Antioxidant activity of a new multiflorane-type triterpene from *Cucurbita argyrosperma* seeds and their protective role in hydrogen peroxide induced oxidative stress

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Submission Date: December 27th, 2019; **Acceptance Date:** February 25th, 2020; **Publication Date:** February 28th, 2020

Citation: Gutierrez R.M.P., Ramirez A.M., Flores J.M.M., Campoy A.H.G. Antioxidant activity of a new multiflorane-type triterpene from *Cucurbita argyrosperma* seeds and their protective role in hydrogen peroxide induced oxidative stress. *Functional Foods in Health and Disease* 2020; 10(2): 95-111. DOI: https:/doi.org/10.31989/ffhd.v10i2.680

ABSTRACT

Background: *Cucurbita argyrosperma* seeds have acquired a reputation as an herbal remedy to treat various diseases because this plant is a predominant source of natural compounds with powerful anti-inflammatory and antioxidant properties, and supplementation with seeds improves oxidative stress. Previous studies indicated that an imbalance between H_2O_2 production and elimination capacity is responsible for β-cell vulnerability thereby making β-cells a susceptible target for pathogens.

The aim of this investigation was to evaluate the protective effects of one new multiflorane-type triterpene 3β-trans-caffeoyloxymultiflor-8-ene- 7α,12β, 18 β-triol (**1**) from MeOH extract from *C. argyrosperma,* on rat pancreatic β cells (INS-1 cells) exposed to hydrogen peroxide (H_2O_2) induced oxidative stress conditions.

Methods: The chemical structure of the novel triterpene, which was identified as 3β-trans-caffeoyloxymultiflor-8-ene- 7α,12β, 18 β-triol (**1**), was established based on the interpretation of spectroscopic analyses. The antioxidant activities of **1** were led by the detected radical scavenging potential of 2,2-dyphenyl-1-picrylhydrazyl (DPPH) and 3.1 2,2′-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) ABTS. The assays were conducted on INS-1 cell lines exposed to increasing concentrations of **1** at 5, 10 and 20 µg/mL and H_2O_2 at 250 µM. Then the experiments, cell viability, cell integrity (LDH; lactate dehydrogenase release), mitochondrial

function (ATP analysis), ROS formation, lipid peroxidation (MDA), and caspase-3, 9 activities were measured in the cells. We also determined the effect of **1** on antioxidant enzyme levels and cytotoxicity in pancreatic β cells under oxidant conditions.

Results: The results showed that triterpene displayed high free-radical-scavenging activity, which is similar to that of standard antioxidants used. Concentrations of 5, 10, and 20 μg/mL protected INS-1 cells against H_2O_2 induced cytotoxicity with a decrease in cell death and a marked increase in cell viability as well as sustained cellular functionality (ATP). Antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reduced (GSH), catalase (CAT), superoxide dismutase (SOD), and the non-antioxidant enzyme (GSH) increased in INS-1 cells with **1** pre-treatment. MDA in pancreatic cells was ameliorated by **1** pre-treatment reducing intracellular reactive oxygen species level. Findings also demonstrated that H_2O_2 induced apoptosis in INS-1 cells and produced modulation of the caspase-3, 9 expressions in INS-1 cells exposed to **1**. Exposure to **1** significantly inhibited ROS and apoptosis, reducing β cell dysfunction under oxidant conditions.

Conclusions: Triterpene consequently could be a promising natural antioxidant for use in maintaining the integrity of pancreatic β-cells exposed to oxidative stress conditions and being able to participate in the control type 2 diabetes.

Keywords: *Cucurbita argyrosperma;* antioxidants; multiflorane; free radical scavenging: oxidative stress

INTRODUCTION

Antioxidants are substances that may prevent or delay some types of cell damage caused by reactive oxygen species (ROS), thereby preventing the appearance of many chronic diseases [1]. The imbalance between ROS production and antioxidant defense results in oxidative stress and the overproduction of reactive ROS can cause lipid peroxidation, protein denaturation and disruption of membrane fluidity, which can lead to cell damage and death [2]. ROS consists mainly of free radicals such as such nitric oxide (NO), superoxide (O2●), lipid peroxyl (LOO●), peroxyl (RO2-) and hydroxyl (OH-) and different form of activated oxygen such as lipid peroxide (LOOH), ozone (O_3), and oxygen (O^2-1). Endogenous ROS in physiologic concentrations regulate cell motility, survival, metabolism, and growth [3]. Accumulated intracellular ROS can cause oxidative stress and cell damage, thereby inducing injury to membranes, DNA, and macromolecules [3]. Thus, in several diseases, oxidative imbalance leads to an increase in the generation of ROS. The oxidative stress induced by hyperglycemia contributes to the damage and dysfunction of pancreatic β cells [4]. It is well studied that chronic exposure to high glucose concentrations leads to apoptosis and damage of pancreatic cells [6]. Thus, natural antioxidants can prevent the damage of oxidative stress associated with diabetes [5]. However, in response to the demand from consumers for food supplements that are free of synthetic antioxidants that have toxic potential, there is an overpowering trend to find natural sources of antioxidants, which is a way to avoid ROS-mediated cellular damage by increasing dietary antioxidant defense ability [7].

Cucurbita argyrosperma, commonly known as calabaza in Mexico, is a native plant of Mesoamerica. It is a member of the Cucurbitaceae family and is used in folk medicine as an

aqueous macerate of seeds in the treatment of symptomatic micturition disorders, diseases of the prostate, diabetes, and inflammatory diseases [8]. In previous studies, it has been demonstrated to act as an inhibitor of the angiogenesis [9] ameliorating oxidative stress in H9c2 cardiomyocytes [10]. The present study aimed to investigate t he potential antioxidant effects of one new triterpenoid that has been isolated from *C. argyrosperma* and to evaluate the protective effect on INS-1 pancreatic β-cells exposed to high glucose and H_2O_2-induced oxidative stress.

METHODS AND MATERIALS

Generals

The IR spectra were obtained on an FT-IR spectrometer (Perkin Elmer). The NMR spectra were recorded on a Brucker AV-400 spectrometer with methyl silane (MS) as an internal standard. ^{1}H and ^{13}C chemical shifts are expressed on the δ scale as parts per million (ppm). High Resolution Mass spectrum (HREIMS*;* 70 ev) was recorded on a Finnigan-MAT95 mass spectrometer. Column chromatography was performed on silica gel (200-300 mesh; Merck, USA) and Sephadex LH-20 (Merck, USA). Fractions were monitored by TLC (Merck, USA) precoated silica gel sheets. All solvents used were purchased from Fermont (Mexico) and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), with an analytical grade.

Plant material

C. argyrosperma, was collected in the vicinity of Amecameca Mexico State, Mexico. The plant was identified and authenticated by Biol. Aurora Chamal, Department of Botany, National School of Biological Sciences, National Polytechnic Institute, where a voucher specimen (No. 8054) has been deposited for further reference.

Extraction and isolation

Powdered air-dried seeds from *Cucurbita argyrosperma* (5 kg) were macerated with hexane (to degrease) and then with MeOH. Each maceration (two) was performed for 5 days (20 L) at room temperature and dried under reduced pressure to yield a green gum (300 g), which was fractionated in water. For that purpose, the methanol extract was dissolved in water following vigorous agitation at 50 °C and allowed to stand for 30 min. Both fractions were evaluated for an antioxidant effect. Aqueous extract (CA) was analyzed by chromatography on a silica gel column (230-400 mesh; 100 x 0.50 cm) with dichloromethane: methanol (50:50 [v/v]) eluent system to provide 6 fractions (fractions CA1−CA6). Fraction CA6 was subjected to chromatography again on a silica gel column with ethyl acetate: methanol (80:20 [v/v]) as a mobile phase to afford 5 fractions (fractions CA61 −CA65). Compound 1 (76 mg) was isolated from subfraction CA65 by subsequent Sephadex LH-20 column chromatography using a chloroform/methanol gradient elution.

3β-trans-caffeoyloxymultiflor-8-ene- 7α,12β, 18 β-triol (1)

Amorphous solid; FTIR ν_{max} cm^{-1} 3377, 1698, 1640, 1610, 1514, 1377, 1231, 1171, 914 cm^{-1}; HREIMS *m/z* 636.4107 (calcd. $C_{39}H_{56}O_7$: 636.4026); ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H 1.74 (1H, m, H-1α), 1.09 (1H, m, H-1β), 1.67 (1H, m, 2 α), 1.67 (1Hβ, m, 2β), 4.62 (1Hβ, t, *J* = 5.5 Hz, H-3), 1.95 (1H, m, H-51.95α), 1.76 (1H, m, 6α), 1.62 (1H, m, 6β), 4.23 (1H, brs, H-7β), 4.89 (1H, d, J

= 5.3 Hz, H-12α), 2.12 (1H, m, H-15α), 1.51 (1H, m, H-15β), 1.52 (1H, m, H-16α), 1.64 (1H, m, H-16β), 1.93 (1H, m, H-19α), 1.57 (1H, m, H-19β), 1.46 (1H, m, H-21α), 1.59 (1H, m, H-21β), 1.85 (1H, m, H-22α), 0.98 (1H, m, H-22β), 0.88 (3H, s, H-23), 0.91 (3H, s, H-24), 0.82 (3H, s, H-25), 1.10 (3H, s, H-26), 1.08 (3H, s, H-27), 1.14 (3H, s, H-28), 1.21 (3H, s, H-29), 1.13 (3H, s, H-30), 7.1 (1H, d, *J* = 1.2 Hz, H-2ʿ), 6.79 (1H, d, J = 8.0 Hz, H-5ʿ), 6.98 (1H, dd, *J* = 8.0, 1.2 Hz, H-6ʿ), 7.51 (1H, d, *J* = 15.4 Hz, H-7ʿ), 6.20 (1H, d, *J* = 15.4 Hz, H-8ʿ); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 36.6 (C-1), 25.3 (C-2), 82.4 (C-3), 36.7 (C-4), 39.2 (C-5), 29.1 (C-6), 65.3 (C-7), 138.4 (C-8), 140.0 (C-9), 38.8 (C-10), 37.6 (C-11), 75.4 (C-12), 44.9 (C-13), 42.1 (C-14), 26.8 (C-15), 34.6 (C-16), 34.9 (C-17), 78.9 (C-18), 28.7 (C-19), 31.5 (C-20), 30.1 (C-21), 35.1 (C-22), 27.1 (C-23), 21.9 (C-24), 19.8 (C-25), 24.9 (C-26), 25.7 (C-27), 31.4 (C-28), 29.2 (C-29), 25.3 (C-30), 129 (C-1ʿ), 123.7 (C-2ʿ), 115.9 (C-3ʿ),146.5 (C-4ʿ),144.2 (C-5ʿ),115.2 (C-6ʿ),143.4 (C-7ʿ),116.7 (C-8ʿ),168.3 (C-9ʿ). For structure (Fig 1A) and key HMBC and COSY correlations, see **Fig. 1B**.

In vitro antioxidant assays

The scavenging activities of **1** were evaluated at various concentrations from 50–1200 µg/ml on DPPH and ABTS radical assays [11, 12].

Cell culture

Rat pancreatic β cells (INS-1 cells) were purchased from the American Type Culture Collection (Manassas, VA, USA), which were maintained in RPMI1640 medium (2 g/L glucose) 1% penicillin/streptomycin solution and supplemented with 10% FBS. Cells were incubated with 95% air and 5% CO_2 at 37°C. The culture medium was replaced every 3 days until the INS-1 cells reached a confluency of 90%. The cells were seeded in 12-well culture plates at 1×10^4 cells per well and incubated for 24h before treatment. All the assays were performed in triplicates.

Cell viability, integrity, and mitochondrial function

The INS-1 cells were cultured at a density of 2000 cells per well in 96-well plates and incubated for 24 h. After INS-1 cells were treated with 250 µM H_2O_2 alone or were pretreated with 5, 10, 20 µg/mL of **1** followed by the addition of 250 µM H_2O_2. After 24 h, 0.5 mg/m L of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis MI, USA) was added to each well and incubated for 2 h at 37°C. The supernatant was then removed, and the formazan crystals were solubilized with 150 µL DMSO. The absorbance was read at 490 nm on a Microplate Reader (Thermo Fisher Scientific, FL, USA). The viability was expressed as the percentage in each treatment group with reference to that of the non-treated control. Cell integrity was measured by LDH assay, determined as lactate dehydrogenase released in the culture medium. Briefly after treatment, 0.2 mL culture medium of each group was evaluated for LDH activity using a commercial kit (Cayman Chemical, MI, USA). Mitochondrial function was measured using cellular ATP analysis. Triterpene **1** was added (5, 10 20 µg/mL) to the culture medium and maintained for 24 h before cellular ATP analysis. 250 mM of $H_2O_2$was incorporated 2 or 4 h before the end of the experiment. Cellular ATP levels were then measured in both cells exposed to ALP and glucose using a fluorometric assay kit (Abcam, Boston, MA, USA) according to the manufacturer's instruction.

Evaluation of intracellular ROS with stimulation H_2O_2

1×10^5 INS-1 cells per well in 96-well plates were treated with 250 μM H_2O_2 alone or were pretreated for 24h with 5, 10, 20 μg/mL of **1** followed by addition of 250 μM H_2O_2. After 24h, the cells were reacted with 1 μM 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, St Louis MI, USA) at 37°C for 30 min. Fluorescence was measured at 485 nm of excitation and 527 nm of emission wavelengths on a microplate reader (Thermo Fisher Scientific, FL, USA).

Antioxidant enzymes and reduced glutathione

INS-1 cells seeded at a density of 5 × 105 cells/well were grown in RPMI-1640 for 24h. Then, the medium was replaced with **1** at 5, 10, and 20 μg/mL in the presence of 250 μM of the pro-oxidant agent H_2O_2. GSH, GPx, CAT, and SOD activities were performed according to manufacturer's assay kit instructions (Cayman Chemical Company, Michigan, USA). The activity was expressed in mg protein for all antioxidant enzymes.

Lipid peroxidation in INS-1 Cell Lines

Lipid peroxidation was measuring to TBARS using a Cayman TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions [13]. Lipid peroxides generated from polyunsaturated fatty acids are decomposed in a complex series of compounds, such as reactive carbonyl species, included MDA, which is used to evaluate lipid peroxidation at an absorbance of 532 nm. Results are expressed as μmol/μg protein.

Measurement of Caspase-3 and 9

To determine the activity of caspase-3 and caspase-9, an ELISA kit was used in accordance with manufacturer's protocol (Sigma-Aldrich, St. Louis, MI, USA). INS-1 cells exposed to H_2O_2 were homogenized in 80/100μl reaction buffer (10% glycerol, 137 mM NaCl, 20mM Tris-HCl (pH 7.5), and 1% NP-40) containing 10 μl of caspase-9 substrate (Ac-LEHDpNA, 2 mM) or caspase-3 substrate (Ac-DEVD-pNA, 2 mM). Then lysates were incubated at 37°C for 2 h. Samples were measured at an absorbance of 405 nm.

Apoptosis Assays

The effects of **1** on INS-1 cells induced by H_2O_2 were evaluated with the methods described by Ho [14]. Briefly, INS-1 cells in 96-well plates were treated with compound **1** and H_2O_2 and non-treated cells were used as controls. The cells were then harvested after incubating for 72 h, washed, and resuspended in cold phosphate- buffered saline (PBS). The cells in the wells were stained with Annexin V fluorescein/isothiocyanate and propidium iodide double staining which was analyzed using an Annexin V/FITC Apoptosis Detection Kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions and the apoptotic rates were assayed by a flow cytometer using BD FACSDiva Software (BD FACS Arial II, USA).

Statistical Analysis

The data are expressed as the mean ± SEM. The results were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. The values of $P<0.05$ were considered statistically significant

RESULTS AND DISCUSSION

Characterization of multiflorane-type triterpene

Repeated column chromatography on silica gel and Sephadex LH-20 from MeOH extract from *C.*

argyrosperma led to the identification of one new multiflorane-type triterpene which was characterized as 3β-trans-caffeoyloxymultiflor-8-ene- 7α,12β, 18 β-triol by comparing its spectral and physical data with literature. Compound **1** was obtained as an amorphous solid and its molecular formula was assigned as $C_{39}H_{56}O_7$ by HREIMS at *m/z* 636.4107 $[M]^+$ (calcd. for 636.4026) and ^{13}C NMR data. The molecular formula indicated an index of hydrogen deficiency of twelve which revealed the presence of five olefins and six double bonds deduced from the carbonyl. This data suggested that the compound is hexacyclic. The IR spectrum of **1** showed absorption bands for OH (3377 cm-1) and α,β-carbonyl, (1698 cm-1), aromatic (1640 and 1514 cm^{-1}) and double bond (1610 cm^{-1}) functionalities. The compound **1** nature was determined by DEPT 90 and 135 experiments, revealing the presence of 39 carbon atoms, including 8 methylenes, 9 methines, 9 methenyl groups and 13 quaternary carbons. ^1H spectrum showed signals for eight tertiary methyl groups, each 3H as a singlet, (δ_H 0.88, 0.91, 0.82, 1.10, 1.08, 1.14, 1.21 and 1.13), two hydroxymethine protons at δ_H 4.23 (1H, brs) and δ_H 4.89 (1H, d, J = 5.3 Hz), a 1,4-disubstituted aromatic ring δ_H 7.1 (1H, d, J = 1.2 Hz), δ_H 6.79 (1H, d, J = 8.0 Hz) and δ_H 6.98 (1H, dd, J = 8.0, 1.2 Hz), and an olefinic double bond at δ_H 6.20 (1H, d, J = 15.4 Hz) and δ_H 7.51 (1H, d, J = 15.4 Hz). ^{13}C NMR spectrum displayed signals of an olefinic double bond at δ_C 143.4 and δ_C 116.7, an ester carbonyl carbon at δ_C 168.3, a tetrasubstituted olefin at δ_C 138.4 and δ_C 140.0, three hydroxymethine carbons at δ_C 65.3, δ_C 75.4 and δ_C 78.9, and a carbon bearing oxygen at δ_C 82.4. Thus, it was suggested to have a pentacyclic skeleton, and the spectral characteristi c was similar to those of derivatives of multiflorane-type (Fig 1A) previously isolated from the other Cucurbitas [15, 16].

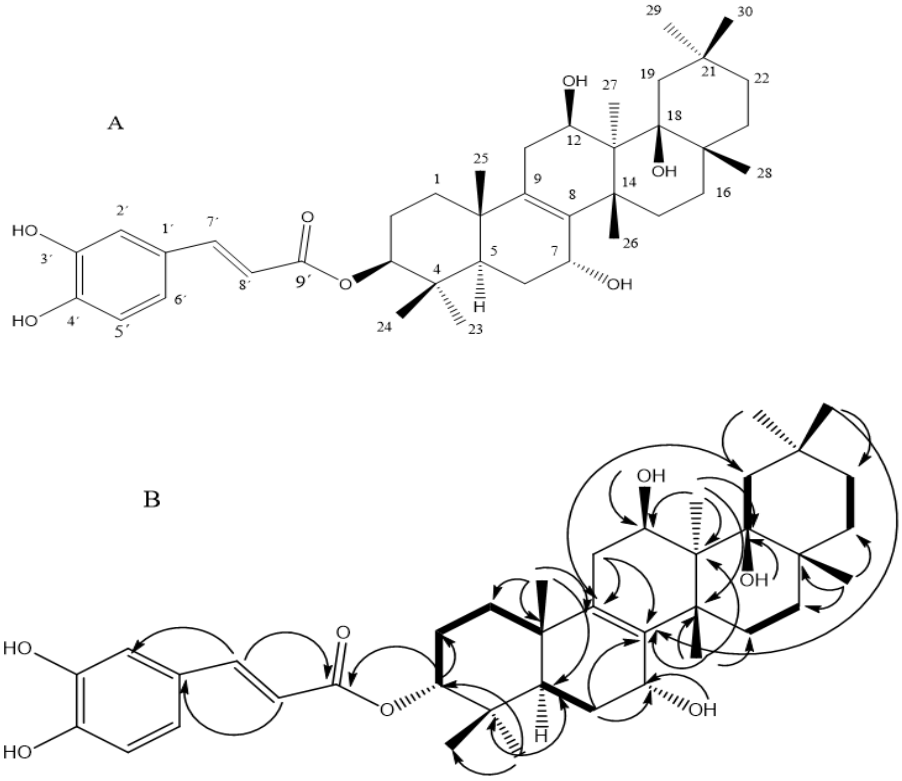

Fig. 1. Key HMBC H-C (⟶) and (B) ^1H-^1H COSY (▬) correlations of the multiflorane-type triterpene isolated from *Cucurbita argyrosperma*

In the HMBC experiment (Fig. 1B), the following correlations were observed: Me-23 to C-3; C-4, C-5 and C-24; Me-24 to C-3, C-4, and C-5; Me-23; Me-25 to C-1, C-5, C-9, and C-10; Me-26 to C-8, C-13, C-14, and C-15; Me-27 to C-12, C-13, C-14, and C-18; Me-28 to C-16, C-17, C-18, and C-22; Me-29 to C-19, C-20, C-21, C-30, Me-30 to C-19, C-20, C-21 and C-29; Me-30 to C-8, Me-19 to C-9; H-5α, H-6α, to C-7; H-6α and H_2-11 to C-8; Me-26 to C-8; Me-25 to C-9; Me-27, and Me-28 to C-18. The ^{1}H NMR spectrum of 1 including two olefinic double bond proton signals at δ_H 7.51 (1H, d, J = 15.4 Hz, H-7′), δ_H 6.20 (1H, d, *J* = 15.4 Hz, H-8′) which was assigned to the trans form from the coupling constant value (*J* = 15.4 Hz) of H-7'and H-8'. In addition, the cross peak between the oxygenated methine proton signal (δ_H 4.62, H-3) and the carbonyl carbon signal (δ_C 168.3, C-9′) in the heteronuclear multiple bond correlation (HMBC) spectrum confirmed that cafferoyl moiety was etherified with the C-3 position of multiflorane triterpene (Fig. 1B) and also indicated that H-3 correlated with C-23, C-24, and C-1' established as β-orientation, and also due to coupling constant of H-3 [4.62 (1Hβ, t , *J* = 5.5 Hz)] and NOEs correlation between H-3 and H3-23, while the coupling constants for the aromatic protons suggested a 1,3,4-trisubstituted benzene at δ_H 7.1 (1H, d, *J* = 1.2 Hz, H-2′), δ_H 6.79 (1H, d, J = 8.0 Hz, H-5′), and δ_H 6.98 (1H, dd, *J* = 8.0, 1.2 Hz, H-6′). These data suggested a caffeoyl moiety [17]. This was confirmed by the HMBC spectrum which indicated long-range correlations between H-2′, H-6′ to C-7′; H-2′, H-5′ to C-4′; and H-2′, H-5′ H-6′, and C-4′. All long-range correlations were consistent with the structure of **1**.

The position of the OH-12 group was suggested by the correlations from the HMBC spectrum of H_3-27 to C-12 and OH-18 and by the correlations of H_3-27 and H_3-28 to C-18 (Fig 1B), which was confirmed by NOE spectrum indicated correlations for H-12 to H_3-27 and H-12 to H-19α. Thus, locating H-12α at δ_H 4.89 supported the beta orientation of OH-12 (Fig 1B). ^{I}H NMR spectrum also showed another signal by a carbinol methine at δ_H 4.23 (1Hβ, brs), which was assigned to C-7. In the ^{1}H-^{1}H COSY assay, H-7 [δ_H 4.23 (brs)] correlated with H_2-6 [δ_H 1.76, 1.62]. The following significant NOE interactions were observed in 1: H-15α/Me-27; H-5/15α; H-18/Me-28; Me-25/ H-18; Me-26/H-7 and Me-27/H_2-29 (Fig. 1B) established that the OH at C-7 is in an α orientation. The ^{13}C NMR spectrum displayed two olefinic carbon signals at δ_C 138.4 and δ_C 140.0. The HMBC spectrum of H_3-30 to C-8 and H_3-19 to C-9 indicated that the tetrasubstituted olefin was assigned a bond between C-8 and C-9. Therefore, this compound **1** was identified as 3β-trans-caffeoyloxymultiflor-8-ene- 7α,12β, 18 β-triol (Fig 1A).

Evaluation of antioxidant capacity in vitro

The antioxidant activities of the triterpene were expressed as half maximal inhibitory concentration (EC_{50}). In order to measure the antioxidant ability, the following tests were performed: DPPH radical scavenging and ABTS radical. Ascorbic acid, EDTA, curcumin, trolox, BHA, and gallic acid were used as positive standards. In the DPPH assay, the results indicated that the compound had significant antioxidant activity with an EC_{50} value of 7.6 μg/mL compared to ascorbic acid (4.5 μg/mL). In ABTS•+ scavenging activity, the EC_{50} value was 11.2 μg/mL. Results have shown that it has significant chelating ability towards the ABTS•+ cation radical in comparison with BHA (EC_{50} 9 μg/mL) and trolox (EC_{50} 7.2 μg/mL) (Fig 2).

Radical scavenging potential plays an important role in the damage caused by free radicals generated in biological systems and foods. The main mechanisms through which antioxidants can act are single electron transfer, hydrogen atom transfer, and metal chelation [18].

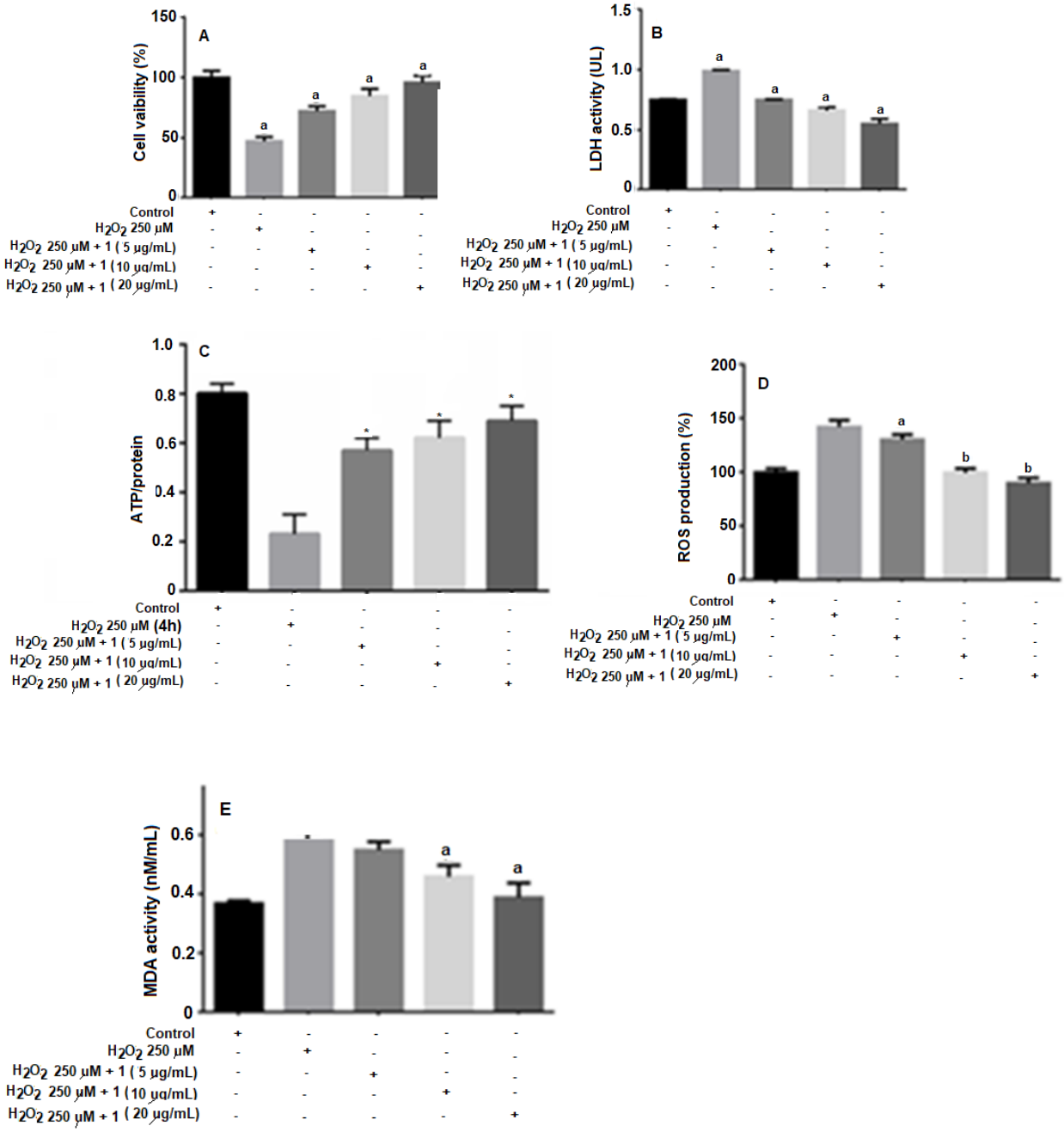

Fig 2. Antioxidant activity of **1** (**A**); Compound **1** protects INS-1 cells from H_2O_2 induced cytotoxicity: (**B**) Cell viability measured using the MTT assay; (**C**) Cytotoxicity was evaluated by an LDH assay (**D**). Treatment with H_2O_2 (250 μM, 4 h) lowered ATP concentration (E) ROS production measured using a DCFH-DA assay; (**F**) Measured MDA activity. The data represent the mean of three independent experiments. ##P < 0.01, compared with untreated control cells, **P < 0.01, and compared with cells treated with H_2O_2 only.

Among these studies, the highest triterpene content was found in *Ilex cornuta*, which has protective effects against H_2O_2-induced myocardial cell injury [19]. It was also reported that oleic acid, ginsenosides, and other triterpenes are effective at scavenging ROS [20, 21, 22]. Glycyrrhizic acid prevents oxidative stress [23]. Other studies found the antioxidant activity of triterpenes from *ficus* and *Agrimonia Pilosa* [24, 25] , these findings clearly show high values for **1** antioxidant scavenging activities in the assays of DPPH followed by ABTS. The antioxidant effect of **1** is consistent with those reported for other triterpenes.

Cell viability, integrity, and mitochondrial function

Exposition of INS-1 cells to H_2O_2 (250 µM) for 24 h led to significant cytotoxicity, which was measured using an MTT assay and an LDH leakage test. Observed cell death was different from H_2O_2 (250 µM) compared to control cells. The maximum inhibition in cell viability was observed with 49.1% at the highest concentration of 250 µM H_2O_2 ($P< 0.05$ vs control). In order to evaluate the protective effect of **1** against H_2O_2-induced pancreatic cell damage, INS-1 cells were pre-treated with 5, 10 and 20 µg/mL of **1** over 2 hours, followed by treatment in absence or presence of H_2O_2 for 24 hours. The protective effect of **1** was observed at 68.5% with a dose of 5 µg/mL and 98% with a pre-treatment of 20 µg/mL (Fig. 2A).

Afterwards, we evaluated the cytotoxicity of **1**. LDH enzyme activity was performed to verify the membrane damage. In INS-1 cells exposed to H_2O_2 (250 µM) for 24 hours, the LDH activity was measured. As shown in Fig. 2B, a 33.3% increase in LDH release was observed in comparison to control cells. However, pre-treatment with **1** reduced LDH release 1.75-fold. Pre-treating the INS-1 cells with **1** significantly inhibits the increase of LDH levels in a dose-dependent manner.

In this study, cellular ATP in INS-1 cells exposed to H_2O_2 for 4 hours was examined, which led to a reduction of the cellular ATP level by 71.25% in comparison to the control. At concentrations of 5, 10 and 20 µg/mL, compound **1** significantly ($p<0.05$) increased ATP levels after 4 hours of H_2O_2 treatment. The values thereof were 71.2, 77.5, and 86.7 % respectively (Fig. 2C). Restored cellular ATP reflects enhanced mitochondrial function.

MTT assays were carried out to evaluate pancreatic cell viability. The exposure of these INS-1 cells to high H_2O_2 levels (250 µM) for 48 hours significantly ($P < 0.05$) inhibited their viability. Treatment with **1** prevented cell death. This indicated that **1** protected the INS-1 cells against oxidative stress conditions. When INS-1 cells were exposed to high H_2O_2 treatment cytotoxicity in a dose-dependent manner for a period of 24 hours, it led to intensive membrane damage with subsequent release of LDH, which can be used as a marker for membrane damage. In this study, the observed LDH enzyme release after treatment with **1** was significantly inhibited.

Mitochondrial function plays an important role in the progression of type 2 diabetes due to its role in controlling cellular redox potential, oxidative phosphorylation, the release of caspase-activating proteins, ATP production, and electron transport. Interruption of these functions leads to cell death [26]. Triterpene **1** preservation of mitochondrial function was examined in pancreatic cell exposed to H_2O_2. We observed that treatment with **1** induces a significant increase in ATP in a dose-dependent manner reflecting mitochondrial function and therefore increases cell viability with important inhibition in the activation of death signals.

Effect of 1 on ROS in β cells exposed to H_2O_2

To evaluate the potential antioxidant effect of **1,** pancreatic β cells were used to determine the level of ROS. The ability of H_2O_2 to produce intracellular ROS was determined using a non-fluorescent precursor of DCF (DCFH2-DA), used as a test for oxidative stress. As shown in Fig. 2D, when the INS-1 cells were exposed to 500 μM of H_2O_2, the generation of ROS increased significantly by 41% in comparison with the control group. Instead, pre-treatment with **1** inhibited DCF fluorescence intensity, which is an indicator of ROS accumulation in a concentration-dependent manner in INS-1 cells compared to the control. Pre-treatment with 20 μg/mL of **1** decreased ROS accumulation by 1.83-fold ($p < 0.01$) compared with the levels in the control which resulted in high protection of H_2O_2 induced cell injury.

It is widely known that H_2O_2-induced stress conditions produce high ROS levels in pancreatic β-cells [27]. Consequently, we evaluated the role of **1** on ROS production in H_2O_2 induced stress conditions. Results indicated that H_2O_2 treatment induces a reduction of ATP generation with a parallel increment in ROS levels as an early event in the apoptotic pathway leading to cell death [28]. In our studies, we observed that **1** attenuated levels of ROS generation and maintained mitochondrial function in the INS-1 cells exposed to oxidative stress, thereby improving β-cell function and destruction.

Previous studies have demonstrated that *C. argyrosperma* seeds ameliorate oxidative stress in H9c2 cardiomyocytes by suppressing production of intracellular reactive oxygen species [29]. For more information on the mechanism, we isolated and studied the effect of **1** using INS-1 pancreatic β-cells.

Effect of 1 on lipid peroxidation

Lipid peroxidation was determined by evaluating the level of MDA, which is considered to be an end-product of lipid peroxidation. Exposure of the INS-1 cells to H_2O_2 produced a significant ($P< 0.05$) increase of 1.57-fold in the MDA content compared to the control cells (0.38 nM/mL). Compound **1** pre-treatment produced significantly ($P< 0.05$) reduced MDA activity in the cells pre-treated with 20 μg/mL of **1** (1.53-fold), supporting that triterpene alleviated H_2O_2 induced lipid peroxidation (Fig. 2E).

Lipid peroxidation is the main mechanism of free radical damage to membrane-bound enzymes and cellular organelles caused by mitochondrial ROS, which produce reactive aldehydes as cytotoxic products [30]. Therefore, lipid peroxidation can induce cell apoptosis, which is involved in numerous pathophysiological disorders [31]. Findings indicated that H_2O_2 treatment induced lipid peroxidation in pancreatic cells and that triterpene displays an effective reduction in TBARS production. In our studies, we observed that **1** brought about a significant reduction of mitochondrial ROS generation, consequently with decreased levels of lipid peroxidation under oxidative stress conditions. The protective effect of **1** on TBARS generation can be accredited to its antiperoxidative effects.

Effect of triterpene on antioxidant enzymes

In this study we used H_2O_2 toxicity in INS-1 cells as a model to measure the antioxidant defense system in INS-1 cells treated with **1** prior to exposure to H_2O_2. The improvement of **1** on neutralizing ROS-mediated oxidative stress was evaluated in levels of antioxidant enzymes such as SOD, CAT, and GSH-Px. The activity of antioxidant enzymes decreased in the presence of H_2O_2 for the ROS generation ($P<0.05$; Fig.3 (A-C)) compared with the control model. Triterpene

pre-treatment with a dose of 20 µg/mL increased the expression of SOD, CAT, GSH, and GSH-Px, maintaining the antioxidant enzyme activities.

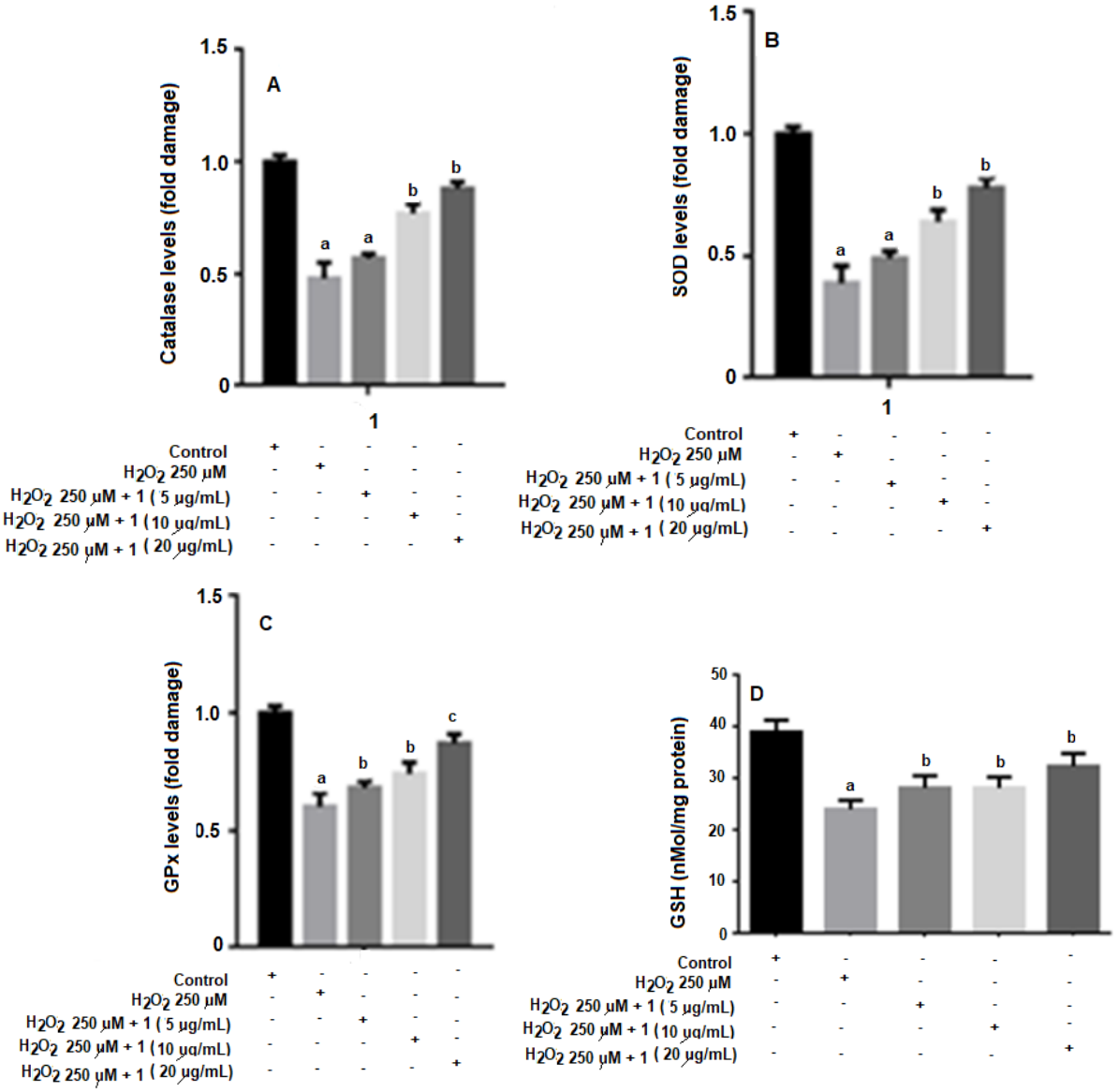

Fig 3. Effect of **1** on accumulation and CAT, SOD, GPx, and GSH (**A**-**D**) activities in INS-1 cells. The data represent the mean of three independent experiments. ##P < 0.01, compared with untreated control cells, **P < 0.01, and compared with cells treated with H_2O_2 only.

The antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and the non-antioxidant enzyme (GSH) can act together to decrease the ROS generated in β-pancreatic cells following H_2O_2 exposition. SOD catalyzes the transformation of the superoxide anion (O2●) to H_2O_2 and molecular oxygen. These are then metabolized into molecular oxygen and water by GPx and CAT. Treatment with **1** provides significant (P<0.05) conservation of the GPx activity, allowing the normal content of GSH to remain. The weak antioxidative defense status in the distinct subcellular organelles causes the β-cells to be susceptible and vulnerable to peroxisomal, mitochondrial and ER stress [32]

***Effect of 1 on caspase-3, 9 expressions in the INS-1 cells exposed to* H_2O_2**

Findings indicate that in INS-1 cells of the model group exposed to H_2O_2, caspase-3 and 9 activity increased significantly ($P< 0.05$), by 50% and 60.46% respectively. However, treatment with **1** decreased the activity of both caspase-3 and 9 significantly ($P< 0.05$), in the INS-1 cells exposed to H_2O_2 (Fig. 4). Triterpene at a concentration of 20 µg/mL significantly ($P< 0.05$) decreased the activity of caspase-3 and 9 in the INS-1 cells by 42.10% and 39.53% respectively. However, **1** significantly ($P< 0.05$) ameliorated the activity of caspase-3 and 9 in the INS-1 cells exposed to oxidative stress.

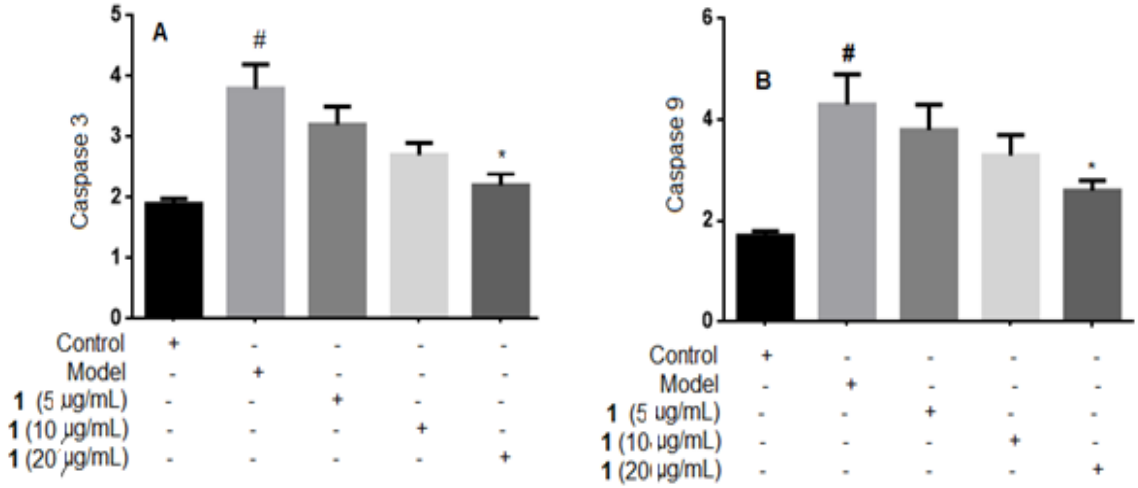

Fig. 4. Effect of **1** on the caspase-3 and 9 activity in INS-1 cells exposed to H_2O_2 induced oxidative stress. INS-1 cells were pre-treated with concentrations of 50, 100 and 200 µg/mL for 24 hours before incubation with H_2O_2 for 24 hours. The activity of INS-1 cell apoptosis protein (A) caspase-3 and (B) caspase-9 was evaluated by an ELISA kit. When data are compared with the control group, ##P<0.05; When data are compared with model group, **P<0.05.

Different concentrations of **1** (5, 10 and 20 µg/mL) were added to pancreatic cells to evaluate whether it can provoke expression of proteins caspase-3 and 9 related to H_2O_2-induced apoptosis. The imbalance of expression pro and anti-apoptotic protein is the final destination of the apoptotic process [33]. It is well known that the exposure of INS-1 cells to H_2O_2 generate an increase in the cell apoptosis rate [34] which is mediated by oxidative stress and is involved in the process of cell apoptosis. Findings indicate that exposure to H_2O_2 results in an increase in pro-apoptotic caspase-3 expression. However, the INS-1 cells exposed to **1** showed a reduction in caspase-3 expression compared with H_2O_2 treated cells. Additionally, the caspase-9 expression level following exposition with **1** was markedly reduced.

Protective effect of 1 against apoptosis

In this study, we explored the anti-apoptotic effects of **1** using H_2O_2 treated pancreatic β cells resulting in oxidative stress, cellular damage, and apoptosis, which all play an important role in the progress of insulin resistance, β cell dysfunction, and diabetic complications [35]. Fig. 5 shows

the microphotograph of INS-1 cells of the control group (A) with great transparency, and a lower percentage of apoptotic cells (Q1 3.42%; Q2 1.42%; Q3 0.64% and Q4 92.5%).

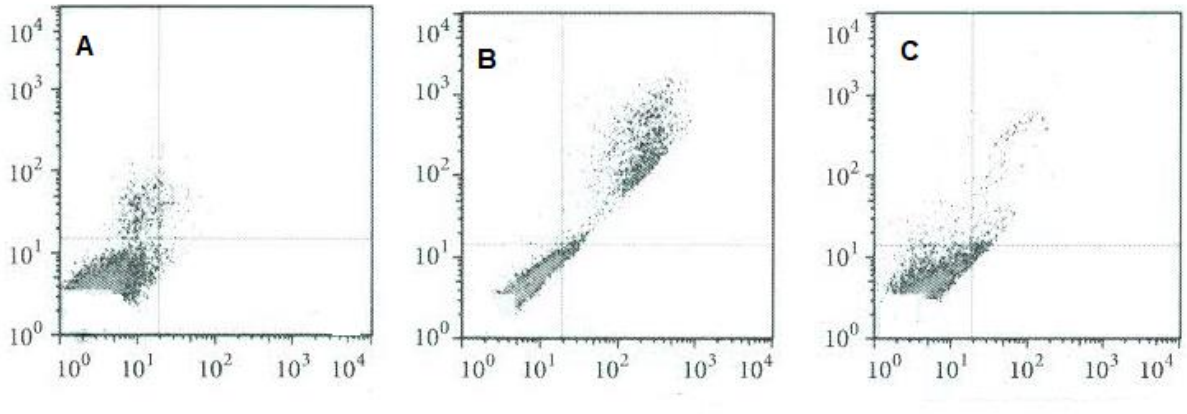

Fig 5. Protective effect of **1** against H_2O_2 induced apoptosis in INS-1 cells. Apoptosis was assessed using flow cytometry with Annexin-V/PI staining. The quantification histograms indicate the percentages of apoptotic cells.

The group treated with H_2O_2 (B) was used as an oxidative stress model cell, which showed cytoplasmic vacuoles, irregular shape, and an increase in the percentage of apoptotic cells compared with the control group (Q1 0.134%; Q2 14.3%; Q3 3.03% and Q4 79.8%). The cells treated with H_2O_2 and **1** (C) show better condition and decreased apoptosis rate (Q1 1.32%; Q2 3.84%; Q3 1.20% and Q4 90.3%). Nevertheless, there was no significant difference between the control and the H_2O_2 + 1 groups in terms of the cell apoptosis rate, suggesting that **1** can protect INS-1 cells from H_2O_2 induced apoptosis. INS-1 cells indicated clear structural evidence of apoptosis after treatment with **1**. The addition of 0.5 μg/mL led to morphological changes of cells involving a decrease in the size of INS-1 cells. However, physiologically changes of cells including detachment from the base of the culture plate indicated a discontinuation of the extracellular matrix and reduction of cell-cell contact. The cells turn become shrunken and flat with an appearance of apoptotic bodies (small vesicle bodies).

It is well known that H_2O_2 exposure to INS-1 cells caused a significant increase in intracellular ROS, MDA content, a deficit in antioxidant enzymes, apoptosis, and mitochondrial dysfunction suggestive of oxidative stress. Cumulative evidence suggests that extra ROS could alter the structural and functional integrity of cells through a variety of mechanisms [36]. Further studies have shown that antioxidant compounds could suppress the increase of LDH, decrease of viability, attenuated production of ROS, decreased concentrations of MDA, reduced cell apoptosis, and significantly suppressed and depleted endogenous antioxidants (SOD, CAT, and GSH) as a result of H_2O_2 exposure [37]. The decline in cellular ATP produced to higher oxidant levels would involve caspase activation for implementation of apoptosis thereby contributing to cell death patterns and taking into consideration that the caspases are directly controlled by the cellular redox state. Accordingly, in Jurkat cells, caspase-3 activity was regulated depending on the levels of H_2O_2 induced ROS generation. Higher levels inhibited activity whereas lower concentrations increased activity [38]. In this study we examined whether **1** had antioxidants effects and, as we expected, findings indicated that **1** significantly attenuate H_2O_2 induced intracellular oxidant stress

by avoiding the intense depletion of antioxidant enzymes or through the scavenging of ROS. Consequently, **1** was able to attenuate the responses to the generation of ROS.

CONCLUSION

In this study a new multiflorane-type triterpene was identified in the seeds of *C. argyrosperma* by RMN as 3β-trans-caffeoyloxymultiflor-8-ene- 7α,12β, 18 β-triol (1). Compound **1** scavenged DPPH and ABTS free radicals. H_2O_2 treatment could increase in INS-1 cell cytotoxicity, oxidative stress, and apoptosis rate. In parallel, it could further damage pancreatic β-cell function, including increasing levels of lipid peroxidation (MDA), reducing levels of CAT, SOD, GPx, GSH, caspase-3, 9 expressions, and cellular ATP. Our findings provide direct evidence that pre-treatment with **1** protected INS-1 cells against apoptosis and oxidative stress induced damage. This protection can be seen through the increased ability to prevent lipid peroxidation, scavenge oxygen free radicals, increase ATP, reduce cell apoptosis, caspase-3, 9 expressions, and by avoiding the intense depletion of antioxidant enzymes.

List of Abbreviations:

DPPH - 2-dyphenyl-1-picrylhydrazyl
ABTS - 2′-Azino-bis(3-Ethylbenzothiazoline-6-Sulfonic Acid
LDH - lactate dehydrogenase
GPx - glutathione peroxidase
CAT - catalase
MS - mathyl silane
ppm - parts per million
SOD - superoxide dismutase
ROS - reactive oxygen species
NO - nitric oxide
GSH - non-antioxidant enzyme
INS-1 cells - rat pancreatic beta cells

Author Contributions: Rosa Martha Perez Gutierrez contributed the ideas and the evaluation of overarching research. Alethia Muñiz Ramirez contributed to the development and the design of methodology. Jose Maria Mota Flores performed the experiments. Abraham Heriberto Garcia Campoy collected data evidence and completed the application of statistical analysis. All authors participated in writing, reviewing and editing of the article.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgement: This work was financially supported by Instituto Politecnico Nacional, Department of Investigation

REFERENCES

1. Cetkovic GS, Djilas SM, Canadanovic-Brunet JM, Tumbas VT: Antioxidant properties of marigold extracts. Food Res Intern 2002, 37: 643-650.
2. Galcin L, Oktay J: Determination of antioxidant activity of *Lichen cetroria* (**L**.). Archive J Ethopharmacol 2002, 79: 325-329.
3. Ray PD, Huang BW, Tsuji Y: Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal 2012, 24: 981-990.
4. Rashid MA, Lee S, Tak E, Lee J, Choi TG, Lee JW, Kim JB, Youn JH, Kang I, Ha Ha J, Kim SS: Carbonyl reductase 1 protects pancreatic β cells against oxidative stress induced apoptosis in glucotoxicity and glucolipotoxicity. Free Rad Biol Med 2010, 49: 1522–1533.
5. Qian J, Wang WB: Ophiopogonin D prevents H_2O2-induced injury in primary human umbilical vein endothelial cells. J Ethnopharmacol 2010, 128: 438-445.
6. Mandrup-Poulsen T, Helqvist S, Wogensen LD, Molvig J, Pociot F, Johannesen J, Nerup J: Cytokine and free radicals as effector molecules in the destruction of pancreatic β cells. Current Topics Microbiol Immunol 1990, 164:169–193.
7. Lee SH, Han JS, Heo SJ, Hwang JY, Jeon YJ: Protective effects of dieckol isolated from *Ecklonia cava* against high glucose-induced oxidative stress in human umbilical vein endothelial cells. Toxicol in Vitro 2010, 24: 375–381.
8. Ghlissia Z, Sayarib N, Kallel R, Bougatef A, Sahnoun Z: Antioxidant, antibacterial, anti-inflammatory and wound healing effects of *Artemisia campestris* aqueous extract in rat. Biomed Pharmcol 2016, 84: 115-122, http://dx.doi.org/ 0.1016/j.biopha.2016.09.018.
9. Perez-Gutierrez RM, Estrella-Mendoza MF, Estrada JF, Marure RL: *Cucurbita argyrosperma* seed extract ameliorating oxidative stress in H9c2 cardiomyocytes through suppression of intracellular reactive oxygen species production. Phcog Mag 2019: 15: 327-334.
10. Estrella-Mendoza MF, Jiménez-Gómez F, López-Ornelas A, Pérez-Gutiérrez RM, Flores-Estrada J: *Cucurbita argyrosperma* seed extracts attenuate angiogenesis in a corneal chemical burn model, Nutrients, 2019. doi: 10.20944/preprints 201904.0193.v1.
11. Lalhminghlui K, Jagetia GC: Evaluation of the free-radical scavenging and antioxidant activities of Chilauni, *Schima wallichii* Korth in vitro. Future Sci OA, 2018, 4: FSO272,. doi: 10.4155/fsoa-2017-0086.
12. Mandel P, Misrat K, Ghosal M: Free radical scavenging activity and phytochemical analysis in the leaf and steam of *Drymara diandra* Blue. Intern J Integrative Biol 2008, 7: 80-84.
13. Yagi K: Simple procedure for specific assay of lipid hydroperoxides in serum or plasma. Methods Mol Biol 1998,108: 107-110.
14. Park MH, Han J: *Padina arborescens* extract protects high glucose-induced apoptosis in pancreatic β cells by reducing oxidative stress. Nutr Res Practice 2014, 8: 494-500.
15. Kikuchi T, Takebayashi M, Shinto T, Yamada T, Tanaka R: three new multiflorane-type triterpenes from Pumpkin (*Cucurbita maxima*) seeds. Molecules 2013, 18: 568-5579, doi:10.3390/molecules18055568.

16. Tanaka R, Kikuchi T, Nakasuji S, Ue Y, Shuto D, Igarashi K Okada R, Yamada T: A novel 3α-p-nitrobenzoylmultiflora-7:9(11)-diene-29-benzoate and two new triterpenoids from the seeds of Zucchini (*Cucurbita pepo* L). Molecules 2013, 18:7448-7459, doi:10.3390/molecules18077448.
17. Hazra B, Biswas S, Mandal N: Antioxidant and free radical scavenging activity of *Spondias pinnata BMC Complemen Alter Med* 2008, 8: 63-68, doi: 10.1186/1472.6882-8-63.
18. Leopoldini M, Russo N, Toscano M: The molecular basis of working mechanism of natural polyphenolic antioxidants. 2011, Food Chem 125: 288–306.
19. Li S, Zhao J, Liu Y, Chen Z, Xu Q, Khan IA, Yang S: New triterpenoid saponins from *Ilex cornuta* and their protective effects against H_2O_2-induced myocardial cell injury. J Agric Food Chem 2014, 62: 488-96, doi: 10.1021/jf4046667
20. Hu XG, Wang M, Bei WJ, Han ZY, Guo J: The Chinese herbal medicine FTZ attenuates attenuates insulin resistance via IRS1 and PI3K in vitro and in rats with metabolic syndrome. J Trans Med 2014, 12: 47.
21. Ley SH, Hamdy O, Mohan V, Hu FB: Prevention and management of type 2 diabetes: dietarycomponents and nutritional strategies. Lancet 2014, 383: 1999–2007.
22. Fernández-Aparicio A, Schmidt-Rio VJ, Perona JS, Correa-Rodríguez M; Castellano JM, González-Jiménez E: Potential Protective Effect of oleanolic Acid on the components of metabolic syndrome: a systematic review. J Clin Med 2019, 8: pii: E1294, doi:10.3390/jcm8091294.
23. Umar SA, Tanveer MA, Nazir LA, Divya G, Vishwakarma RA, Tasduq SA: Glycyrrhizic acid prevents oxidative stress mediated DNA damage response through modulation of autophagy in ultraviolet-b-irradiated human primary dermal fibroblasts. Cell Physiol Biochem 2019, 53: 242-257, doi: 10.33594/000000133.
24. Ogunlaja OO, Moodley R, Baijnath H, Onnalagadda SB: Chemical constituents and in vitro antioxidant activity of crude extracts and compounds from leaves and stem bark of ficus BURTT-DAVYI. Acta Pol Pharm 2016, 73:1593-1600.
25. Liu X, Zhu L, Tan J, Zhou X, Xiao L, Yang X, Wang B; Glucosidase inhibitory activity and antioxidant activity of flavonoid compound and triterpenoid compound from *Agrimonia Pilosa* Ledeb. BMC Complemen Altern Med 2014, 10: 12-16, doi: 10.1186/1472-6882-14-12.
26. Yu L, Wan H, Yang J, Yin F, Zhou H, Fang H, Li C, He Y: Astragaloside IV alleviates ischemia reperfusion-induced apoptosis by inhibiting the activation of key factors in death receptor pathway and mitochondrial pathway. J Ethnopharmacol 2019, 19: 112319, doi: 10. 1016/j.jep. 2019.112319.
27. Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H: Glucose toxicity in β-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes 2003, 52: 581–587.
28. Supale S, Ning L, Brun T, Maechler P: Mitochondrial dysfunction in pancreatic b cells. Trends Endocrinol Metab 2012, 23:.477–487.
29. Pérez-Gutiérrez RM, Estrella-Mendoza MF, Flores-Estrada J, Marure RL: *Cucurbita argyrosperma* seed extract ameliorating oxidative stress in h9c2 cardiomyocytes through

suppression of intracellular reactive oxygen species production. Phcog Mag 2019, 15: 327-334, DOI: 10.4103/pm.pm_432_18

30. Esterbauer H, Schaur RJ, Zollner H: Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Rad Biol Med 1991, 11: 81-128.
31. Kappus H: Oxidative stress in chemical toxicity. Archives Toxicol 1987, 60: 144-149
32. Jörns A, Mehmeti I,. Lenzen S: Comparative analysis of cytoprotective enzyme equipment in human pancreatic beta-cells and non-beta-cells. Diabetologia 2016, 59: Suppl 1, S218.
33. Antonsson B, Martinou JC: The Bcl-2 protein family. Experimental Cell Res 2000, 256: 50-57.
34. Porter AG, Jänicke RU: Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999, 6: 99-104.
35. Evans JL, Goldfine LD, Maddux BA, Grodsky GM: Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocrinol Review 2002, 23: 599-622.
36. Zhoua,E, Yang W, Lia,Z, Luoa, D, Lia,W, Zhanga Y, Wanga X, Fangd M, Chend Q, Jina X: *Moringa oleifera* stem extract protect skin keratinocytes against oxidative stress injury by enhancement of antioxidant defense systems and activation of PPARα. Biomed Pharm 2018, 107: 44-53.
37. Dhalla NS, Elmoselhi AB, Hata T, Makino N: Status of myocardial antioxidants in ischemia reperfusion injury. Cardiovasc Rev 2000, 47: 446–456.
38. Mareninova OA, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I., Gukovskaya AS: Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. J Biol Chem 2006, 281: 3370-3381