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Alternanthera paronychioides protects pancreatic β-cells from glucotoxicity by its antioxidant, antiapoptotic and insulin secretagogue actions

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ABSTRACT

The antioxidant and antiglucotoxic effects of *Alternanthera paronychioides* on pancreatic β-cell were investigated. Antioxidant assays demonstrated that ethanol extracts of *A. paronychioides* (EEAP) exhibited the highest antioxidant activity, which also had the highest phenolic and flavonoid contents. Two major polyphenolics, ferulic acid and quercetin, were identified from EEAP by HPLC-DAD. Effects of EEAP, ferulic acid and quercetin on high glucose (25 mmol/L)-induced pancreatic β-cell apoptosis and dysfunction were further evaluated. Results showed that EEAP and quercetin but not ferulic acid protected β-cells from glucotoxicity through several mechanisms, including: (1) maintaining β-cell viability; (2) suppressing reactive oxygen species production; (3) reducing characteristic features of apoptosis; (4) inhibiting the activation of caspase-9 and caspase-3 and the cleavage of poly (ADP-ribose) polymerase; (5) upregulating pancreatic and duodenal homeobox 1 gene expression and the insulin secretagogue action of pancreatic β-cells. These findings may shed light on the preventive actions of *A. paronychioides* on diabetic glucotoxicity.

1. Introduction

Diabetes is a systemic metabolic disease often associated with symptoms such as weight loss, polydipsia, polyphagia, and polyuria. Without appropriate treatment and therapeutic intervention, diabetic patients may develop multiple complications, including acute ones such as hyperglycaemia, diabetic ketoacidosis and nonketotic hyperosmolar coma, and chronic ones such as cardiovascular disease, renal failure, neuropathy, and compromised wound healing. Many reports have confirmed that the levels of oxidative stress in diabetic patients is markedly higher than that observed in healthy people (Lowell & Shulman, 2005). Chronic hyperglycaemia in diabetic patients accelerates glucose autoxidation in mitochondria, which generates excess free radicals that elevate oxidative stress (Piro et al., 2002). Pancreatic β-cells are responsible for secreting insulin to maintain blood glucose levels in a normal range. Under hyperglycaemic conditions, the detrimental effects of excessive glucose concentrations, known as glucotoxicity, induce β-cell apoptosis and a gradual cell mass deficiency that leads to an insulin secretion deficiency and results in diabetes (Lowell & Shulman, 2005). Thus, there is a close association among the elevation of hyperglycaemia-induced oxidative stress, insulin secretion deficiency and apoptosis in pancreatic β-cells.

To improve the insulin secretory function of islet cells, drug therapy is frequently used in clinics to treat diabetes. However, drug therapy is usually associated with multiple side effects. Therefore, many studies have been aimed at finding natural resources with preventive effects on diabetes. *Alternanthera paronychioides*, a perennial evergreen herb belonging to the *Amaranthaceae* family, originates from tropical Central and South America and is used as a food ingredient. According to the records of traditional Chinese medicine, *A. paronychioides* has therapeutic effects on hyperuricemia, gout, rheumatic arthritis, nephritis, cystitis, uremia, diabetes, and systemic neuralgia. A previous study has shown that the *Amaranthaceae* species are rich in flavonoids, which suggests that they have antioxidant activities (Salvador & Dias, 2004). Ethanol extracts from *Alternanthera maritima* contain

Abbreviations: MEAP, methanol extracts from *Alternanthera paronychioides*; EEAP, ethanol extracts from *Alternanthera paronychioides*; WEAP, water extracts from *Alternanthera paronychioides*; TEAC, Trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; CAA, cellular antioxidant activity; HPLC-DAD, high performance liquid chromatography-diode array detector; HG, high glucose; NG, normal glucose; ROS, reactive oxygen species; PARP, cleavage of poly (ADP-ribose) polymerase; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; MTT dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; PDX1, pancreatic and duodenal homeobox 1.

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flavonoids and inhibit H_2O_2-induced ROS production (Souza et al., 2007). In addition, extracts from *Amaranthus esculentus* promote insulin secretion and have the ability to alleviate hyperglycemic conditions in diabetic mice (Kim, Kim, Cho, Kim, & Shin, 2006). Taken together, these data suggest that phenolic compounds and *Amaranthaceae* plants may have physiological, protective roles in preventing diabetes. However, the protective effects of *A. paronychioides* and its active antioxidant components on pancreatic β-cells have not yet been reported.

Several pancreatic β-cell lines, including HIT-T15, RIN-m5F, βTC, MIN6, and INS-1 cells, have been clearly demonstrated to have the ability to secrete insulin (Poitout, Olson, & Robertson, 1996). The evidence has indicated that two pancreatic β-cell lines, HIT-T15 and RIN-m5F, are hypersensitive to oxidant stress and susceptible to free radical-induced oxidative cell damage (Zhang, Ollinger, & Brunk, 1995). Kaneto et al. (2001) reported that oxidative stress inhibits insulin gene transcription in pancreatic β-cells. They also found that in HIT-T15 cells and primary mouse cells, ROS reduces the binding affinity between pancreatic and duodenal homeobox 1 (PDX1) and the insulin gene, which causes a decrease in insulin mRNA levels and leads to a decrease in insulin synthesis. Previous animal studies have demonstrated that the *Amaranthaceae* plants have positive effects for treating diabetes (Kim et al., 2006). However, the protective effects of the *Amaranthaceae* plants on hyperglycemia-induced pancreatic β-cell damage remain unclear. Therefore, this study investigated the antioxidant activity of *A. paronychioides* and analysed its active antioxidant components, such as polyphenols, by high performance liquid chromatography. The effects of *A. paronychioides* on pancreatic β-cell function in HIT-T15 and RIN-m5F grown under high glucose (HG) conditions that mimic physiological hyperglycaemic conditions were examined. Finally, we also investigated whether *A. paronychioides* and its potential active components inhibited hyperglycaemia-induced cell damage and the possible molecular mechanisms of these actions.

2. Materials and methods

2.1. Chemicals

D-Glucose, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), ferulic acid, gallic acid, quercetin, mannitol, MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], β-phycoerythrin, propidium iodide (PI) and Trolox were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 glucose-free medium and foetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Dimethyl sulphoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany). L-Glutamine, penicillin–streptomycin solution and sodium pyruvate solution were purchased from Hyclone (Logan, UT, USA). Anti-β-actin, anti-Bax, anti-Bcl-2, anti-caspase-3, anti-PARP [poly-(ADP-ribose) polymerase], and anti-p53 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl-XL and anti-Bid antibodies were purchased from BioSource (Camarillo, CA, USA). The anti-caspase-9 antibody was obtained from BioVision (Mountain View, CA, USA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA). Polyvinyl difluoride (PVDF) membranes for Western blot analysis were obtained from Millipore (Bedford, MA, USA). Trizol RNA isolation kit was obtained from Life Technologies (Rockville, MD, USA). The primers for RT-PCR, dNTPs, reverse transcriptase, and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). Waters Oasis HLB extraction cartridge was purchased from Waters (Milford, MA, USA). All other chemicals were of analytical grade and all solvents were of high-performance liquid chromatography (HPLC) grade from Merck (Darmstadt, Germany).

2.2. Sample preparation

Raw materials of *A. paronychioides* were obtained from Fengshan Tropical Horticultural Experiment Branch, Taiwan. A 20 g powder of *A. paronychioides* was extracted with methanol, ethanol or water (160 mL) on a rotary shaker at room temperature for 24 h. The extracts from *A. paronychioides* were filtered through Whatman No. 2 filter paper, dried by a vacuum evaporator and stored at −20 °C until use. The methanol, ethanol and water extracts from *A. paronychioides* were named MEAP (methanol extract of *A. paronychioides*), EEAP (ethanol extract of *A. paronychioides*) and WEAP (water extract of *A. paronychioides*), respectively.

2.3. Determination of total phenolic and total flavonoid contents

The concentration of total phenolics was measured according to the method described by Hsu, Hong, Yu, and Yen (2010) and calculated using gallic acid as a standard. A sample (0.1 mL) was added to 2.0 mL of 0.02 g/mL Na_2CO_3. After 2 min, 50% Folin–Ciocalteu reagent (100 μL) was added to the mixture. After 30 min, the absorbance was measured at 750 nm using a spectrophotometer (BMG Labtechnologies, Offenburg, Germany). The total phenolics were calculated as a gallic acid equivalent using the regression equation between the gallic acid standard and absorbance. The total flavonoid content of the extracts was determined according to the method of Shih, Chan, Liao, Wang, and Yen (2010). In general, different concentrations of extracts or standard (quercetin) were mixed with 5% (v/v) $NaNO_2$, 10% $AlCl_3$ and 1 M NaOH. After mixing well, the absorbance was read at 510 nm and flavonoid contents were expressed as milligram of quercetin equivalents per gram of extracts.

2.4. HPLC analysis for polyphenolic compositions of A. paronychioides extracts

Polyphenolic components in MEAP, EEAP, and WEAP were determined by using a solid-phase extraction with HPLC-diode array detection (HPLC-DAD). In brief, sample extracts (20 mg) were refluxed with 2 N HCl at 85 °C for 30 min, after cooling, the hydrolysates were extracted with a 1:1 (v/v) mixture of diethyl ether and ethyl acetate and then re-extracted as above for three times. The sample solutions were combined and evaporated to dryness. Dried extracts were then applied to a Waters Oasis HLB extraction cartridge and washed with water and 5% aqueous methanol. The phenolics fractions were then eluted and evaporated under a nitrogen stream according to manufacturer's instructions. The residue was dissolved in glacial acetic acid/water mixture (27:983, v/v), and 20 μL was submitted to HPLC based on the method of Schieber, Keller, and Carle (2001). The HPLC analysis was performed with a Hitachi L-6200 intelligent pump equipped with a photodiode array detector Hitachi L-7455 (Hitachi, Tokyo, Japan) and a Mightysil RP-18 column (250 × 4.6 mm; 5 μm) (Kanto Chemical Co., Tokyo, Japan). The quantitative measurement was performed by integrating the peak area at 280 nm. Polyphenolic compounds were identified by comparisons of their retention time values and UV–visible spectra with those of known standards and were quantified by peak areas from the chromatograms.

2.5. Trolox equivalent antioxidant capacity (TEAC) assay

The determination of TEAC was carried out using the method described by Hsu et al. (2010). $ABTS^{\cdot+}$ was generated by the interaction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)