

30 **ABSTRACT**

31 Progressive decline in β cell function and reduction in the β cell mass is important in type 2
32 diabetes. Here, we tested the hypothesis that madecassoside's previously demonstrated in vivo
33 protective effects on the β cell in experimental diabetes were exerted directly. We investigated
34 the effects of madecassoside in protecting a β cell line (INS-1E) against a variety of agents.
35 INS-1E cells were treated with madecassoside in the presence of high glucose (HG), a cytokine
36 mixture, hydrogen peroxide (H_2O_2), or streptozotocin (STZ). HG, the cytokine mixture, H_2O_2
37 and STZ each produced a significant decrease in cell viability; this was significantly reversed
38 by madecassoside. Pre-treatment with madecassoside reduced the number of apoptotic cells
39 induced by HG, the cytokine mixture, H_2O_2 , and STZ, and concentration-dependently reduced
40 ROS production. Madecassoside also significantly enhanced glucose-induced insulin
41 secretion. The results suggest that madecassoside's in vivo effects are exerted directly on the
42 β cell.

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44 **3. Experimental**

45 **3.1. Materials**

46 **3.1.1. Drugs**

47 Madecassoside (M6949, Sigma, USA); Resveratrol (PI28587, PI Chemicals, Shanghai,
48 China); Streptozotocin (**U9889**, Santa Cruz Biotechnology Inc., USA); Nicotinamide
49 (**SC208096**, Santa Cruz Biotechnology Inc., USA)

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51 **3.1.2. Experimental work**

52 The following materials were used and obtained from the suppliers named in parentheses.
53 Optimized RPMI-1640 medium (Cat #C0004-02, Addexbio, USA); Fetal bovine serum (Tico
54 Europe, Netherlands); β -mercaptoethanol (Sigma, USA); Penicillin and streptomycin
55 solution (Nacalai Tesque, Japan); 2.5 g/L-Trypsin/ 1 mmol/L- EDTA solution (Nacalai
56 Tesque, Japan); D-(+)-glucose solution (Sigma, USA); 10x phosphate buffered saline; MTT
57 powder (Sigma, USA); Dimethyl sulfoxide (Sigma, USA); Hydrogen peroxide (Sigma, USA);
58 1L-1 β , TNF α and IFN γ (Merck Millipore, Germany); H2-DCF-DA (Invitrogen, USA),
59 TiterTACS TUNEL assay kit (R&D Systems, USA); Rat insulin ELISA kit (Mercodia, USA);

60 T75 cell culture flasks (SPL Life Sciences, Korea); 96-well plates (SPL Life Sciences,
61 Korea); 12-well plates (SPL Life Sciences, Korea); 0.22-micron sterile filters; 10 mL
62 serological pipettes (SPL Life Sciences, Korea); 5 mL serological pipettes (SPL Life
63 Sciences, Korea); 15 mL conical tubes (SPL Life Sciences, Korea); 50 mL conical tubes
64 (SPL Life Sciences, Korea); syringes (Terumo, USA)

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66 **3.2. Methodology**

67 **3.2.1. Cell culture**

68 Rat insulinoma cells (INS-1E) were acquired from Addexbio, USA. The cells were grown in
69 RPMI-1640 culture medium and supplemented with, 2 mmol/L L-glutamine, 1 mmol/L
70 sodium pyruvate, 10 mmol/L HEPES, 1500 mg/L sodium bicarbonate, 2000 mg/L glucose
71 10% FBS, 50 µmol/L β-mercaptoethanol, 100 µg/mL streptomycin and 100 U/mL penicillin,
72 at 37°C in a humidified (5% CO₂, 95% air) atmosphere. The cells were subcultured into T75
73 flasks in 1:2 ratios when they reached 70 – 80% confluence. The culture medium was
74 replaced every two to three days.

75 No animals were used in this study. The experiments were performed in agreement with the
76 guidelines set by IMU-Joint Committee (IMU-JC) on Research and Ethics with internationally
77 accepted principles. The date of IMU-JC approval is 29 September 2016 and the reference
78 number is 4.16/JCM-125/2016.

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80 **3.2.2. Cell viability assay**

81 INS-1E cells were seeded onto a 96-well plate at 2×10^4 cells per well in 100 µL culture
82 medium for 24 hours, and then exposed individually to different toxicants in the presence or
83 absence of madecassoside (10, 30, 60 µM); these toxicants were a high glucose (HG)
84 concentration (30 mM, 72 hours), hydrogen peroxide (H₂O₂) (50 µM, 24 hours),
85 streptozotocin (STZ) (400 µM, 24 hours) and a cytokine mixture comprising 1L-1β (0.044
86 ng/mL), TNFα (0.087 ng/mL) and IFNγ (0.103 ng/mL), for 48 hours. Followed by the
87 incubation period, 10 µL of MTT solution (5 mg/mL in PBS buffer) was added per well and
88 incubated in a humidified atmosphere for 3 hours. The medium was aspirated and 100 µL
89 of DMSO was added per well to dissolve the formazan crystal (Morgan 1998). The colour

90 intensity of the solution reflects the cell growth condition, which was recorded at 570 nm
91 (reference filter: 690 nm) using a microplate reader (Infinite M200, Tecan). The experiments
92 were performed in triplicate (n=3), and the entire experimental procedure was repeated
93 three times consecutively (n=9).

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95 **3.2.3. Cell apoptosis assay**

96 Cell apoptosis was measured using the TiterTACS TUNEL protocol. It provides the detection
97 of DNA fragmentation and quantitation of apoptotic cells colorimetrically (Kyrylkova et al.
98 2012). In brief, 2×10^4 cells per well were plated in a 96-well plate for 24 hours, and then
99 exposed to different toxicants as described above in the presence or absence of
100 madecassoside. The plate was centrifuged at 1000 x g for 3 minutes at room temperature.
101 The media was removed and washed once with PBS. The wells were filled with 3.7%
102 buffered formaldehyde solution and left at room temperature for 7 minutes. Then the plate
103 was centrifuged at 1000 x g for 3 minutes at room temperature. The fixative was discarded
104 and washed with PBS. Samples were then post-fixed with 100% methanol for a period of 20
105 minutes at room temperature. The cells were washed twice with PBS with centrifugation
106 between washes. This was followed by a labelling procedure which was performed as per
107 the manufacturer's protocol. Lastly, absorbance was recorded at 450 nm using a microplate
108 reader (Infinite M200, Tecan) within 30 minutes of acid addition. The experiments were
109 performed in triplicate (n=3), and the entire experimental procedure was repeated two times
110 consecutively (n=6).

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112 **3.2.4. Glucose-stimulated insulin secretion**

113 INS-1E cells (1×10^5) were plated in 12-well plates and pre-incubated with glucose (5.5 or
114 30 mM) for 48 hours, then followed by incubation in the absence or presence of
115 madecassoside (30 and 60 μ M) for 24 hours. Thereafter, media were discarded, and the
116 cells were washed carefully with PBS. Fresh medium containing 3 mM glucose,
117 supplemented with 2% FBS was added and incubated for 5 hours. Afterwards, the cells
118 were stimulated using Krebs-Ringer buffer (KRB) (4.75 mM KCl, 119 mM NaCl, 2.54 mM
119 CaCl_2 , 5 mM NaHCO_3 , 1.2 mM KH_2PO_4 , and 20 mM HEPES, pH 7.4) containing either 5 or

120 25 mM glucose for 1 hour at 37°C (Arya et al. 2012). The culture medium was then collected
121 for the detection of insulin using a rat insulin ELISA kit (Merckodia, USA). The experiments
122 were performed in triplicate (n=3), and the entire experimental procedure was repeated
123 three times consecutively (n=9). The assay was performed according to the manufacturer's
124 protocol.

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126 **3.2.5. Measurement of ROS production**

127 The measurement of intracellular ROS levels was carried out using the dichlorofluorescein
128 assay (X. Wang and Roper 2014). The 2', 7'-dichlorodihydrofluorescein diacetate (H₂-DCF-
129 DA) can be deacetylated in cells where it will quantitatively react with intracellular radicals
130 and convert into fluorescent product DCF that will be retained within the cells. 2 x 10⁴ cells
131 per well were seeded in a black opaque 96-well plate for a period of 24 hours. The cells
132 were incubated with high glucose (30 mM, 72 hours), H₂O₂ (50 µM, 24 hours), STZ (400
133 µM, 24 hours) or the cytokine mixture (described in 2.2.2 above) for 48 hours, and then
134 incubated in the absence or presence of madecassoside (10, 30 and 60 µM) for 24 hours.
135 The cells were then washed with PBS to remove traces of the medium. 100 µL of H₂-DCF-
136 DA dye medium (1 µM) was added to each well and incubated in the dark for 30 minutes at
137 37°C in a humidified atmosphere. The plate was read using a fluorescence plate reader
138 (Spectramax M3, Molecular Devices, USA) at an excitation wavelength of 485 nm and an
139 emission wavelength of 535 nm. The experiments were performed in triplicate (n=3), and
140 the entire experimental procedure was repeated three times consecutively (n=9).

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142 **3.3. Statistical analysis**

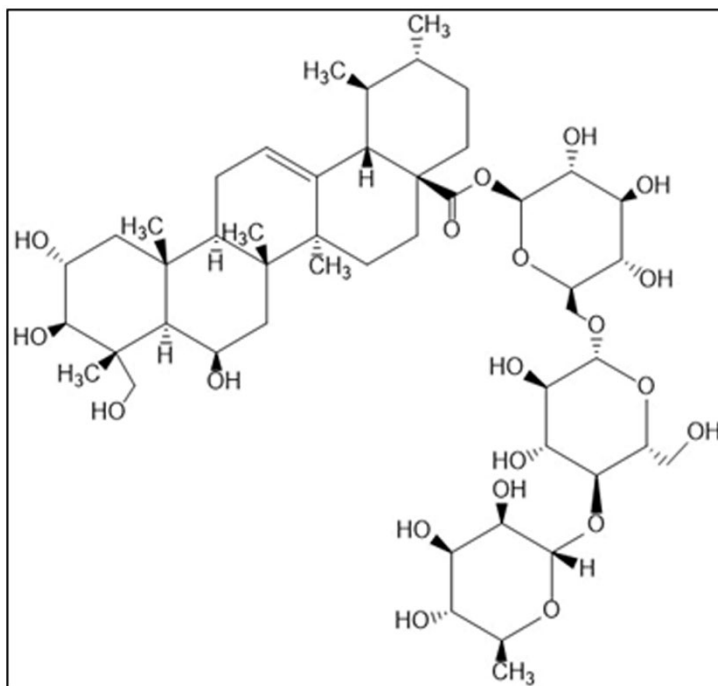
143 The data was normalised to the mean of the control groups, which was randomly set to 100%,
144 and the relative changes were presented as percent changes over the control. Mean ±
145 standard error mean (SEM) was used to present other data. Statistical analysis was done
146 using the SPSS (Statistical Package for Social Science) with one-way analysis of variance
147 (ANOVA) and Tukey HSD post-hoc test. A p-value of less than 0.05 was considered to be
148 statistically significant.

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Figures

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Figure S1. Chemical structure of madecassoside (C₄₈H₇₈O₂₀)

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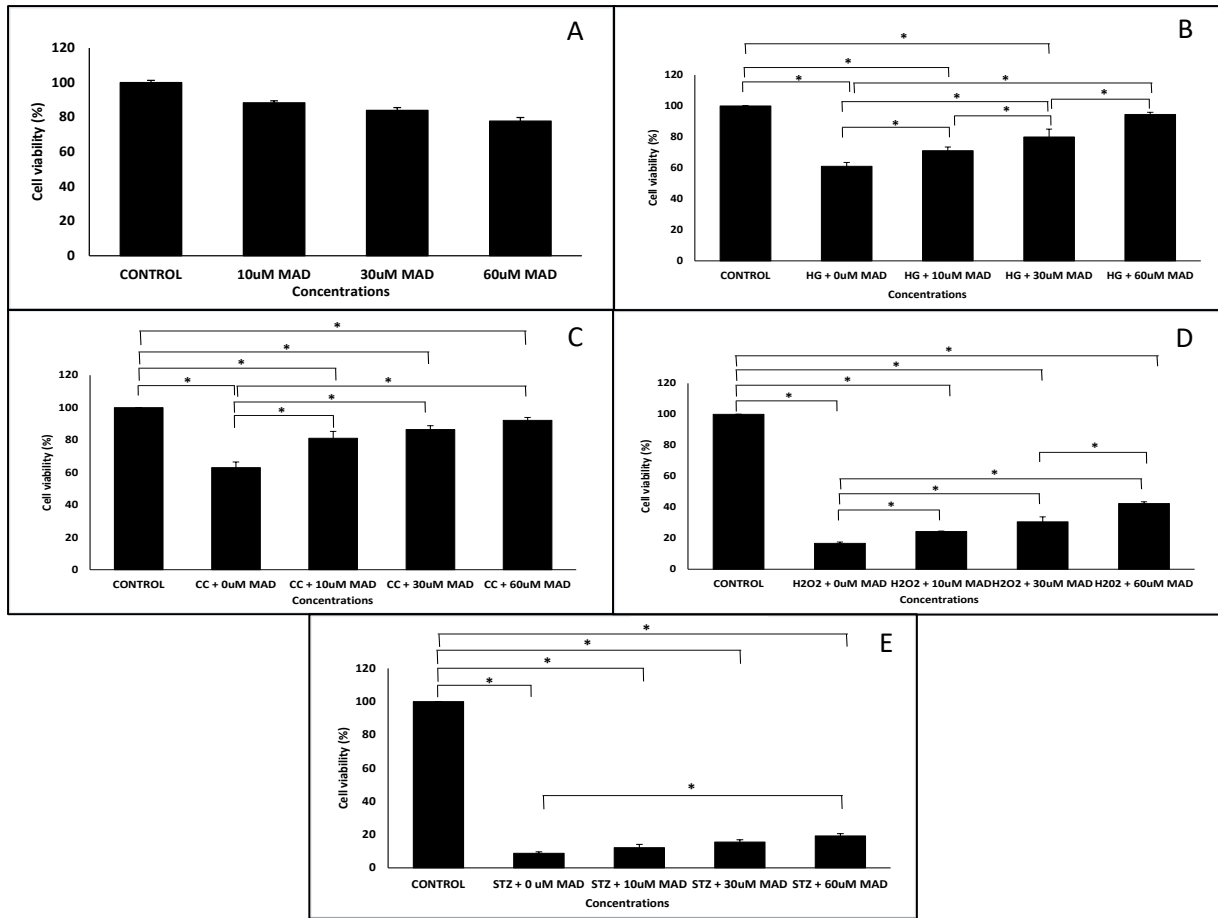
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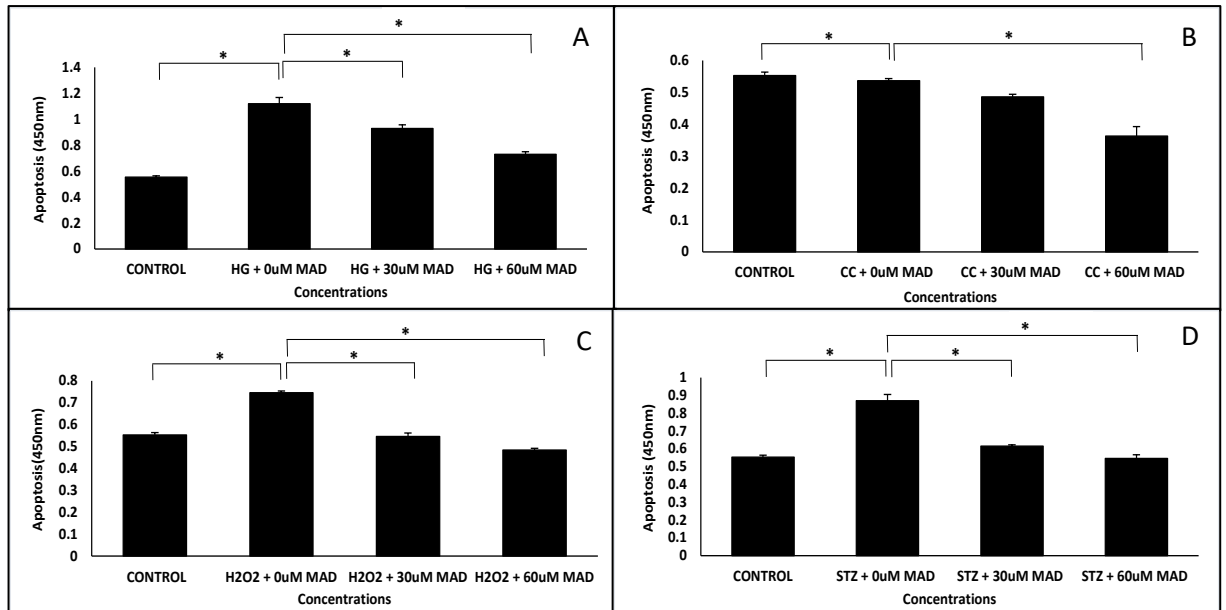
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Figure S2. Effect of madecassoside (MAD) on the viability of INS-1E cells as influenced by (A), high glucose (B), a cytokine cocktail (C), hydrogen peroxide (D) or (E) streptozotocin. The control represents cells incubated in a normal glucose concentration (5.5 mM). Data are presented as mean \pm SEM (n=9). One-way ANOVA with post-hoc Tukey's test. * p-value less than 0.05 was considered to be statistically significant.

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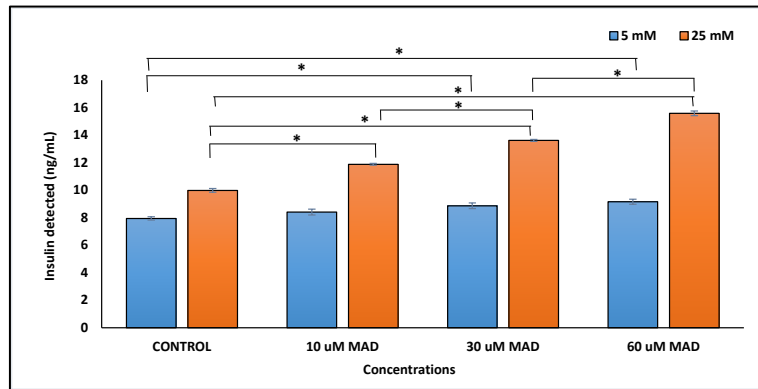
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Figure S3. Effect of madecassoside (MAD) on apoptosis of INS-1E cells induced by high glucose (A), cytokine cocktail (B), hydrogen peroxide (C) or streptozotocin (D) apoptosis. The control represents cells in normal glucose level (5.5 mM). Data are presented as mean \pm SEM (n=6). One-way ANOVA with post-hoc Tukey's test. * p-value less than 0.05 was considered to be statistically significant.



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201 Figure S4. Effect of madecassoside (MAD) on glucose-induced insulin secretion by INS-1E cells. The
202 cells were exposed to 30 mM glucose for 48 hours before the experiment. Data are expressed as mean
203 \pm SEM (n=9). One-way ANOVA with post-hoc Tukey's test. * p-value less than 0.05 was considered to
204 be statistically significant.

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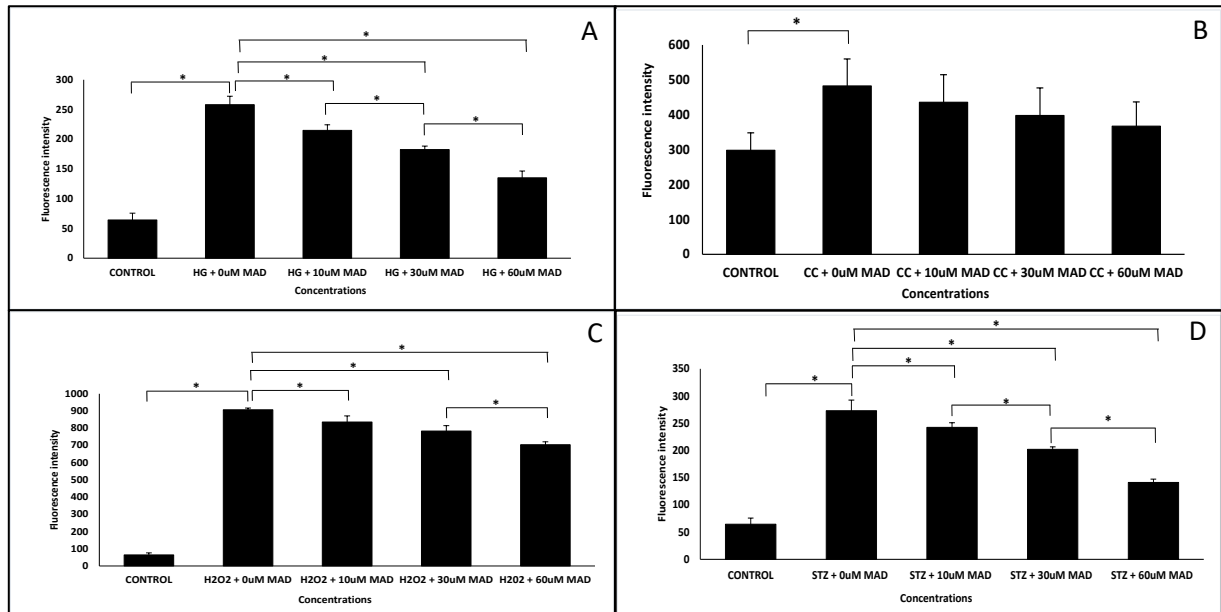
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Figure S5. Effect of madecassoside (MAD) on the production of reactive oxygen species evoked by 30 mM glucose (A), a cytokine cocktail (B), hydrogen peroxide (C) or streptozotocin (D) in INS-1E cells. The control represents cells in normal glucose level (5.5 mM). Data are presented as mean \pm SEM (n=9). One-way ANOVA with post-hoc Tukey's test. * p-value less than 0.05 was considered to be statistically significant.