Inhibitory Activities of Guaiananolides 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 guaiananolides. Structural elucidation was established on the basis of spectral data as, byrsonima C (1) to byrsonima 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. Guaiananolides 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 guaiananolides 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 crassifolia, Guaiananolides, 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 groups in proteins. They are a group of complex and heterogeneous 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 guaiananolides 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 FTIR 1720X. NMR experiments were obtained on a Bruker DRX-400. The NMR data were processed using UTXNMR 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 rotary 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-F6) 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 (F111-1 to F111-6). The F111-1 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).

Byrsonima 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 C23H26O6); 1H NMR (500 MHZ, CDCl3) δ: 1.36 (1H, m, 7 (80 mg), and 8 (59 mg)).

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H-1), 1.74 (2H, m, H-2), 1.48 (2H, m, H-3), 5.30 (1H, dd, 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, dd, 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’), 6.11 (1H, t, J=7.6 Hz, H-4’), 2.20 (3H, s, COOME); \(^1^C\) NMR (100 MHz, CDCl\(_3\)) \& 58.21 (C-1), 22.88 (C-2), 32.12 (C-3), 68.17 (C-4), 50.65 (C-5), 57.51 (C-6), 68.18 (C-7), 30.41 (C-8), 86.51 (C-9), 137.65 (C-11), 173.56 (C-12), 121.03 (C-13), 16.06 (C-14), 167.88 (COO), 129.21 (C-1), 130.98 (C-2’, 6’), 132.47 (C-4’), 21.10 (COOME), 173.45 (COOMe).

**Figure 1a:** Extraction, isolation and characterization of the sesquiterpene lactones.
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); 13C NMR (100 MHz, CDCl3) δ: 149.23 (C-1), 197.72 (C-2), 83.20 (C-4), 48.89 (C-5), 68.62 (C-6), 58.97 (C-7), 68.15 (C-8), 77.45 (C-10), 135.24 (C-11), 173.56 (C-12), 124.21 (C-13), 16.08 (C-14), 22.86 (COOMe), 173.31 (C-16), 20.51 (C-5').

**Byrsonima J (8):** IR (KBr) vmax 3440, 1749 (γ-lactone), 1731 (benzyol), 1652 (C=C), 1459, 1373, 1238 (acetate), 1163, 1097, 1045, 756 cm⁻¹; HR-ESIMS: m/z 404.432 (calcd., 404.765 for C22H28O7): 1H 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=10.0 Hz, H-6), 2.63 (1H, ddd, J=3.0,13 Hz, H-7), 4.62 (1H, ddd, J=8.0, 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'); 13C 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), 68.62 (C-6), 58.97 (C-7), 68.15 (C-8), 77.45 (C-10), 135.24 (C-11), 170.99 (C-12), 120.83 (C-13), 168.13 (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 (0.0 mg/ml) was incubated at 37°C in 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 wavelength 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 phosphogluconol 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 l 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 HbA₁ were determined by ion exchange resin method glyco hemoglobin kit (Pariksha Biotech, India).

**Na-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 were diluted with an equal volume of sodium dodecyl sulfate (SDS), terpenes and buffer (2% w/v SDS, 30% w/v glycerol, 0.25M Tris hydroxymethylamine, 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 sample was combined (1:1) with sinapic acid matrix solution 5 mg/ml. Mass spectra were obtained 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 C₁₆H₁₈O₁₀ 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 8C 173.36 (C-12), 137.65 (C-H), 68.39 (C-6) and also by the characteristic 1H NMR.
Correlations observed between H-13 and H-6, revealed that they are oriented toward β-face. The coupling constant $J_{1,3}$, $J_{1,4}$ and $J_{1,6}$ (9.4 Hz) show that has an A.B cis-fused guaianolide skeleton with trans-diaxial disposition of H-6 (β) and H-7 (α). One acetoxy and one benzoxylo group were clearly distinguishable in the NMR spectrum. The relative downfield shift of H-4 (δ 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 δ C 167.89. NOE 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-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 δ C 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 δ 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 δ H 6.06 (H-3', brq, J=7.5 Hz) and at δ C 1.85 (H-5', br, s). The position of the angeloyloxy group was deduced to be at C-8 (δ C 68.18) by correlation between H-8 (6.06) and C-1' (δ C 167.83) in the HMBC spectrum. The configuration of the angatele 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 δ C 4.55 so, angeloyloxy moiety must be at C-8 since the small coupling constant value of $J_{2,3}$ 3 Hz required an α-orientation of H-8, which was further confirmed by NOE experiments. The position of the angatele group was confirmed at C-8 (δ C 68.18) by the correlation between H-8 (δ C 4.55) and C-1' (δ C 167.83) in the HMBC spectrum. A singlet at δ C 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 δ C 62.27 exhibited that the methyl and acetyl groups were attached to the same carbon. Moreover, correlations between the quaternary carbons, C-4 (δ C 137.80) and C-10 (δ C 86.43) and their neighbours made clear the total skeleton of guaianolide [21]. Structure 2 was further supported by ‘$^1$H’$^1$C COSY data while NOESY cross-peaks between H-6 and H-7, between H-8 and H-5 and H-9 and between H-7 and H-9a supported the relative configuration. Consequently, the structure of sesquiterpene lactone was elucidated as byronisone D.

Compound 3 was related to the molecular formula of C$_{23}$H$_{24}$O$_{6}$ by HR-ESIMS. Its NMR data was closely similar to that compound 1 except for a signal corresponding to an acetyl group at C-2. The C-2 carbon experiencing lowfield shift from δ C 22.88 to δ 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 δ C 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 byronisone E.

The molecular formula C$_{24}$H$_{30}$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 (δ C 139.92 and 134.81), an exomethylen (δ C 135.24 and 170.99), three carbonyl (δ C 121.03, 168.78 and 124.21) a carboxyl ester signal of an acetyl group (δ 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$_5,10$ 10.2 Hz) which have 5H-α, 6H-β, 7H-α and 8H-β orientation closely resembled those for 4-O-methylsesquioxyaceticrinic [20]. The values obtained for the vicinal coupling between H-7, 3.8 Hz and H$_{5,6}$ 2.3 Hz suggest a configuration of the C-8 trans-diaxial, since no vicinal coupling larger than 9 Hz has been observed.

| Inducer          | Treatment | AGEs (µM) | IC$_{50}$ (µM) |
|------------------|-----------|-----------|---------------|
| Glucose          |           |           |               |
| 1                | 619 ± 8.32** |
| 2                | 593 ± 7.45*  |
| 3                | 545 ± 10.43 |
| 4                | 712 ± 15.21** |
| 5                | 1003 ± 25.28*  |
| 6                | 815 ± 7.87*  |
| 7                | 1065 ± 23.65*  |
| 8                | 512 ± 18.23  |
| Aminoguanidine   |           |           |               |
| 1                | 482 ± 43.21 |
| 2                | 553 ± 29.42 |
| 3                | 1169 ± 12.45*  |
| 4                | 1032 ± 21.67 |
| 5                | 986 ± 19.54  |
| 6                | 1305 ± 46.28** |
| 7                | 1701 ± 52.34*  |
| 8                | 1659 ± 46.64** |
| 9                | 1824 ± 38.90** |
| 10               | 875 ± 19.76  |
| Aminoguanidine   |           |           |               |
| 1                | 910 ± 20.37  |
| 2                | 1019 ± 32.48  |
| 3                | 47.2 ± 5.43*  |
| 4                | 51.9 ± 4.86  |
| 5                | 57.7 ± 3.98  |
| 6                | 42.5 ± 4.65*  |
| 7                | 36.3 ± 4.75*  |
| 8                | 30.9 ± 1.98*  |
| 9                | 29.8 ± 3.56*  |
| 10               | 60.3 ± 2.74  |
| Phloroglucinol   |           |           |               |
| 1                | 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. *p<0.05, **p<0.01, ***p< 0.001; vs control aminoguanidine or *p<0.05, **p<0.01, ***p<0.001; vs control controlphloroglucinol or phloridzinexamine n.

| Inducer          | Treatment | AGEs (µM) | IC$_{50}$ (µM) |
|------------------|-----------|-----------|---------------|
| Lysozyme/ribose  |           |           |               |
| 1                | 47.2 ± 5.43*  |
| 2                | 51.9 ± 4.86  |
| 3                | 57.7 ± 3.98  |
| 4                | 42.5 ± 4.65*  |
| 5                | 36.3 ± 4.75*  |
| 6                | 30.9 ± 1.98*  |
| 7                | 29.8 ± 3.56*  |
| 8                | 60.3 ± 2.74  |
| Pyridoxamine     |           |           |               |
| 1                | 58.3 ± 5.10  |
be 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_14/C8, H_14/C9, H_14/C1, H_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 1H NMR and 13C NMR data gave evidence that compound 6 was analogue of 1. They only significant differences in the missing of signals at δ_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 δ_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 1H NMR and 13C 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 1H NMR of 7 showed a quite different chemical shift at δ_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 δ_C 134.46 and C-2 ketone function at δ_C 194.35, but lacking the signals due to the acetyl or angeloyl moiety. One double bond at C-2 to C-3. 1H NMR resonances at δ_H 1.46, 5.92 (d, J=10.0 Hz, H-2) and δ_H 6.02 (d, J=10.0 Hz, H-3) indicated a double bond at C-2 to C-3. 1H NMR resonances at δ_H 1.77 (s), 1.91 (brd, J 7.5, H-2) and 6.06 (brq, J 7.5) suggest an angelate moiety. The position of the angeloyl group was confirmed at C-8 (δ_C 68.73) by the correlation between H-8 (δ_H 4.62) with the correlation between H-8 (δ_H 4.62) and C-1 (δ_C 167.74) in the HMBC spectrum. Two sharp three-proton singlet at δ_H 1.24, (s, H-14) and δ_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 the chemical shifts at δ_C 77.45 assigned to C-10 in the HMBC spectrum. A cross-peaks of the proton at δ_H 2.68 (H-9b) with the hydroxyl substituted carbon at δ_C 77.45 (C-10), and C-1 were also observed. A singlet at δ_H 1.42 could be assigned to a methyl tertiary group attached to a quaternary carbon (C-4) these assignments were supported by HMBC H-3 to C-4, C-3 and C-5. The structure of this guaianolide, designated therein as 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. 13C NMR and 1H NMR signals at δ_C 132.55 (C-2), δ_C 140.87 (C-3), δ_H 5.92 (d, J=10.0 Hz, H-2) and δ_H 6.02 (d, J=10.0 Hz, H-3) indicated a double bond at C-2 to C-3. 1H NMR of 7 showed a quite different chemical shift at δ_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 δ_C 134.46 and C-2 ketone function at δ_C 194.35, but lacking the signals due to the acetyl or angeloyl moiety. One double bond at C-2 to C-3. 1H NMR resonances at δ_H 1.46, 5.92 (d, J=10.0 Hz, H-2) and δ_H 6.02 (d, J=10.0 Hz, H-3) indicated a double bond at C-2 to C-3. 1H NMR resonances at δ_H 1.77 (s), 1.91 (brd, J 7.5, H-2) and 6.06 (brq, J 7.5) suggest an angelate moiety. The position of the angeloyl group was confirmed at C-8 (δ_C 68.73) by the correlation between H-8 (δ_H 4.62) with the correlation between H-8 (δ_H 4.62) and C-1 (δ_C 167.74) in the HMBC spectrum. Two sharp three-proton singlet at δ_H 1.24, (s, H-14) and δ_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 the chemical shifts at δ_C 77.45 assigned to C-10 in the HMBC spectrum. A cross-peaks of the proton at δ_H 2.68 (H-9b) with the hydroxyl substituted carbon at δ_C 77.45 (C-10), and C-1 were also observed. A singlet at δ_H 1.42 could be assigned to a methyl tertiary group attached to a quaternary carbon (C-4) these assignments were supported by HMBC H-3 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).

![Figure 1b: Compounds isolated from Byrsonima crassifoli.](image-url)
**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. Amadori product which are considered an important route to AGEs formation that has been implicated in diabetes complications. Terpenes may act to inhibit protein glycation.

**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 (GHb) is formed by non-enzymatic process of reaction glucose-

| Groups | GHB (%) | HbA1c  | 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* | 13.6 ± 1.59* | 19.1 ± 2.04* |
| 2 | 16.2 ± 0.36* | 12.6 ± 0.83* | 18.0 ± 3.11* |
| 3 | 15.6 ± 0.19* | 11.9 ± 0.52* | 18.8 ± 1.57* |
| 4 | 18.4 ± 0.41* | 14.4 ± 0.73* | 19.8 ± 1.43* |
| 5 | 20.8 ± 0.38* | 15.1 ± 0.31* | 20.3 ± 1.43* |
| 6 | 22.3 ± 0.59* | 16.0 ± 1.86* | 20.9 ± 2.28* |
| 7 | 23.9 ± 0.87* | 16.9 ± 1.25* | 21.7 ± 1.19* |
| 8 | 14.7 ± 1.34* | 11.0 ± 0.17* | 17.4 ± 1.64* |
| Aminoguanidine | 15.1 ± 0.67* | 13.1 ± 0.45* | 20.4 ± 1.87* |

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

Glycation process and potential sites where compounds 1-4 and 8 may act to inhibit 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.

**Table 2:** The inhibitory effects of 1-8 on glycosylated protein, hemoglobin GHb and HbA1c.

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

**References:**

[22] G. M. and R. A. M. (2015) Inhibitory Activities of Guaiianolides from the Seeds of Byrsonima Crassifolia against Protein Glycation In Vitro. Med chem 5: 217-225. doi:10.4172/2161-0444.1000267

**Citation:** Perez-Gutierrez RM, Muñiz-Ramirez A, Anaya-Sosa I, Cruz-Victoria T, Mota-Flores JM (2015) Inhibitory Activities of Guaiianolides from the Seeds of Byrsonima Crassifolia against Protein Glycation In Vitro. Med chem 5: 217-225. doi:10.4172/2161-0444.1000267
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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.
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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 3, 8, 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 HbA1c formation. Carboxymethyl-lysine (CML) is an advanced glycation end product formed in 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 ion MALDI linear TOF MS with the sinapic acid matrix (b). In Figure 6, we can observe a typical spectrum of glycated BSA by the conjugate with a protonated form. The small band at m/z 6470 of AG corresponds to the protonated monoglycated form. The small band at m/z 6470 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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