (ɣ-lactone), 1740 (benzoyl), 1657 (C=C), 1460, 1376, 1240 (acetate), 1160, 1098, 723 cm⁻¹; HR-ESIMS: *m/z* 446.5413 (calcd., 446.2391 for C24H30O8) ¹H NMR (500 MHZ, CDCl3) δ: 2.74 (1H, brt, $J$=9.0 Hz, H-1), 5.44 (1H, dd, $J$=2,3 2.0; $J$=2,15 0.5 Hz, H-2), 5.71 (1H, dq, $J$=1.1 Hz, H-3), 2.80 (1H, m, H-5), 4.44 (1H, dd, $J$=10, 10.3 Hz, H-6), 2.63 (1H, ddd, $J$=3,10,13 Hz, H-7), 4.51 (1H, ddd, $J$=4.2, 10.9, 1.5 Hz, H-8), 2.51, 2.69 (2H, dd, $J$=15.8, 4.5 Hz, H-9), 6.22 (1H, br d, $J$=3.5 Hz, H-13a), 5.56 (1H, br d, $J$=3.5 Hz, H-13b), 1.52 (3H, s, H-14), 1.70 (3H, s, H-15), 2.27, (3H, s, COOMe) OAng: 6.06 (1H, brq, $J$=7.51 Hz, 3'), 1.97 (3H, br d, $J$=7.5 Hz, H-4'), 1.85 (3H, br, s, H-5'); $^{13}$C NMR (100 MHZ, CDCl3) δ: 57.20 (C-1), 76.65 (C-2), 129.10 (C-3), 137.81 (C-4), 52.45 (C-5), 68.92 (C-6), 58.97 (C-7), 68.15 (C-8), 30.72 (C-9), 86.40 (C-10), 135.24 (C-11), 170.99 (C-12), 120.83 (C-13), 16.08 (C-14), 22.86 (COOMe), 173.31 (COOMe) OAng: 167.89 (C-1'), 129.06 (C-2'), 139.62 (C-3'), 15.52 (C-4'), 20.51 (C-5').

*Byrsonina F* (4): IR (KBr) vmax 1760 (ɣ-lactone), 1749 (benzoyl), 1644 (C=C), 1460, 1377, 1262 (acetate), 1162, 1098, 801, 722 cm⁻¹; HR-ESIMS: *m/z* 396.432 (calcd., 396.765 for C23H24O6); ¹H NMR (500

MHZ, CDCl3) δ: 1.75 (2H, m, H-2), 1.48 (2H, m, H-3), 5.31 (1H, ddd, $J$=11.2, 7.9, 3.5 Hz, H-4), 2.70 (1H, m, H-5), 4.22 (1H, dd, $J$=10.5, 10.2 Hz, H-6), 2.19 1H, ddd, $J$=12.0, 10.5, 10.2 Hz, H-7), 4.92 (1H, ddd, $J$=8.0, 3.8, 3.2 Hz, H-8), 2.45 (1H, dd, J 16.3, 3.8 Hz, H-9a), 2.68 (1H, dd, J 16.3, 2.3 Hz, H-9b), 6.79 ( 1H, d, $J$=3.4 Hz, H-13a), 5. 23 (1H, d, $J$=3.0 Hz, H-13b), 1.78 (3H, s, H-14), 7.70 (2H, d, $J$=7.6 Hz, H-2',6'), 7.49 (2H, d, $J$ 7.6 Hz, H-3',5'), 7.61 (1H, t, $J$ 7.6 Hz, H-4'), 2.11 (3H, s, COOMe) ; 13C NMR (100 MHZ, CDCl3) δ: 139.92 (C-1), 22.88 (C-2), 32.12 (C-3), 68.17 (C-4), 50.65 (C-5), 68.39 (C-6), 57.51 (C-7), 74.9 (C-8), 34.53 (C-9), 134.81 C-10), 137.65 (C-11), 173.56 (C-12), 124.21 (C-13), 16.06 (C-14), 168.78 (COO), 129.24 (C-1'), 130.98 (C-2',6'), 128.87 (C-3',5'), 132.47 (C-4'), 20.90 (COOMe), 170.71 (COOMe).

*Byrsonina G* (5): IR (KBr) vmax 1745 (ɣ-lactone), 1736 (benzoyl), 1652 (C=C), 1469, 1373, 1261 (acetate), 1162, cm⁻¹; HR-ESIMS: *m/z* 276.432 (calcd., 276.765 for C16H20O4); ¹H NMR (500 MHZ, CDCl3) δ: 1.75 (2H, m, H-2), 1.48 (2H, m, H-3), 1.50 (2H, m, H-4), 1.58 (1H, m, H-5), 4.22 (1H, dd, $J$=10.5, 10.2 Hz, H-6), 2.19 1H, ddd, $J$=12.0, 10.5, 10.2 Hz, H-7), 4.92 (1H, ddd, $J$=8.0, 3.8, 3.2 Hz, H-8), 2.45 (1H, dd, J=16.3, 3.8 Hz, H-9a), 2.68 (1H, dd, J=16.3, 2.3 Hz, H-9b), 6.79 ( 1H, d, $J$=3.4 Hz, H-13a), 5. 23 (1H, d, $J$=3.0 Hz, H-13b), 1.78 (3H, s, H-14), 2.11 (3H, s, COOMe); 13C NMR (100 MHZ, CDCl3) δ: 139.92 (C-1), 22.88 (C-2), 32.12 (C-3), 33.11 (C-4), 50.65 (C-5), 68.39 (C-6), 57.51 (C-7), 74.9 (C-8), 34.53 (C-9), 134.81 C-10), 137.65 (C-11), 173.56 (C-12), 124.21 (C-13), 16.06 (C-14), 20.90 (COOMe), 170.71 (COOMe).

*Byrsonina H* (6): IR (KBr) vmax 1738 (ɣ-lactone), 1729 (benzoyl), 1661 (C=C), 1470, 1326, 1265 (acetate), 1244, 1183, 803, 717 cm⁻¹; HR-ESIMS: *m/z* 456.432 (calcd., 456.765 for C25H28O8); ¹H NMR (500 MHZ, CDCl3) δ: 1.36 (1H, m, H-1), 1.74 (2H, m, H-2), 1.48 (2H, m, H-3), 5.30 (1H, ddd, J 11.2, 7.9, 3.5 Hz, H-4), 2.70 (1H, m, H-5), 4.22 (1H, dd, $J$=10.5, 10.2 Hz, H-6), 2.19 1H, ddd, $J$=12.0, 10.5, 10.2 Hz, H-7), 4.92 (1H, ddd, $J$=8.0, 3.8, 3.2 Hz, H-8), 2.45 (1H, dd, J 16.3, 3.8 Hz, H-9a), 2.68 (1H, dd, J=16.3, 2.3 Hz, H-9b), 6.79 (1H, d, $J$=3.4 Hz, H-13a), 5.23 (1H, d, $J$=3.0 Hz, H-13b), 1.59 (3H, s, H-14), 7.70 (2H, d, $J$=7.6 Hz, H-2',6'), 7.49 (2H, d, $J$=7.6 Hz, H-3',5'), 7.61 (1H, t, $J$=7.6 Hz, H-4'), 2.20 (3H, s, COOMe); $^{13}$C NMR (100 MHZ, CDCl3) δ: 58.21 (C-1), 22.88 (C-2), 32.12 (C-3), 58.17 (C-4), 50.65 (C-5), 68.39 (C-6), 57.51 (C-7), 68.18 (C-8), 30.41 (C-9), 86.51 (C-10), 137.65 (C-11), 173.56 (C-12), 121.03 (C-13), 16.06 (C-14), 168.78 (COO), 129.24 (C-1'), 130.98 (C-2',6'), 128.87 (C-3',5'), 132.47 (C-4'), 21.10 (COOMe), 173.45 (COOMe).

*Byrsonina I* (7): IR (KBr) vmax 1770 (ɣ-lactone), 1710 (benzoyl), 1644 (C=C), 1460, 1377, 1262 (acetate), 1162, 1098, 801 cm⁻¹; HR-ESIMS: *m/z* 232.432 (calcd., 232.765 for C14H16O3); ¹H NMR (500 MHZ, CDCl3) δ: 2.73 (2H, dd, J=16.8, 4.7 Hz, H-3), 1.50 (2H, m, H-4),

1.58 (1H, m, H-5), 4.22 (1H, dd, $J$=10.5, 10.2 Hz, H-6), 2.19 1H, ddd, $J$=12.0, 10.5, 10.2 Hz, H-7), 1.46 (2H, m, H-8), 2.90 (2H, m, H-9), 6.79 ( 1H, d, $J$=3.4 Hz, H-13a), 5.23 (1H, d, $J$=3.0 Hz, H-13b), 1.78 (3H, s, H-14); $^{13}$C NMR (100 MHZ, CDCl3) δ: 149.23 (C-1), 197.72 (C-2), 32.12 (C-3), 33.11 (C-4), 50.65 (C-5), 68.39 (C-6), 57.51 (C-7), (C-8), 34.53 (C-9), 127.65 (C-10), 137.65 (C-11), 173.56 (C-12), 124.21 (C-13), 16.06 (C-14).

*Byrsonina J* (8): IR (KBr) νmax 3440, 1749 (γ-lactone), 1731 (benzoyl), 1652 (C=C), 1459, 1373, 1238 (acetate), 1163, 1097, 1045, 756 cm$^{-1}$; HR-ESIMS: $m/z$ 404.432 (calcd., 404.765 for C22H28O7); $^1$H NMR (500 MHZ, CDCl3) δ: 2.74 (1H, m, H-1), 5.92 (d, J=10.0 Hz, H-2), 6.02 (d, J=10.0 Hz, H-3) 2.80 (1H, m, H-5), 4.44 (1H, dd, $J$=10, 10.3 Hz, H-6), 2.63 (1H, ddd, $J$=3,10,13 Hz, H-7), 4.62 (1H, ddd, $J$=8.0, 3.8, 3.2 Hz, H-8), 2.45 (1H, dd, J 16.3, 3.8 Hz, H-9a), 2.68 (1H, dd, J 16.3, 2.3 Hz, H-9b), 6.22 (1H, br d, $J$=3.5 Hz, H-13a), 5.56 (1H, br d, $J$=3.5 Hz, H-13b), 1.24 (3H, s, H-14), 1.42 (3H, s, H-15), 2.30, (3H, s, COOMe), OAng: 6.06 (1H, brq, $J$=7.51 Hz, 3'), 1.97 (3H, br d, $J$=7.5 Hz, H-4'), 1.85 (3H, br, s, H-5'); $^{13}$C NMR (100 MHZ, CDCl3) δ: 57.54 (C-1), 132. 55 (C-2), 140.87 (C-3), 83.20 (C-4), 48.89 (C-5), 68.92 (C-6), 58.97 (C-7), 68.15 (C-8), 30.72 (C-9), 77.45 (C-10), 135.24 (C-11), 170.99 (C-12), 120.83 (C-13), 16.08 (C-14), 22.86 (COOMe), 173.31 (COOMe), OAng: 167.74 (C-1'), 129.06 (C-2'), 139.62 (C-3'), 15.52 (C-4'), 20.51 (C-5').

### Bovine serum albumin (BSA)-glucose assay

The methodology was based on that of Brownlee et al. [13]. BSA (10 mg/ml) was incubated at 37°C with 500 mM of glucose in phosphate buffered-saline (PBS) (5 ml total volume, pH 7.4) and compound containing 0.02% sodium azide. All reagents and samples were sterilized by filtration through 0.2 μm membrane filters. The protein, the sugar and the prospective inhibitor were included in mixture simultaneously. Aminoguanidine (AG) was used as an inhibitor positive control. After 15 days of incubation the fluorescence intensity was measured at an excitation wavelength 370 nm and emission wave-length 440 nm in the test solutions.

### BSA-methylglyoxal assay

This assay was based on a published method by Rahbar and Figarola [14], establish the inhibition of protein glycation at an intermediary. BSA and methylglyoxal were dissolved in phosphate buffer (100 mM, pH 7.4) to a concentration of 20 mg/ml and 60 mM, respectively. Isolated were dissolved in the same phosphate buffer. One milliliter of the BSA solution was mixed with 1ml of methylglyoxal solution and 1 of ml compounds. The mixture was incubated at 37°C with sodium azide (0.2 g/l) as an aseptic agent. Phosphate buffer was used as a blank. AG and phloroglucinol was used as positive control inhibitor. Fluorescence of the samples was measured after seven days of incubation, using an excitation wavelength 340 nm and an emission 420 nm.

### Amadorin activity

Amadorin activity was determined using a post-Amadori screening assay [15]. 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 4 1 of 0.1M 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 procedure [16]. Dialysed ribated lysozyme (10 mg/ml) was reincubated with 10 mg/ml of either 1-8 and AG in 0.1M sodium phosphate buffer containing 3 mM sodium azide, pH 7.4 at 37°C for 15 days.

### Glycation of hemoglobin

Glucose (Sigma, 2 mg/dl), hemoglobin (Sigma, 12 mg/dl), compounds (5 mg/dl), and glutathione (Sigma, 20 mM) were dissolved in distilled and sterilized water. The amount of glycated hemoglobin (%GHb) and amount of hemoglobin HbA$_{1c}$ were determined by ion exchange resin method glyco hemoglobin kit (Pariksha Biotech, India).

### Nα-acetyl-glycyl-lysine methyl ester-o-ribose test

Assay was used to determine the ability of terpenes to inhibit the cross-linking of G.K. peptide in the presence of D-ribose [17]. G. K. peptide (80 mg/ml) was incubated with D-ribose (0.8M) and sodium phosphate buffer (0.5M, pH 7.4) under sterile conditions at 37°C for 24 h. Compounds 1-8 were added to a final concentration of 1 mM, however AG was used at 10 and 50 mM. The fluorescence intensity was measured at an excitation wavelength of 330 nm and an emission wavelength of 415 nm.

### Analysis of protein conformation changes by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was made according to Laemmli [18]. Protein samples was diluted with an equal volume of sodium dodecyl sulfate (SDS), terpenes and buffer (2% w/v SDS, 30% w/v glycerol, 0.25M Tris hydroxyaminomethane, pH 6.8), and then boiled for 5 min; 10 μL was loaded on a 10% gradient acrylamide slab gel (Multigel 10/20, Daiichi Pure Chemicals, Tokyo) and electrophoresed at a current of 1 mA/lane for 1 h. After that, the gel was fixed with 60% ethanol followed by Coomassie Brilliant Blue (CBB) R-250 staining.

### MALDI linear TOF MS

After BSA-glucose assay with guaianolides were initially reconstituted in trifluoroacetic acid (TFA 0.1%). An aliquot of each sample was combined (1:1) with sinapic acid matrix solution 5 mg/ml in acetonitrile (ACN) at 50%/TFA 0.1% and applied onto well MALDI plates [19]. Instrument was calibrated by adding compounds (12.0 ng; 0.5 μl) to each calibration spot. Mass spectra were obtained with a MALDI-TOF/TOF mass spectrometer (4800 Proteornics Analyzer: Applied Biosystems, Foster City, CA) in the positive ion reflector mode with a mass range between 800 and 7000 Da. Manual interpretation of tandem mass spectra was performed through the Data Explorer software TM ver. 4.4 (Applied Biosystems).

### Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using commercially software sigmastat 3.5. For multiple comparisons, one way ANOVA was used followed by Tuckey and/Dunnet's test. $p$-values less than 0.05 were considered to be statistically significant.

## Results and Discussion

### Characterization of the guaianolides

Positive HRFABMS provided an ion at m/z 398.5461, indicating the molecular formula $C_{23}H_{26}O_6$, of 1 confirmed with the 13C NMR spectrum and DEPT experiment. The IR spectrum revealed absorption bands for γ-lactone (1743 cm-1), 1734 and 1268 cm$^{-1}$ (acetyl group). The 13C NMR and DEPT spectra exhibited 23 carbons resonances, consisting of two tertiary methyls, five methylenes, ten methines and six quaternary carbons (one lactone carbonyl, and one ester groups). Compound 1, appeared to be a sesquiterpene lactone with a α-methylene-γ-lactone moiety by the carbon signals at δC 173.56 (C-12), 137.65 (C-H), 68.39 (C-6) and also by the characteristic 1H NMR

for H-13a and H-13b ($\delta$H 6.23 and $\delta$H 5.56 each d, J=3.5 Hz) were consistent with a skeleton of a guaianolide [20] (Table 1).

Correlations observed between H-13 and H-6, revealed that they are oriented toward β-face. The coupling constant $J_{1,5}$, $J_{5,6}$ and $J_{6,7}$ (9.4 Hz) show that 1 has an A.B *cis*-fused guaianolide skeleton with *trans*-diaxial disposition of H-6 (β) and H-7 (α). One acetoxy and one benzoyloxy groups were clearly distinguishable in the NMR spectrum. The relative downfield shift of H-4 ($\delta_H$ 5.23) was indicative that has a benzoyl moiety that was confirmed by HMBC correlations between H-4 and the carbonyl ester signal at $\delta_C$ 167.89. NOESY correlations between H-4, H-5α as well as between H-5α, H-1α, H-3α and H-7α confirmed the α-configuration of H-4. COSY spectrum showed correlations between H-1, H-2, H-5; H-2, H-3; H-5, H-6; H-7, H-6; H-8, H-9. The HMBC spectrum exhibited correlations between H-2 with C-4, C-5; H-5 with C-3; H-13 with C-7, C-12, H-6 with C-8; H-9 with C-7, C-14. A singlet at $\delta_H$ 1.29 could be assigned to a methyl tertiary group attached to a quaternary carbon (C-10) further substituted by an oxygen function (acetyl group) these assignments were supported by HMBC $H_3$-14 to C-10, C-9 and C-1. The presence of this quaternary carbon at $\delta_C$ 66.51 indicated that the methyl and acetyl groups were attached to the same carbon. Combined observed NOE contacts and vicinal J (H-H) we could determine the relative configuration in positions 1,4,5 in relation to those at position 7 and 8 suggest that were on the same face of the molecule. NOE correlation between H-14 and H-7 and the methyl of the acetyl group and H-7 confirming the 10α-acetyl orientation. Therefore, guaianolide compound 1 was named as byrsonina C.

Comparison of IR and $^1$H NMR data of compound 1 and 2 indicated that both have typical signals of the guaianolide sesquiterpene lactone. Terpene 2 have two esters groups identified as angeloyloxy group by typical signals characteristic at $\delta_H$ 6.06 (H-3', brq, J=7.5 Hz) and at $\delta_H$ 1.85 (H-5', br, s). The position of the angeloyloxy group was deduced to be at C-8 ($\delta_C$ 68.18) by correlation between H-8 (6.06) and C-1' ($\delta_C$ 167.83) in the HMBC spectrum. The configuration of the angelate double bond were assigned as Z on the basis of a NOESY cross-peak from the H-3' to the methyl 5' pairs. The second ester was essentially identical with those of compound 1 at C-10. H-8 appeared at $\delta_H$ 4.55 so, angeloxy moiety must be at C-8 since the small coupling constant value of $J_{7,8}$ 3 Hz required an α-orientation of H-8, which was further confirmed by NOE experiments. The position of the angeloxy group was confirmed at C-8 ($\delta_C$ 68.18) by the correlation between H-8 ($\delta_H$ 4.55) and C-1" ($\delta_C$ 167.83) in the HMBC spectrum. A singlet at $\delta_H$ 1.30 could be assigned to a methyl tertiary group attached to a quaternary carbon (C-10) further substituted by an oxygen function (acetyl group) these assignments were supported by HMBC $H_3$-14 to C-10, C-9 and C-1. The presence of this quaternary carbon at $\delta_C$ 62.27 exhibited that the methyl and acetyl groups were attached to the same carbon. Moreover, correlations between the quaternary carbons, C-4 ($\delta_C$ 137.80) and C-10 ($\delta_C$ 86.43) and their neighbours made clear the total skeleton of guaianolide [21]. Structure 2 was further supported by $^1$H-H$^1$ COSY data while NOESY cross-peaks between H-6 and H-7, between H-8 and H-5 and H-9β and between H-7 and H-9α supported the relative configuration. Consequently, the structure of sesquiterpene lactone was elucidated as byrsonina D.

Compound 3 was related to the molecular formula of $C_{24}H_{30}O_8$ by HR-ESIMS. Its NMR data was closely similar to that compound 2, except for a signal corresponding to an acetyl group at C-2. The C-2 carbon experiencing lowfield shift from $\delta_C$ 22.88 to $\delta_C$ 76.68 on acetylation. The C-15 protons and the C-6 and C-8 protons all correlations to each other, indicating that they have syn-orientations to each other. As evident

from the occurrence of one-proton $\delta_H$ 5.44 coupled according to COSY to H-1 and H-3, characteristic for an acetyl proton. Acetylation of C-2 modified multiplicity of the signals of H-3 (dq, J=1.1 Hz,) and H-1 (brt, J=9.0 Hz). Therefore, compound 3 was named as byrsonina E.

The molecular formula $C_{23}H_{24}O_6$, of the compound 4 was established by HRESIMS *m/z* 549.2483). The $^1$H and $^{13}$C NMR spectra were very similar to those of compound 1 (byrsonina C), signals unlike for C-8, C-9 and C-10. Angeloyloxy group was replaced by an acetyl group. Its $^{13}$C NMR spectrum revealed the occurrence of a tetra-substituted double bond ($\delta_C$ 139.92 and 134.81), an exomethylene ($\delta_C$ 135.24 and 170.99), three carbonyl ($\delta_C$ 121.03, 168.78 and 124.21) a carboxylic ester signal of an acetyl group ($\delta_C$ 170.71). The NOE correlation between the methyl of the acetyl group and H-6 indicated that the acetyl group is substituted at C-8. NOE cross peaks between H-6 and H-8 suggest that they have a β-orientation. The spectrum COSY showed correlation between H-8 and H-9a, H-8 and H-9, H-9a and H-14 methyl signal. The coupling constants for 4 ($J_{5,6}$ 10.5 Hz, $J_{7,8}$ 10.2 Hz) which have 5H-α, 6H-β, 7H-α and 8H-β orientation closely resembled those for 8-O-methylsenecioylaustricin [20]. The values obtained for the vicinal coupling between H-$_{8\beta,9a}$ 3.8 Hz and H$_{8\beta,9\beta}$ 2.3 Hz suggest a configuration of the C-8 *trans*-diaxial, since no vicinal coupling larger than 9 Hz has

| Inducer | Treatment | AGEs $IC_{50}$ (μM) |
|---|---|---|
| Glucose | 1 | 619 ± 8.32[a***] |
| | 2 | 593 ± 7.45[b] |
| | 3 | 545 ± 10.43 |
| | 4 | 712 ± 15.21[c**] |
| | 5 | 1003 ± 25.28[c*] |
| | 6 | 815 ± 7.87[a**] |
| | 7 | 1065 ± 23.65[c*] |
| | 8 | 512 ± 18.23 |
| | Aminoguanidine | 482 ± 43.21 |
| | Phloroglucinol | 553 ± 29.42 |
| Methylglyoxal | 1 | 1169 ± 12.45[a] |
| | 2 | 1032 ± 21.67 |
| | 3 | 986 ± 19.54 |
| | 4 | 1305 ± 46.28[b**] |
| | 5 | 1701 ± 52.34[a**] |
| | 6 | 1659 ± 48.64[c***] |
| | 7 | 1824 ± 38.90[b*] |
| | 8 | 875 ± 19.76 |
| | Aminoguanidine | 910 ± 20.37 |
| | Phloroglucinol | 1019 ± 32.48 |
| | | % |
| Lysozyme/ribose | 1 | 47.2 ± 5.43[c] |
| | 2 | 51.9 ± 4.86 |
| | 3 | 57.7 ± 3.98 |
| | 4 | 42.5 ± 4.65[a] |
| | 5 | 36.3 ± 4.75[a] |
| | 6 | 30.9 ± 1.98[b] |
| | 7 | 29.8 ± 3.56[a] |
| | 8 | 60.3 ± 2.74 |
| | Pyridoxamine | 58.3 ± 5.10 |

Data are mean ± standard deviation of triplicate tests. The fluorescence intensity was measured at ex 370 nm and em 470 nm. The intensity of each blank was subtracted from the intensity of each sample. Concentration of an inhibitor required to inhibit 50% of the control. Calculated from linear regression equation. [a]p<0.05, [b]p<0.01, [c]p< 0.001; vs control aminoguanidine or [*]p<0.05, [**]p<0.01, [***]p<0.001; vs control controlphloroglucinol or orpyridoxamine n.

**Table 1:** The inhibitory effects of 1-8 and aminoguanidine on the formation of advanced glycation end products (AGEs), *in vitro* induced by glucose, methylglioxal and ribose.

been observed for 4, so acetyl group is a α-substituent. An HMBC experiment was used to confirm the attachment of acetyl group, a correlation was observed for $H_3$14/C8, $H_3$14/C9, $H_3$14/C1, $H_3$14/C10. Thus, compound 4 was identified as byrsonina F.

Although the NMR spectral data of compound 5 were essentially identical with those for 4 but only two carbonyl groups were detected, suggesting that in 5 the benzoyl group is absent. The structure was assigned as byrsonina G.

The $^1$H NMR and $^{13}$C NMR data gave evidence that compound 6 was analogue of 1. They only significant differences in the missing of signals at $\delta_H$ 1.46 (H-8) in 6. The finding of the HMBC were finally confirmed by the result of COSY experiments, in which coupling between the proton at $\delta_H$ 4.95 (ddd, J=4.1, 10.9, 1.5 Hz, H-8) and H-7 and H-9α were found. This indicated that one acetyl moiety was attached to C-8. Thus, the structure of compound 6 was identified as byrsonina H.

Compound 7 its IR spectrum displayed absorption bands at 1770 and 1710 cm$^{-1}$ assignable to lactone and unsaturated ketone functions. As deduced from HREIMS and $^1$H NMR and $^{13}$C NMR data, 7 had a molecular formula of $C_{14}H_{16}O_3$, which gave very similar chemical shifts with 5, however, analysis of $^1$H NMR of 7 showed a quite different chemical shift at H-2 ($\delta_H$ 2.21, m for 5) this signal was not present in 7, and, instead the compound was another guaianolide with a C-1 at $\delta_C$ 134.46 and C-2 ketone function at $\delta_C$ 194.35, but lacking the signals due to the acetyl or angeloyl moiety. One double bond at C-1 and C-10 was confirmed in the HMBC spectrum, with correlations between H-14 and C-1, C-2; H-3 with C-1 ($\delta_C$ 134.48), C-2 ($\delta_C$ 194.35; α,β-unsaturated

carbonyl) established the cyclopentenone fragment. No characteristic acetyl signals were observed in the NMR spectrum of 7. From these data 7 was named byrsonina I.

Compound 8 was obtained as a colourless oil. The molecular formula $C_{22}H_{28}O_7$ was determined by HR-ESIMS analysis. The IR spectrum showed absorption bands at 3440 and 1749 cm$^{-1}$ assignable to hydroxyl and lactone functions respectively. $^{13}$C NMR and $^1$H NMR signals at $\delta_C$ 132. 55 (C-2), $\delta_C$ 140.87 (C-3), $\delta_H$ 5.92 (d, J=10.0 Hz, H-2) and $\delta_H$ 6.02 (d, J=10.0 Hz, H-3) indicated a double bond at C-2 to C-3. $^1$H NMR resonances at $\delta_H$ 1.77 (s), 1.91 (brd, J 7.5), and 6.06 (brq, J 7.5) suggest an angelate moiety. The position of the angeloxy group was confirmed at C-8 ($\delta_C$ 68.73) by the correlation between H-8 ($\delta_H$ 4.62) and C-1" ($\delta_C$ 167.74) in the HMBC spectrum. Two sharp three-proton singlet at $\delta_H$ 1.24, (s, H-14) and $\delta_H$ 1.42, (s, H-4) showed that the methyl groups could be assigned to a methyl group attached to a carbon further substituted by an oxygen function (hydroxyl group) indicating that positions 4 and 10 were fully substituted in accord with correlation of H-14 (1.24) with $\delta_C$ 77.45 assigned to C-10 in the HMBC spectrum. A cross-peaks of the proton at $\delta_H$ 2.68 (H-9b) with the hydroxyl substituted carbon at $\delta_C$ 77.45 (C-10), and C-1 were also observed. A singlet at $\delta_H$ 1.42 could be assigned to a methyl tertiary group attached to a quaternary carbon (C-4) further substituted by an acetyl group these assignments were supported by HMBC $H_3$-15 to C-4, C-3 and C-5. The structure of this guaianolide, designated therein as byrsonina J (all structures are shown in Figure 1b).



Byrsonina C (**1**)

Byrsonina D (**2**)

Byrsonina E (**3**)

Byrsonina F (**4**)

Byrsonina G (**5**)

Byrsonina H (**6**)

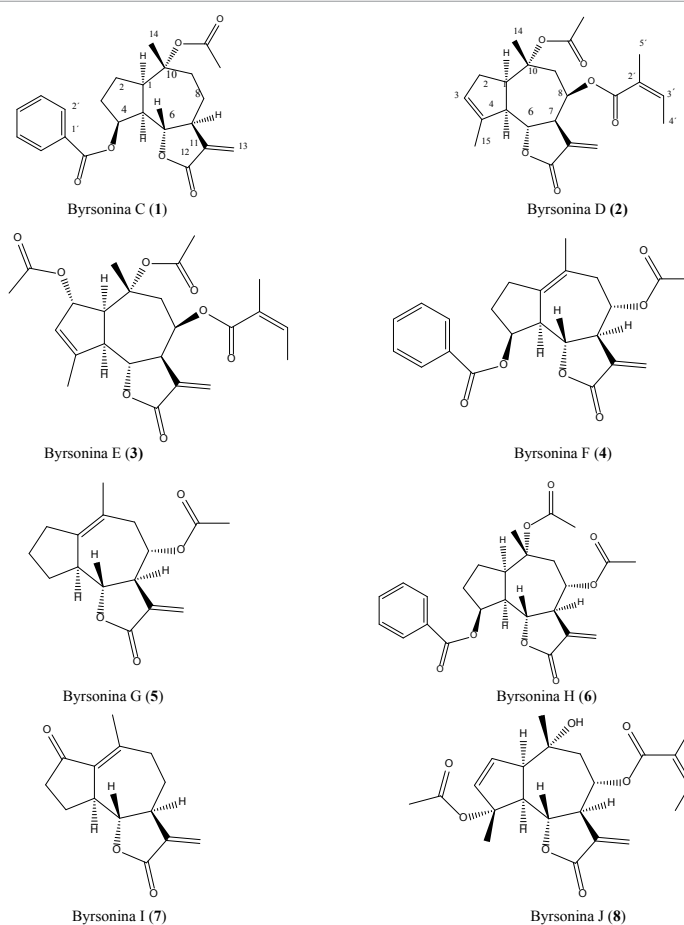Byrsonina I (**7**)

Byrsonina J (**8**)

**Figure 1b:** Compounds isolated from *Byrsonima crassifoli*.

## Effect of guaianolides in protein glycation

In this study, several methods assay have been proposed to determine the inhibitory effect of 1-8 on AGEs formation, including assays based on the inhibition of advanced glycation end products (AGEs) cross-linking and monitoring the production of fluorescent products which is characteristic of AGEs.

Guaianolides, phloroglucinol and AG showed inhibition against AGEs formation of BSA with glucose, with an $IC_{50}$ value range of 0.512 to 1.654 µM /ml (Table 1). Figure 2 exhibit inhibitory activity of compounds 1-8 on BSA glycation at 1 µM. The BSA-glucose assay is a useful tool for determine the effects of guaianolides on the nonenzymatic glycation process. Compounds 1-4 and 8 present inhibitory activity on AGE formation in this model.

Methylglyoxal (MG) is a reactive dicarbonyl, formed during glycolysis is a precursor of AGEs formation and triggers of oxidative stress. Methylglyoxal-BSA glycation inhibition was evaluated for 1-8 which show significant activity ($p<0.05$), compared with phloroglucinol and aminoguanidine (Table 1). However, activity of 1-8 on the formation of AGEs induced by ribose exhibited a significant inhibition ($p<0.05$) compared to AG. In all experiments, the compound 8 was the most active followed by 3, 2, 1, 4 and 6, 5, 7 as the less actives. Oxidative stress and the formation of AGEs contribute to cellular aging, so increased MG and AGEs cause accelerated cellular aging. MG is double-edged sword due that is a potent inducer of oxidative stress [22], and it is a major precursor of AGEs formation. AGEs also induce oxidative stress.

Rearrangement of the Schiff base to form stable complexes called Amadori product which are considered an important route to AGEs formation that has been implicated in diabetes complications. Terpenes act as inhibitors of this conversion of Amadori intermediates to AGEs. Sesquiterpene lactones 8, 3, 2, and 1 show considerable like activity at the AG, however the compounds 4, 5 and 7 have moderate Amadorin activity (Table 1). Table 1 presents the ability of the compounds 1-8 to inhibit the crosslinking of lysozyme in the presence of ribose.

## Glycation of hemoglobin

HbA1c, is an Amadori product of the reaction glucose-hemoglobin, is used as an indicator of metabolic control of diabetes being that glycohemoglobin levels increased in diabetics [23]. Glycated hemoglobin (GHb) is formed by non-enzymatic process of reaction of aldehyde group of hexoses with the amino-terminal group of hemoglobin [24]. Table 2 shows the amount of GHb and HbA1c. These results indicates that 1-4 and 8 have a potent glycation inhibition activity
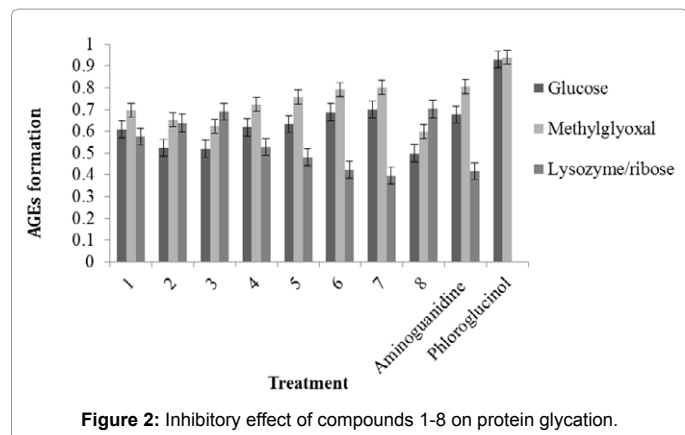
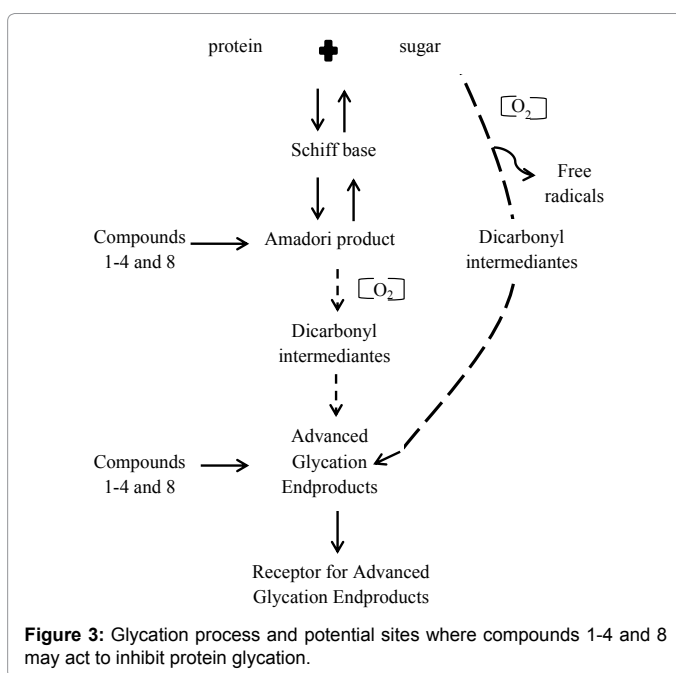| Groups | GHb (%) | HbA$_{1c}$ | Glycated protein (nmol/mg protein) |
|---|---|---|---|
| Negative Control | 8.9 ± 0.06 | 7.9 ± 0.98 | 15.3 ± 1.47 |
| Positive control | 27.6 ± 1.34 | 17.5 ± 1.56 | 23.7 ± 2.19 |
| 1 | 17.1 ± 0.30[a] | 13.6 ± 1.59[a] | 19.1 ± 2.04[a] |
| 2 | 16.2 ± 0.36[a] | 12.6 ± 0.83[a] | 18.0 ± 3.11[a] |
| 3 | 15.8 ± 0.19[a] | 11.9 ± 0.52[a] | 18.8 ±1.57[a] |
| 4 | 18.4 ± 0.14[a] | 14.4 ± 0.73[a] | 19.8 ± 1.43[a] |
| 5 | 20.8 ± 0.38[a] | 15.1 ± 0.31[a] | 20.3 ± 1.43[a] |
| 6 | 22.3 ± 0.59[a] | 16.0 ± 1.86[a] | 20.9 ± 2.28[a] |
| 7 | 23.9 ± 0.87[a] | 16.9 ± 1.25[a] | 21.7 ± 3.19[a] |
| 8 | 14.7 ± 1.34[a] | 11.0 ± 0.17[a] | 17.4 ± 1.64[a] |
| Glutathione | 8.1 ± 0.08[a] | 9.0 ± 0.67[a] | - |
| Aminoguanidine | 15.1 ± 0.67[a] | 13.1± 0.45[a] | 20.4 ± 1.87[a] |

Negative control: Incubation with hemoglobin (0.468 mMol/ml), positive control: Incubation with hemoglobin (0.468 mMol/ml)+glucose (0.278 mM), guaianolides Incubation with hemoglobin (0.468 mMol/ dl)+glucose (0.278 mM)+guaianolides (6.57 mMol/ml), glutathione: Incubation with hemoglobin (0.468 mMol/ dl)+glucose (2.7 mM)+glutathion (0.5 mM). Data as expressed as ± SD; [a]P<0.05 vs positive control values.
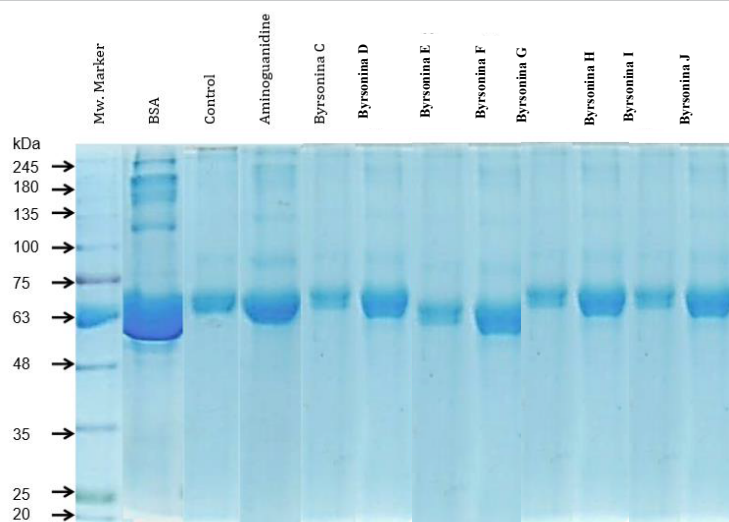
**Table 2:** The inhibitory effects of 1-8 on glycosylated protein, hemoglobin GHb and HbA$_{1c}$.

| Compound | Fluorescence (340/430 nm) |
|---|---|
| Control | |
| 1 | 1019 ± 32.18[b] |
| 2 | 1001 ± 24.66 |
| 3 | 943 ± 19.56 |
| 4 | 1167 ± 34.12 [b,] |
| 5 | 1282 ± 41.28[c] |
| 6 | 1201± 32.17 [a,] |
| 7 | 1310 ± 28.17 [b] |
| 8 | 876 ± 25.76 |
| AG | 942 ± 16.51 |

Data are mean ± standard deviation of triplicate tests. Fluorescence of samples was measured at excitation 330 and emission 415 nm. AG (aminoguanidine) positive control at 10 µM. [a]p< 0.05, [b]p< 0.01, [c]p< 0.001; vs control aminoguanidine.

**Table 3:** Inhibitory effect of 1-8 on the last stage of protein glycation.



**Figure 2:** Inhibitory effect of compounds 1-8 on protein glycation.



**Figure 3:** Glycation process and potential sites where compounds 1-4 and 8 may act to inhibit protein glycation.

**Figure 4:** SDS-PAGE Coomassie stained gel profile of BSA incubated with/without glucose and in the presence or absence of aminoguanidine, Byrsonina C to Byrsonina J.
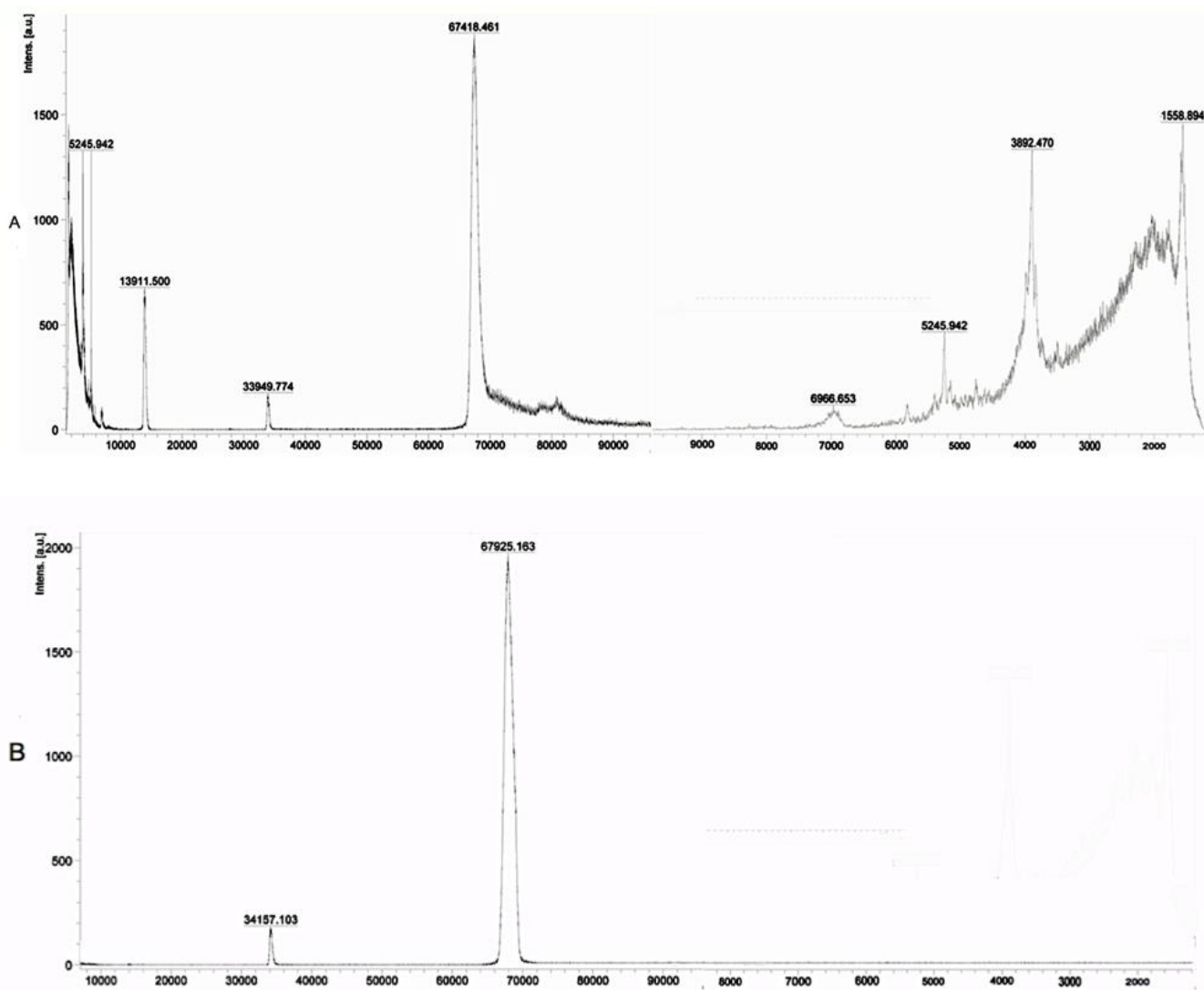


**Figure 5:** Positive ion MALDI linear TOF MS, with the standard (sinapic acid) (a) SEM detector and (b) BSA.
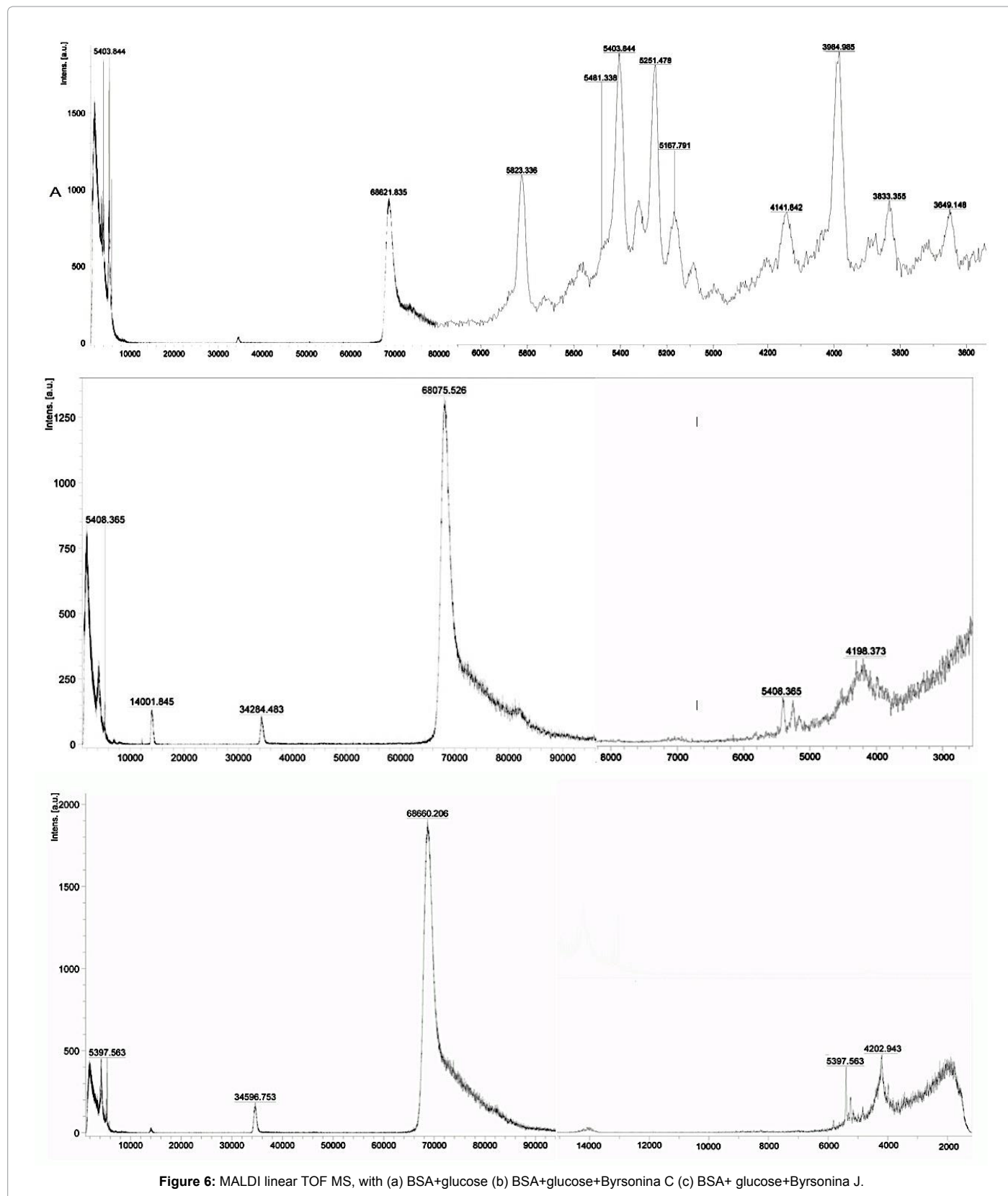
**Figure 6:** MALDI linear TOF MS, with (a) BSA+glucose (b) BSA+glucose+Byrsonina C (c) BSA+ glucose+Byrsonina J.

at the early stage of hemoglobin glycation at a concentration, of 10 mM/ml. Compounds 8, 3, and 2 had the most potent inhibitory effect with values of 37.1%, 32% and 28% respectively compared with the positive control ($p<0.05$). These compounds were more effective than that of AG (25.1%, 10 mM), indicating that can effectively prevent GHb and $HbA_{1C}$ formation. Carboxymethyl-lysine (CML) is an advanced glycation end product formed on protein as glyco-oxidation product which is not fluorescent and not reactive [25]. CML is an indicator of the advanced stages of the Maillard reaction and are biomarkers of more extensive underlying glycative and oxidative damage to the protein. As shown in Table 3, compound 8 exhibited a potent anti-cross-linking activity in G.K. peptide-D-ribose model system. Potential sites where the most effective compounds inhibit protein glycation were indicated in Figure 3. These observations suggested that compounds 1-4, and 8 can inhibit the glycoxidative modification of proteins.

## SDS-Page profile of guaianolides on protein

SD-PAGE profiles for control, BSA-guaianolides and BSA-AG. BSA-glucose produces cross-linking to give dimers into high molecular weight polymers which are shown in Figure 4.

BSA cross-linking by glucose was inhibited by the addition of guaianolides. Polymers of high molecular weight were formed in a very small amount in the presence of AG and guaianolides as compared to the control inhibiting crosslinked advanced glycation endproducts causing a reduction in intensity of the dimerised peaks. This study confirms the capacity of guaianolides to avoid formation of crosslinked advanced glycation end-products *in vitro*. Due to the above suggest that guaianolides can be mainly attributed to the ability of compounds to react with carbonyls groups. While AG can inhibit through competitive binding to glucose as well as inhibiting of carbonyl formation [26].

## Glycation of guaianolides monitored by MALDI linear TOF MS

Glycation of guaianolides was monitored by MALDI linear TOF MS. (Figure 5) exhibit mass spectra of BSA (a) and shows the typical mass spectra of positive of ion MALDI linear TOF MS with the sinapic acid matrix (b). In Figure 6, we can observe a typical spectrum of glycated BSA where glycation of the protein was performed by incubation with glucose and Byrsonines C and J shows a different mass profile, with a relative abundance of each species. The main band at *m/z* 67925.163 correspond to native BSA whereas the second peak at *m/z* 3999.656 corresponds to a diglycated form of BSA. A third minor band at *m/z* 5397.565 may indicated a protonated monoglycated form. The small band at *m/z* 4202.943 correspond to the protonated diglycated and triglycated forms. Through MALDI linear TOF MS is established that guaianolides inhibit the glycation process likely through competitive binding to glucose.

## Conclusions

The guaianolides isolated from seeds of *Byrsonima crassifolia* were effective for glycation inhibitory activity using different in vitro glycation assays. The antiglycation effect was due to the ability to react with dicarbonyl intermediates being the major mechanism for protein glycation inhibition. However, our results showed that compounds 1-4 and 8 also react with Amadori adducts blocking their conversion to AGEs. Based in these results, we suggest that the terpenes may prevent or improve the AGE associated chronic conditions as diabetes complications.

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