

THE PROTECTIVE EFFECT OF SOME EXTRACTS AND ISOLATED COMPOUNDS FROM *EUPHORBIA HIRTA* ON PANCREATIC β -CELLS MIN6

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ABSTRACT

Euphorbia hirta L., a species belonging to genus *Euphorbia*, Euphorbiaceae family, has been found a lot in the Mekong Delta. From the ethyl acetate and methanol extracts of this plant, four pure compounds were isolated and identified their chemical structures by spectroscopic techniques including quercitrin (1), luteolin (2), quercetin (3) and caffeic acid (4). The effect of crude ethanolextract and some fractioned extracts as well as four isolated compounds from *E. hirta* L., collected in the Mekong Delta, on endoplasmic reticulum stress-induced cell death in mouse β -cell lines was investigated. The results showed that methanol extract and an isolated compound, quercitrin gave strong cell-protective effect with the cell viability of 69 % and 78 % at the dose of 100 μ g/mL and 10 μ g/mL, respectively. This proves that *E. hirta* L. might have antidiabetic effect and worth conducting further pharmacological investigations related to type 2 diabetes mellitus.

Keywords: *Euphorbia hirta* L., ER Stress, MIN6.

1. INTRODUCTION

Endoplasmic reticulum (ER) is the organelle functionally responsible for folding proteins, calcium storage and lipid synthesis. Protein misfolding in the ER contributes to the pathogenesis of many diseases. Recent studies suggested that protein misfolding in the ER might also lead to

insulin resistance [1]. ER stress plays an important role in the pathogenesis of diabetes, contributing to pancreatic β -cell loss and insulin resistance in type 2 diabetes mellitus (T2DM). In T2DM, chronic insulin resistance and a progressive decline in β -cell function result in β -cell apoptosis and dysfunction. Therefore, it has been suggested that preserving β -cell function is an essential therapeutic target for the treatment of T2DM [2, 3].

Vietnamese traditional medicine plays a major role in the health care system and many of the plants, especially found in the Mekong Delta, having been proved to possess medicinal functions. Among them, *Euphorbia hirta* L. (Eup) has been much of concerns recently because of their bioactivities, especially relating to T2DM. Eup is known to contain polyphenols, among which flavonoids and tannins, sterols, alkaloids, glycosides and triterpenoids. There were several researches on pharmaceutical application of Eup such as anti-inflammatory, antifungal, antibacterial, antidiarrheal, and antioxidant [4, 5].

Glucose-stimulated insulin secretion, glucose transport, glucose phosphorylation, and glucose utilization have been characterized in the insulinoma cell line MIN6, which is derived from a transgenic mouse expressing the large T-antigen of SV40 in pancreatic β -cell.

Despite the known chemical constituents and bioactivities of this medicinal plant, the protective effects on MIN6 cells from ER stress-induced apoptosis have not been yet reported. Therefore, in this study, we investigated the effect of a Vietnamese sample of *Euphorbia hirta* L. (from the Mekong Delta) on ER stress-induced cell death in the β -cell lines MIN6.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Solvents utilized including *n*-hexane, chloroform, ethyl acetate, butanol, methanol (purity $\geq$ 99.0 %), and ethanol 96 % were purchased from Chemsol company (Vietnam). Silica gel 60 (0.063–0.200mm, Merck) and Bondesil C-18 were used for column chromatograph. TLC F₂₅₄ plate (Merck) and TLC RP-18 F₂₅₄ plate (Merck) were used for thin layer chromatography.

HR-ESI-MS and 1D, 2D-NMR spectra were recorded on a Bruker AVANCE (600 MHz) in Kyoto Institute of Technology, Japan.

Fetal bovine serum (FBS) and the cell culture medium Dulbecco's Modified Eagle Medium (DMEM) were purchased from Wako (Japan). WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt), dimethyl sulfoxide (DMSO), thapsigargin, trypsin, and trypan blue solution were supplied by Sigma (St. Louis, MO).

2.2. Sample treatment and preparation

The whole plant was collected at the end of February 2017 in Can Tho city, Vietnam. The plant sample was authenticated by Dr. Dang Minh Quan, Department of Biology Education, Can Tho University where a voucher specimen (EUPH1032017) was deposited. The sample was then washed away from muds and dust; the rotten and damaged parts were also discarded. The raw materials were left to dry in the shade at room temperature for some days and then dried in an oven at about 50 °C until well-dried.

2.3. Extraction and isolation

The well-dried plant was ground into powder (8 kg) which was then soaked in 96 % ethanol at room temperature for four times (4×20 L) and filtered. The filtrate was concentrated under reduced pressure to give brown residue as crude ethanol extract (CE, 700 g). This crude extract was then fractionated on flash column chromatography successively with hexane, ethyl acetate, butanol, and methanol, respectively to yield the corresponding extracts of hexane (HE, 160 g), ethyl acetate (EE, 95 g), butanol (BE, 185 g), and methanol extracts (ME, 172 g). The crude and extracts were kept at -20 °C for the following experiments.

The methanol extract was subjected to a silica gel column chromatography (CC) and eluted with EtOAc:MeOH:H₂O (200:1:1 to 10:1:1, v/v) to obtain six fractions (ME1-6). Fraction ME2 was further separated on a silica gel CC and eluted with EtOAc:MeOH (from 50:1 to 10:1, v/v) to yield seven subfractions (ME2.1-7). Subfraction ME2.2 was subjected repeatedly many times on silica gel CC to yield subfraction M2.2.4.3 and then was further chromatographed on silica gel CC Rp18, eluted with MeOH:H₂O (1:3, v/v) to afford subfraction (ME2.2.4.3.2) which was then recrystallized in MeOH to obtain compound **1** (40 mg).

The ethyl acetate extract was also subjected to flash column chromatography on silica gel and eluted with various proportions of hexane and ethyl acetate (100:0 – 0:100) to obtain eight fractions (EE1-8). Fraction EE5 was further separated on a silica gel column, eluted with CHCl₃:MeOH (from 50:1 to 1:1, v/v) to yield fourteen subfractions (EE5.1-14). Subfraction EE5.11 was further chromatographed on silica gel CC, eluted with CHCl₃:MeOH (10:1 to 1:1, v/v) to obtain seven subfractions (EE5.11.1-7). Finally, compound **2** (8 mg) and compound **3** (10 mg) were obtained from subfraction EE5.11.5 and EE5.11.6 by evaporation.

Similarly, fraction EE6 was separated by a silica gel column and eluted with CHCl₃:MeOH (from 50:1 to 5:1, v/v) to yield 11 subfractions (EE6.1-11). Subfraction EE6.5 was further chromatographed on silica gel CC, eluted with CHCl₃:MeOH (10:1 to 1:1, v/v) to obtain eight subfractions (EE6.5.1-8). At last, compound **4** (5 mg) - light yellow crystals - was obtained from silica gel CC Rp18 with MeOH:H₂O (1:1, v/v) as eluent from subfraction EE6.5.6.

Quercitrin (1): amorphous yellow powder. HR-ESI-MS: m/z = 447.0494 [M-H]⁻ (calculated: 447.0927). ¹H-NMR (500 MHz, DMSO), δ_H (ppm): 7.29 (1H, *d*, 2.0 Hz, H-2''); 7.24 (1H, *dd*, 2.0 Hz *vs* 8.5 Hz, H-6'); 6.85 (1H, *d*, 8.5 Hz; H-5'); 6.38 (1H, *d*, 2.0 Hz, H-8); 6.19 (1H, *d*, 2.0 Hz; H-6); 5.25 (1H, *s*, H-1''); 3.97 (1H, *d*, 1.0 Hz, H-2''); 3.51 (1H, *dd*, 3.0 Hz *vs* 9.5 Hz, H-3''); 3.24 (1H, *m*, H-4''); 3.16 (1H, *m*, H-5''); 0.82 (3H, *d*, 6.0 Hz; H-6''). ¹³C-NMR (125 MHz, DMSO), δ_C (ppm): 177.6 (C-4); 164.2 (C-7); 161.2 (C-5); 157.2 (C-2); 156.4 (C-9); 148.4 (C-4'); 145.1 (C-3'); 134.1 (C-3); 121.0 (C-6'); 120.6 (C-1'); 115.5 (C-2'); 115.3 (C-5'); 103.9 (C-10); 101.7 (C-1''); 98.6 (C-6); 93.5 (C-8); 71.1 (C-4''); 70.5 (C-3''); 70.3 (C-2''); 69.9 (C-5''); 17.4 (C-6'').

Luteolin (2): yellow powder. HR-ESI-MS, [M-H]⁻ m/z = 285.0347 (calculated: 285.0399). ¹H-NMR (600 MHz, DMSO), δ_H (ppm): 7.40 (1H, *d*, 8.4 Hz, H-6'); 7.39 (1H, *s*, H-2'); 6.90 (1H, *d*, 8.4 Hz; H-5'); 6.65 (1H, *s*, H-3); 6.44 (1H, *s*, H-8) and 6.19 (1H, *s*, H-6). ¹³C-NMR (150 MHz, DMSO), δ_C (ppm): 182.1 (C-4); 164.6 (C-2); 164.4 (C-7); 162.0 (C-5); 157.8 (C-9); 150.2 (C-4'); 146.2 (C-3'); 122.1 (C-1'); 119.4 (C-6'); 116.5 (C-5'); 113.9 (C-2'); 104.2 (C-10); 103.4 (C-3); 99.3 (C-6); 94.3 (C-8).

Quercetin (3): pale yellow powder. HR-ESI-MS, [M-H]⁻ m/z = 301.0388 (calculated: 301.0348). ¹H-NMR (600 MHz, DMSO), δ_H (ppm): 7.68 (1H, *s*, H-2'); 7.54 (1H, *d*, 9.0 Hz, H-6'); 6.88 (1H, *d*, 9.0 Hz; H-5'); 6.41 (1H, *s*, H-8); 6.19 (1H, *s*, H-6). ¹³C-NMR (150 MHz,

DMSO), δ_C (ppm): 176.3 (C-4); 164.4 (C-7); 161.2 (C-5); 156.6 (C-9); 148.2 (C-2); 147.3 (C-4'); 145.5 (C-3'); 136.2 (C-3); 122.4 (C-1'); 120.4 (C-6'); 116.1 (C-5'); 115.5 (C-2'); 103.5 (C-10); 98.7 (C-6); 93.8 (C-8).

Caffeic acid (**4**): yellow amorphous solid. HR-ESI-MS, $[M-H]^-$ m/z = 179.0614 (calculated: 179.0344). 1H -NMR (600 MHz, DMSO), δ_H (ppm): 7.41 (1 H, *d*, 15.6 Hz, H-7), 7.02 (1 H, *s*, H-2), 6.96 (1 H, *d*, 8.4 Hz, H-6), 6.76 (1 H, *d*, 8.4 Hz, H-5), 6.17 (1 H, *d*, 15.6 Hz, H-8). ^{13}C -NMR (150 MHz, DMSO), δ_C (ppm): 168.3 (C-9), 148.6 (C-4), 146.0 (C-7), 145.0 (C-3), 126.2 (C-1), 121.6 (C-6), 116.2 (C-5), 115.6 (C-8), 115.1 (C-2).

2.4. Bioactive assay

MIN6 mouse β -cell line was a kind gift from Professor Kaeko Kamei, Department of Biomolecular Engineering, Kyoto Institute of Technology. MIN6 cells were cultured in DMEM containing 25 mM glucose supplemented with 12 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin. Exponentially growing cultures were maintained at 37°C under a humidified atmosphere containing 5 % CO₂.

The ability of the extracts to protect cultured cells from ER stress-induced cell death was evaluated by published method with modifications [6]. The cells were cultured at a density of 2×10^4 cells/mL in 96-well plates for one day, then pre-treated with samples. All the tested samples were prepared with dimethyl sulfoxide (DMSO) of which the maximum DMSO concentration was 1 %. After addition of extracts, 2 μ M thapsigargin (TG) solution was added to the wells with the exception of the control group and re-incubated for 24 hours to induce cell death. WST-8 reagent was added to each well after the media were removed, and then the plate was incubated at 37 °C for further 4 hours. Absorbance of each well was then measured at 450 nm using a 96-well microplate absorbance reader. Cell survival is calculated in % compared to the blank control consider as 100 %.

2.5. Statistical analysis

The variation in a set of data has been estimated by performing one way analysis of variance (ANOVA). Results were calculated from three independent experiments and repeated five times at each experiment, and are shown as mean $\pm$ SD, $n = 3$. Results were considered as statistical significant when p value was < 0.05 .

3. RESULTS AND DISCUSSION

3.1. Structural elucidation

The crude extract was then fractionated on flash column chromatography successively with hexane, ethyl acetate, butanol, and methanol. The methanol extract was chromatographed on silica gel, and octadecylsilane column to give compound **1** (40 mg). The same procedure was applied to ethyl acetate extract to give compound **2** (8 mg), compound **3** (10 mg), and compound **4** (5 mg). Based on their spectral data together with comparison with literature data, these isolated compounds were identified as quercitrin (**1**) [7], luteolin (**2**) [8], quercetin (**3**) [9] and caffeic acid (**4**) [10] (Figure 1).

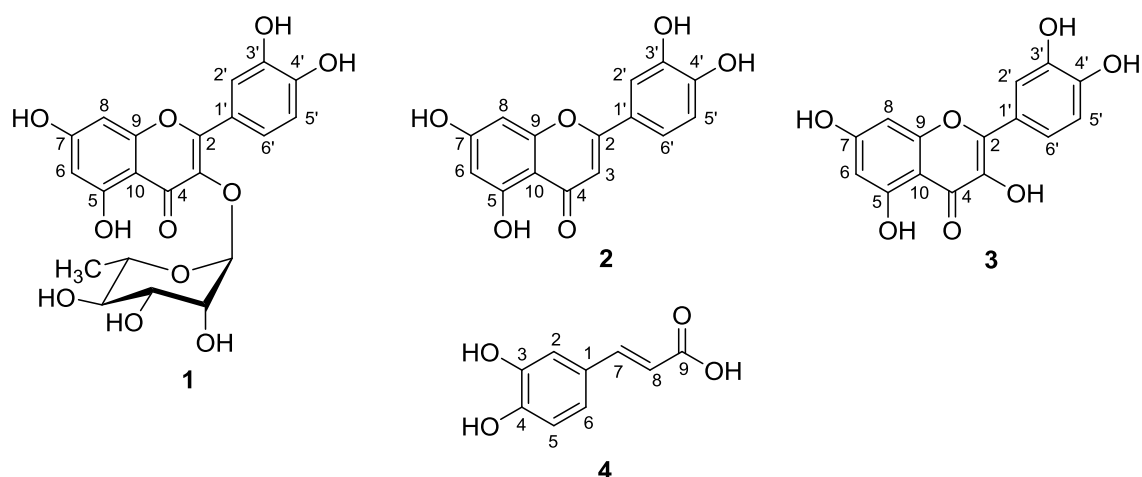


Figure 1. Chemical structures of compounds 1-4.

3.2. Effects of plant extracts on cell viability

3.2.1. Effects of crude and fractioned extracts on cell viability

ER stress is one of the main causes of T2DM, which leads to β -cell apoptosis and dysfunction [11]. In this study, TG was used as a model to induce ER stress and β -cell death. Crude ethanol extract and some fractioned extracts including *n*-hexane (HE), ethyl acetate (EE), *n*-butanol (BE), and methanol extracts (ME) were tested for protective effect on ER stress-induced MIN6 cells. The results are presented in Figure 2.

The cells were pretreated with different samples for 24 hours and then 2 μ M TG (a concentration inducing about 60 % cell death) was added for 24 additional hours. CCK-8 assay was performed to determine the cell viability. TG decreased cell viability compared to control, but pre-treatment with plant extracts (0.01–10 mg/mL), the percentage of cell viabilities was improved.

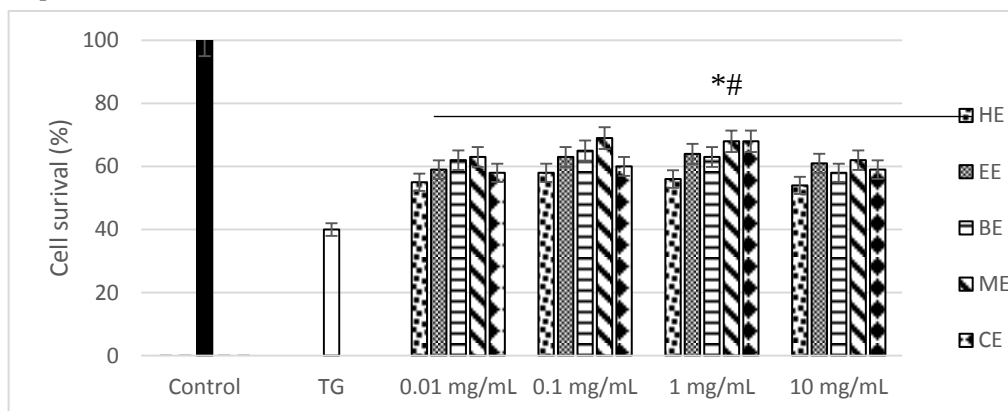


Figure 2. Cell-protective effect of crude and different fractioned extracts on TG-induced cytotoxicity in cultured MIN6 cells. Compared with blank control group, * $p < 0.05$; compared with TG model group, # $p < 0.05$.

The ME (0.1 mg/mL) showed the strongest protective effect giving a maximum cell viability of 69 %. The CE with the concentration 1.0 mg/mL gave the lower cell viability of 68 %. The values of % cell viability of EE, BE and HE treated groups ranged (59-64) %, (58-65) %, and (54-58) %, respectively at concentration of (0.01–10) mg/mL.

The above results indicated that the crude extract and all the fractioned extracts have enhanced the protective effect against toxicity in MIN6 cells.

3.2.2. Effects of isolated compounds on cell viability

The four compounds protected MIN6 cells from TG toxicity from 1.0 to 1000 $\mu\text{g/mL}$ (Figure 3). All compounds promoted cell survival against TG-induced cytotoxicity of MIN6 cells. The highest protective effect against TG was observed for quercitrin (78 %, 10 $\mu\text{g/mL}$). Depending on the concentration (1.0–1000 $\mu\text{g/mL}$), the % cell viability of luteolin and quercetin treated groups ranged from 66 % to 69 %, from 62 % to 70 %, respectively. As a result, quercetin and luteolin have proved to enhance the protective effect against toxicity in MIN6 cells and showed their maximum efficacies at the concentration of 10 $\mu\text{g/mL}$, and caffeic acid also showed the highest cell-protective activity (68 %) at this concentration. These results were in accordance with the observed phytochemical results of this medicinal plant.

In this study, three flavonoids were isolated from the EE and ME. It is noteworthy that the ME and quercitrin possessed the cell protection activity against TG-induced toxicity significantly. In addition, it has been reported that Eup extract possessed significant antihyperglycemic activity instreptozotocin (STZ)-induced diabetic mice and rat [12,13]. In the other report, the antidiabetic nature in rats of the Eup leaves extract has been also indicated [14]. It has also been announced that quercitrin has the ability to increase glucose utilization and normalize the carbohydrate metabolic enzymes in STZ-induced diabetic rats [15]. A study by Mona F. Mahmoudet *al.*[16] demonstrated that quercetin is potential candidate to prevent diabetic vascular complications in both insulin deficiency and resistance via its inhibitory effect on inflammatory pathways. Our results, based on ER stress-induced apoptosis in mouse β -cell lines, support the potential antidiabetic effect of Eup from the Mekong Delta of Vietnam.

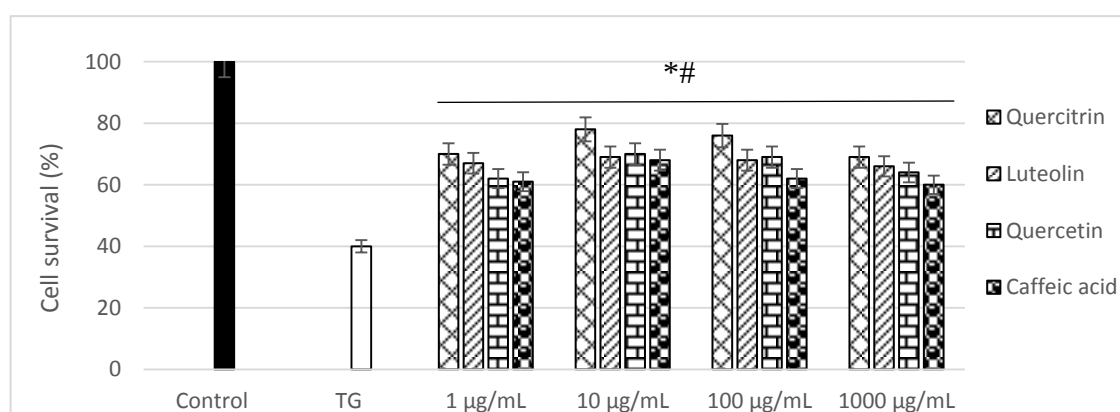


Figure 3. Cell-protective effect of isolated compounds on TG-induced cytotoxicity in cultured MIN6 cells. Compared with blank control group, * $p < 0.05$; compared with TG model group, # $p < 0.05$.

In this study, for the first time, the cell-protective effects of fractioned extracts and isolated compounds from Vietnamese *E. hirta* L. had been conducted on pancreatic β -cells MIN6 survival exposed to thapsigargin-ER stress conditions.

4. CONCLUSIONS

The cell-protective effects of crude ethanol extract and some fractioned extracts as well as some isolated compounds from *Euphorbia hirta* L. collected in the Mekong Delta on ER stress-induced cell death in mouse β -cell lines were investigated. The results of this study revealed that methanol extract and quercitrin isolated from *Euphorbia hirta* L. were able to significantly reduce thapsigargin-induced toxicity in MIN6 cells, indicating some potential antidiabetic effects or giving an explanation of the previously observed effects. Nevertheless other assays should be performed in order to develop novel drug for T2DM.

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