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Original Article

Acute oral toxicological evaluation in Wistar rats of interruptin-rich extract from *Cyclosorus terminans* and its *in vitro* antidiabetic potential

[Evaluación toxicológica oral aguda en ratas Wistar del extracto de *Cyclosorus terminans* rico en interruptina y su potencial antidiabético *in vitro*]

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Abstract

Context: Interruptins A and B derivatives from edible fern *Cyclosorus terminans* have been reported properties including anti-bacterial, anti-cancer, anti-oxidation, anti-inflammatory, and antidiabetic activities. Unfortunately, studies on its safety are still scarce.

Aims: To evaluate the acute oral toxicity of interruptin-rich extract (IRE) from *C. terminans* in Wistar rats and examine the antidiabetic potential of IRE *in vitro*.

Methods: IRE was evaluated cytotoxicity by MTT assay and potency of glucose consumption in hepatocyte and skeletal muscle cells. IRE was evaluated for acute toxicity in Wistar rats by following OECD 420 guidelines. Wistar rats were single oral administrated of 2000 mg/kg IRE and further observed for 14 days. LCMS was assessed for verifying IRE absorption into the bloodstream. Hematological, biochemical parameters and microscopic examination of heart, liver and kidney were evaluated.

Results: IRE demonstrated no cytotoxicity toward hepatocytes and skeletal muscle cells and facilitated glucose consumption into cells. In the acute toxicity study, on day 14, after a single oral administration of 2000 mg/kg IRE, no mortality and behavioral alterations. There was no change in metabolic parameters. Histopathology of heart, liver and kidney showed normal architecture.

Conclusions: Thus, LD_{50} of IRE was considered superior to 2000 mg/kg. Hence the extract can be utilized safely and could provide a capability for diabetic control.

Keywords: acute toxicity; antidiabetic; *Cyclosorus terminans*; histopathology; Wistar rats.

Resumen

Contexto: Se han informado propiedades de los derivados de las interruptinas A y B del helecho comestible *Cyclosorus terminans*, que incluyen actividades antibacterianas, anticancerígenas, antioxidantes, antiinflamatorias y antidiabéticas. Desafortunadamente, los estudios sobre su seguridad aún son escasos.

Objetivos: Evaluar la toxicidad oral aguda del extracto rico en interruptina (IRE) de *C. terminans* en ratas Wistar y examinar el potencial antidiabético de IRE *in vitro*.

Métodos: Se evaluó la citotoxicidad de IRE mediante ensayo MTT y la potencia de consumo de glucosa en hepatocitos y células de músculo esquelético. Se evaluó la toxicidad aguda de IRE en ratas Wistar siguiendo las directrices de la OCDE 420. A ratas Wistar se les administró por vía oral una sola dosis de 2000 mg/kg de IRE y se observaron durante 14 días. Se evaluó mediante LCMS para verificar la absorción de IRE en el torrente sanguíneo. Se evaluaron parámetros hematológicos, bioquímicos y examen microscópico de corazón, hígado y riñón.

Resultados: IRE no demostró citotoxicidad hacia los hepatocitos y las células del músculo esquelético y facilitó el consumo de glucosa en las células. En el estudio de toxicidad aguda, el día 14, tras una única administración oral de 2000 mg/kg IRE, no hubo mortalidad ni alteraciones del comportamiento. No hubo cambios en los parámetros metabólicos. La histopatología de corazón, hígado y riñón mostró una arquitectura normal.

Conclusiones: Así, la DL50 de IRE se consideró superior a 2000 mg/kg. Por lo tanto, el extracto se puede utilizar de forma segura y proporcionaría una capacidad para el control de la diabetes.

Palabras Clave: toxicidad aguda; antidiabético; *Cyclosorus terminans*; histopatología; ratas Wistar.

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INTRODUCTION

Natural compounds have frequently been applied as alternative medicines for numerous diseases worldwide and novel drugs combined with new technology (Ngo et al., 2013). The common reasons for using traditional medicine include their connection to the patient's tradition, affordability, and the perception of natural products as safer than pharmaceutical drugs (Canter and Ernst, 2004). However, many herbs are capable of producing a wide range of undesirable or toxic effects. Therefore, to ensure the effectiveness of medicinal plants used with safety, toxicity study in animals is initially concerned for supporting clinical use (WHO, 2005).

Diabetes mellitus (DM) is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. DM affects millions of people worldwide, with rapidly increasing incidence and prevalence (Sinnott et al., 2017). Nowadays, many antidiabetic drugs have been used to save lives and alleviate symptoms, however, sub-therapeutic effects still occur and the costs of diabetes treatment are expensive (May et al., 2002). Hence, there is a tremendous need to explore novel alternative antidiabetic substances providing more cost-effectiveness, attenuating side effects, and increasing patient compliance.

In the normal state, plasma glucose homeostasis is ensured by maintaining a balance between the rate of glucose entering the circulation and the rate of glucose removal from the circulation. During the postprandial state, the high blood glucose level is diminished by activating glucose uptake into hepatocytes (up to 60%) and muscle cells (up to 80%) (Aronoff et al., 2004; Kelley et al., 1988). Typically, the glucose level descends to normal levels within 3 h, whereas diabetic patients are ineffective in this process resulting in prolonged hyperglycemia, which consequently leads to severe diabetes-associated complications (Aronoff et al., 2004). Therefore, encouraging glucose uptake by hepatocytes and muscle cells is one of the important glycemic controls of interest in diabetes.

The fern *Cyclosorus terminans* (J. Sm. ex Hook.) K.H. Shing (*Thelypteridaceae*) is one of the lower plants that has been consumed as a vegetable for a long time in Thailand (Kumboonruang, 2009) and has been widely distributed all over part of Thailand, Laos and Cambodia (Tagawa and Iwatsuki, 1988). Even though its traditional usage has not been declared, its isolated interruptin derivatives have been identified and displayed diverse bioactivities. For example, interruptins A and B revealed anticancer properties against MCF-7 human breast and HT-29 human colon cancer cells (Kaewsuwan et al., 2015). Interruptin A demonstrated antibacterial activity against Gram-positive aerobic bacteria, including methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and *Bacillus subtilis* along with an anaerobic bacterium *Propionibacterium acne* (Chaiwong et al., 2018; Kaewsuwan et al., 2015). Interruptins A and B were also tested as anti-inflammatory agents by scavenging nitric oxide (NO) radical and reducing NO production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells as well as antioxidant compounds determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reduction antioxidant power (FRAP) assays (Chaiwong et al., 2019). Moreover, they also revealed a powerful capability for intracellular ROS scavenging and anti-apoptotic impact against extracellular oxidative injury by H_2O_2 (Kaewsuwan et al., 2015). Recently, interruptin C has been indicated as a radioprotective agent on breast MCF-10A and human keratinocyte HaCaT cells (Chumsuwan et al., 2022). Intriguingly, interruptin B was found to encourage brown adipocyte differentiation and induce glucose consumption in adipocytes differentiated from human adipose-derived stem cells (ASCs) by acting as a dual PPARα and γ ligand (Kaewsuwan et al., 2016). At present, interruptins A and B were discovered to enhance glucose uptake and glycogen accumulation in mouse hepatocytes via PPARs regulation (no published data). DM is normally characterized by insulin resistance and chronic inflammation (Shoelson et al., 2006). As mentioned above, interuptins A and B but not C from *C. terminans* delivered both antidiabetic and anti-inflammatory potentials; hence, they could be suggested as naturally antidiabetic agents that provide beneficial effects over inflammation associated with diabetes. Unfortunately, their isolated yields were low as 0.002% and 0.003% (w/w), respectively (Kaewsuwan et al., 2015), which restricted pharmacological application. The enriched extract with a high amount of active agents was therefore considered as an alternative approach. Nevertheless, an understanding of the extract safety *in vivo* is substantially concerned. Therefore, the present study aims to provide a scientific basis to clarify the effect of interruptin-rich extract (IRE) from *C. terminans* regarding *in vitro* antidiabetic on hepatocytes and muscle cells before further pharmacological study *in vivo* and to support the safety application of edible vegetable *C. terminans* by evaluation of the acute oral toxicity of IRE in Wistar rats following the Organization for Economic Cooperation and Development (OECD) guidelines.

MATERIAL AND METHODS

Plant material and extract preparation

The aerial parts of *C. terminans* (J. Sm.ex.Hook) Panigrahi were collected from Phrom Khiri district, Nakornsrithammarat province, Thailand (GPS coordinates are latitude N8°35′12.228″, longitude E99°45′1.987″), and identified by Prof. Dr. Thaweesakdi Boonkerd (Chulalongkorn University, Thailand). A voucher specimen with the number SKP 2080320001 was kept at the Faculty of Pharmaceutical Sciences, PSU, Thailand. The aerial parts were dried and ground into powder. The 5 kg of dried powder was extracted with n-hexane under reflux (each 1 h, 50 L × 3 times). The hexane extract was filtrated using Whatman® filter paper no. 4 and evaporated to yield 48.6 g of extract (0.97% (w/w) of initial powder). The hexane extract was partially purified to obtain high concentrations of active compound interruptins with vacuum column chromatography on silica gel and eluted using a step gradient of n-hexane with an increasing concentration of dichloromethane. The fractions containing interruptins were combined and concentrated with a rotary evaporator (Helidolph Laborata 4000, Germany) to give 31.2 g of interruptin-rich extract (IRE) (yield 64.1% (w/w) of initial hexane extract), which was stored in the refrigerator at 4°C until use.

HPLC analysis of the extract

A comparison of interruptin concentration in hexane extract and IRE from *C. terminans* was measured by HPLC, which consisted of a binary pump, a thermostated column compartment, and a photodiode array (PDA) detector. The analysis was performed on chromaster 5410 (Hitachi, Japan). The separated condition was achieved at 25°C on a 290 mm 4.6 mm i.d. TSK-gel ODS-80Ts column (Tosho Bioscience, Japan). The mobile phase comprised of MeOH and 1% acetic acid in water (85:15) with a flow rate of 1 mL/min. The injection volume was 20 µL (Kaewsuwan et al., 2015; Chaiwong et al., 2018).

Cell cultures and reagents

Mouse hepatocytes (FL83B) and skeletal muscle cells (Sol8) were purchased from American Type Culture Collection (ATCC) then were cultured in Nutrient mixture F12 Ham Kaighn's modification (Sigma-Aldrich, St. Louis, MO, USA) and Hi-glucose DMEM medium (Gibco, Life Technology, Grand Island, NY, USA), respectively. Cells were incubated at 37°C in 5% CO_2 incubator and subcultured every 2-3 days. Fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Gibco (Life Technology, Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), glucose oxidase and peroxidase were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents used were analytical grade.

Cell viability

MTT assay was used for the determination of cell viability in this study. In brief, cell suspension of FL83B and Sol8 cells were counted and seeded at a concentration of 2×10^4 and 2.5×10^3 cells/well, respectively, into 96-well plates in their completed media supplemented with 10% FBS. After 24 h, cells were treated with various concentrations (1-10 µg/mL) of IRE and 0.5%DMSO as a control. Plates were incubated at 37°C in a humidified atmosphere containing a 5% CO_2 incubator for 24 h. Each well was filled with 10 µL of 5 mg/mL MTT and placed in dark condition for 3 h. Then all media were removed and replaced with 100 µL DMSO. The UV absorbance was measured at 570 nm by using a microplate reader (BMG Labtech, Germany). The percentage of cell viability was calculated by comparing it with the control.

Glucose consumption

Sol8 muscle cells (1×10^4 cells/well) were cultured overnight in a 24-well plate with 10% FBS contained in the completed medium. The medium was then changed to 2% FBS contained in the completed medium to differentiate myoblasts into contractile myotubes. FL83B hepatocytes 1.5×10^5 cells/well were seeded in a 24-well plate and cultivated overnight. FL83B and Sol8 cells were treated with 0.5-10 µg/mL IRE and 20-30 µM rosiglitazone in 0.5% and 2% FBS contained in their completed media, respectively. After 24 h, the conditioned medium was sampled for measurement of the residual glucose concentration by glucose oxidation assay (Li et al., 2018). The glucose consumption was calculated by subtracting from the initial glucose concentration in the medium. Results were expressed as percentage glucose consumption as compared to control cells, of which the glucose consumption was assigned as 100%.

Experimental animals

To avoid the estrogenic effects mediated diabetes, male rats would be employed to further verify *in vivo* antidiabetic activity of IRE. Nevertheless, its acute toxicological evaluation *in vivo* was initially concerned. Hence, healthy adult male Wistar rats weighing 180-200 g were used in this study. The animals were purchased from Nomura Siam International (Bangkok, Thailand) and housed in standard environmental conditions at 22 ± 2°C, 50 ± 10% humidity and 12:12 dark/light cycle. Food and water were

available ad libitum. The animals were kept for at least one week in the animal room before the study. The experiments were reviewed and approved by the institutional committee for the Ethical Use of Experimental Animals at Prince of Songkla University, in compliance with National Institutes of Health guidelines (approval no. Ref.60/2018).

Assignment of animals

The study was performed according to the Organization for Economic Co-operation and Development (OECD) guidelines No. 420 (OECD, 2001), in the main study following Annex 3. The rats were randomly divided into two groups, control and treatment groups of five rats each. The rats had fasted for 12 h before treating. IRE at 2000 mg/kg dissolved in a co-solvent consisting of tween 80: PEG 400: distilled water (1:4:5) (Surapanthanakorn et al., 2017) was single orally administered in a constant volume to the treatment group, while the vehicle was given to the control group. All rats were continuously observed over 24 h.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Six hours after the IRE sample administration, 200 µL of blood was collected from the lateral tail vein of each animal. Plasma was pre-treated by single-step protein precipitation with acetonitrile and filtrated with 0.2 µM syringe filter before taking to identify interruptins A and B and compared with standard 1.0 µg/mL interruptins A and B by LC-MS analysis. LC-MS was used for separating the components according to the molecular weight. LC-MS analysis of plasma rats was done using a liquid chromatograph-quadrupole time-of-flight mass spectrometer (LC-QTOF MS) (Agilent Technologies, USA). Mass spectra were recorded within 1 min. The injection volume was 20 µL. The flow rate was set to 20 µL/min. The full scan mass spectra from m/z 100-1500 amu were acquired in negative ion modes. The optimum conditions of the interface were as follows: ESI-negative; ion spray Vcap voltage: 4000 V, Fragmentor: 200 V, Nebulizer: 20 psig, drying gas flow: 5 L/min, Drying gas temperature: 325°C. MassHunter Workstation software (Agilent Technologies, USA) was used for data acquisition and processing.

Cage side observation and body weight measurement

The clinical observation, which included changes in skin and fur, eyes and mucous membrane, respiratory circulation, somatomotor activity, behavioral pattern, tremor, convulsion, salivation, diarrhea, sleep, and coma, were observed for all the experiments. Animals were observed individually after administration, and special attention was given during the first 24 h and even 12 h daily thereafter, for a total of 14 days. The amounts of food and water consumption were documented daily from the quantity of food and water supplied and the amount remaining at 12 h after a meal. The body weight of each rat was recorded throughout the study period.

Hematological and biochemical analysis

After overnight fasting, blood samples of animals were drawn from inferior vena cava puncture. The samples collected in plastic tubes containing EDTA anti-clotting were evaluated for hematological parameters, including red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW) and total white blood cells (WBC) by using automated hematology analyzer Sysmex XS-800i (Sysmex, Japan). Similarly, biochemical analyses were detected from blood collected in sodium fluoride tubes and plain glass tubes. Blood samples were centrifuged at 3000 rpm 4°C for 15 min. The obtained plasma and serum were separated and stored at -20°C until analysis. Plasma in sodium fluoride tubes was determined for glucose, and serum in plain glass tubes was separated for total cholesterol, total triglyceride, total protein, albumin, globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and blood urea nitrogen (BUN) determination with colorimetric assay by automated chemistry analyzer URIT-8031 (URIT, China).

Relative organ weight and histopathology analytical examination

After the rats were euthanized, parts of heart, liver and kidney tissues were collected for relative organ weight and histopathological analytical studies. Each average organ weight was normalized by its average body weight. The tissues from these organs were washed in normal saline and fixed immediately in 10% formalin for a period of at least 24 h, dehydrated with alcohol, embedded in paraffin and cut into 4-5 μm thick sections. The sections were then stained with hematoxylin and eosin (H&E) and examined under a light microscope. The features of each organ from the treatment group were compared with the control group

Statistical analysis

In vitro results are expressed as mean ± SD (n = 3) and *in vivo* data are presented as mean ± SEM (n = 5). Comparisons between groups were performed using

one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test with SPSS statistical software version 22. Values of *p<0.05 and **p<0.01 were considered as significant differences.

RESULTS AND DISCUSSION

Interuptins A and B from *C. terminans* carried various bioactivities (Chaiwong et al., 2018; 2019; Kaewsuwan et al., 2015; 2016). However, their safety profile is still scarce. Therefore, IRE was prepared from *C. terminans* to evaluate the antidiabetic property *in vitro* and toxicity *in vivo* study. IRE exhibited no harmfulness on mouse hepatocytes and skeletal muscle cells and also promoted glucose consumption in both cells. No mortality after oral administration of 2000 mg/kg IRE in Wistar rats along with no signs of toxicity during 14 days of the experiment.

HPLC analysis of the extract

Since naturally low amounts of pure isolated antidiabetic active compounds, interruptins A and B, from *C. terminans* their uses *in vivo*. Therefore, we enriched the interruptin derivatives from *C. terminans* extract by partial purification from originally hexane extract using vacuum column chromatography. As shown in Fig. 1, chromatographic peaks of interruptins A, B and C were detected by the validated HPLC method (Chaiwong et al., 2018). However, only interruptins A and B have been implicated in antidiabetic activity (Kaewsuwan et al., 2016). The amounts of bioactive constituents, interruptins A and B, in the prepared extracts were then analyzed in this study. The concentration of interruptins A and B in IRE were found to be as high as 244.4 and 91.8 µg/mg extract, respectively, while its initial hexane extract demonstrated 84.8 and 41.4 µg/mg, respectively (Fig. 2). IRE contained 2.2-2.9 folds higher contents of interruptins A and B than its original extract. As a result, the IRE was successfully prepared by the vacuum column chromatography method and further brought to the next study.

Cell viability

The effect of IRE on cell viability was initially evaluated by MTT assay. As shown in Fig. 3, 24 h incubation of FL83B hepatocytes and Sol8 muscle cells with IRE applied at 1-10 µg/mL revealed a percentage of cell viability for both cells. Interestingly, treatments of 5 and 10 µg/mL IRE induced statistically significant proliferation of FL83B hepatocytes as well as10 µg/mL IRE was effective in increasing the proliferation of Sol8 muscle cells, when compared to the control. Hence, IRE showed non-toxic to FL83B hepatocytes and Sol8 muscle cells with more than 80% cell viability (percentages above 80% are considered non-cytotoxicity) (ISO 10993-5, 2009).

Glucose consumption

The evaluation of the antidiabetic potential of any medicine or compounds using a cell-based method by determining the amount of glucose that disappeared in the culture medium after incubation with compounds or drugs has widely been established (Gayathri and Gomathi, 2016). Since glucose consumption is a considerable process that maintains blood glucose homeostasis, we designed to use hepatocytes and skeletal muscle cells to determine the glucose consumption affected by IRE.

Fig. 4 provides the percentage glucose consumption achieved in FL83B hepatocytes and Sol8 induced myotubes by IRE treated at 0-5 and 0-10 µg/mL, respectively, for 24 h. Induction of glucose consumption in a dose-dependent manner by IRE was observed in both cells. IRE at 1 and 5 µg/mL exhibited high activity with the response of 137.5% and 163.7% glucose consumption, respectively, into FL83B hepatocytes as compared to 20 µM rosiglitazone standard of which the glucose uptake was 113.5%. Whereas only a high concentration of 10 µg/mL IRE significantly potentiated glucose uptake into Sol8 myotubes with 118.1% glucose consumption comparable to 30 µM rosiglitazone (114.9% glucose consumption). As a result, it can be concluded that IRE exhibited antidiabetic properties by increasing the percentage of glucose consumption into both FL83B hepatocytes and Sol8 muscle cells.

Previous reports indicated that impairment of skeletal muscle and hepatic glucose uptake is associated with the development of hyperglycemia in type 2 diabetes (Iozzo et al., 2003; DeFronzo and Tripathy, 2009). Moreover, studies observed a decrease in glucose transport activity in hepatocytes and skeletal muscles of diabetic patients (Karlsson et al., 2006; Chadt and Al-Hasani, 2020). These results suggested that the ability of IRE to promote glucose consumption into FL83B hepatocytes and Sol8 cells could be a benefit for diabetic patients. However, IRE seemed to induce glucose uptake in the liver more favorable than in muscle cells. It is possible that different organs may have differential sensitivity to IRE. Besides the major organ of glucose homeostasis, the liver collects glucose influx into glycogen form, which is promptly used to sustain blood glucose levels. These provide the benefits of stored hepatic glycogen to serve as essential fuel for the brain and prevent cognitive dysfunction including obtundation and seizures when the level of plasma glucose has fallen (Sprague and Arbeláez, 2011).

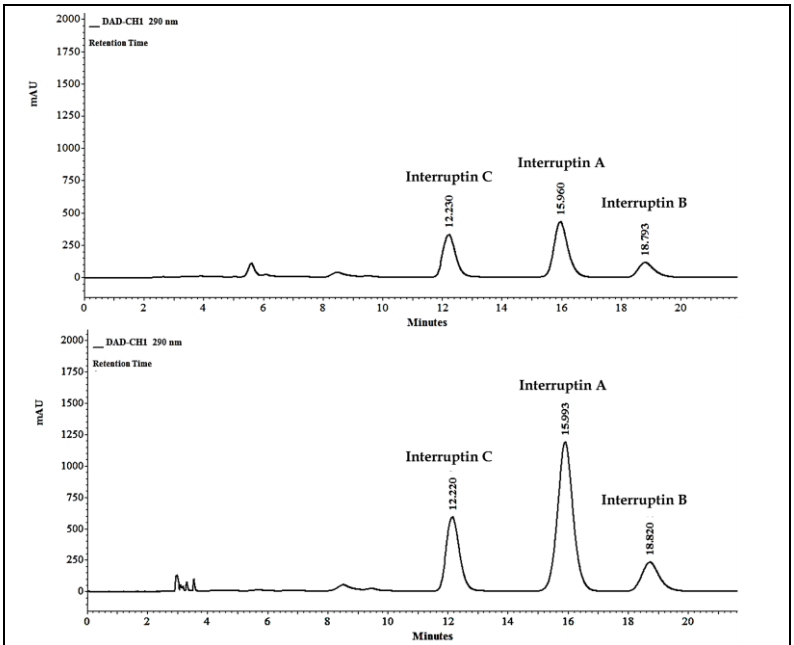

Figure 1. HPLC chromatograms of hexane extract **(A)** and IRE **(B)** from *C. terminans*.

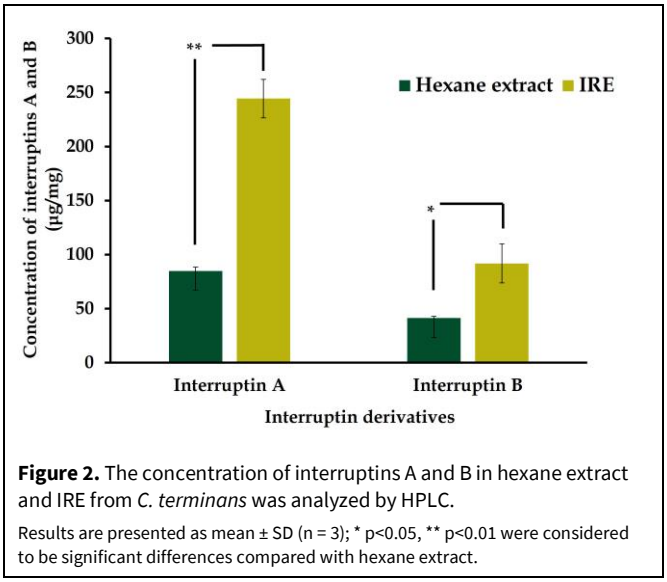

Figure 2. The concentration of interruptins A and B in hexane extract and IRE from *C. terminans* was analyzed by HPLC.

Results are presented as mean ± SD (n = 3); * p<0.05, ** p<0.01 were considered to be significant differences compared with hexane extract.

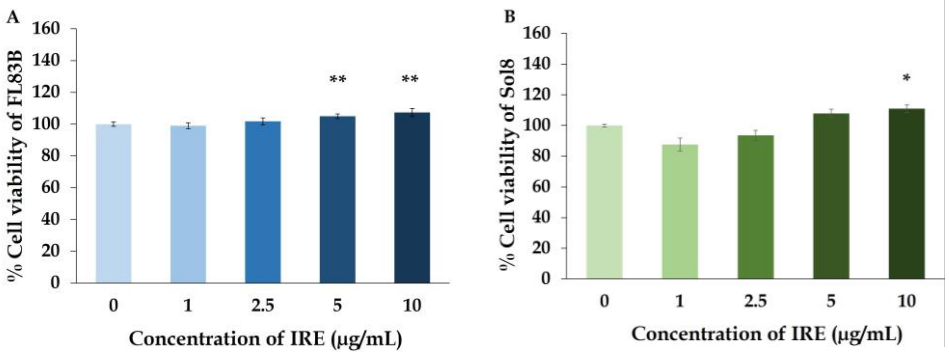

Figure 3. Cell viability of IRE in FL83B hepatocytes (**A**) and Sol8 cells (**B**) after 24 h incubation was determined by MTT assay.

Results are presented as the means ± SD (n = 3). * p<0.05 and ** p<0.01 were considered to be significant differences when compared with the control cells. 0.5%DMSO was used as a control, which presented 100% viability.

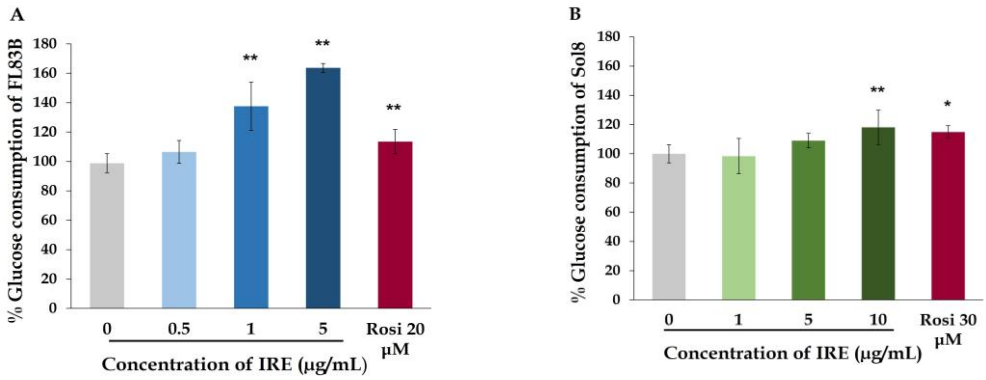

Figure 4. Glucose consumption of IRE and rosiglitazone (Rosi) in FL83B hepatocytes (**A**) and Sol8 cells (**B**) after 24 h incubation was evaluated by glucose oxidase assay.

Results are presented as the means ± SD (n = 3). * p<0.05 and ** p<0.01 were considered to be significant differences compared with the control cells. The starting glucose concentration in 0.5% FBS containing Nutrient mixture F12 Ham Kaighn's modification (for FL83B hepatocytes) and 2% FBS containing Hi-glucose DMEM (for Sol8 cells) media was 1.26 and 4.5 mg/mL, respectively.

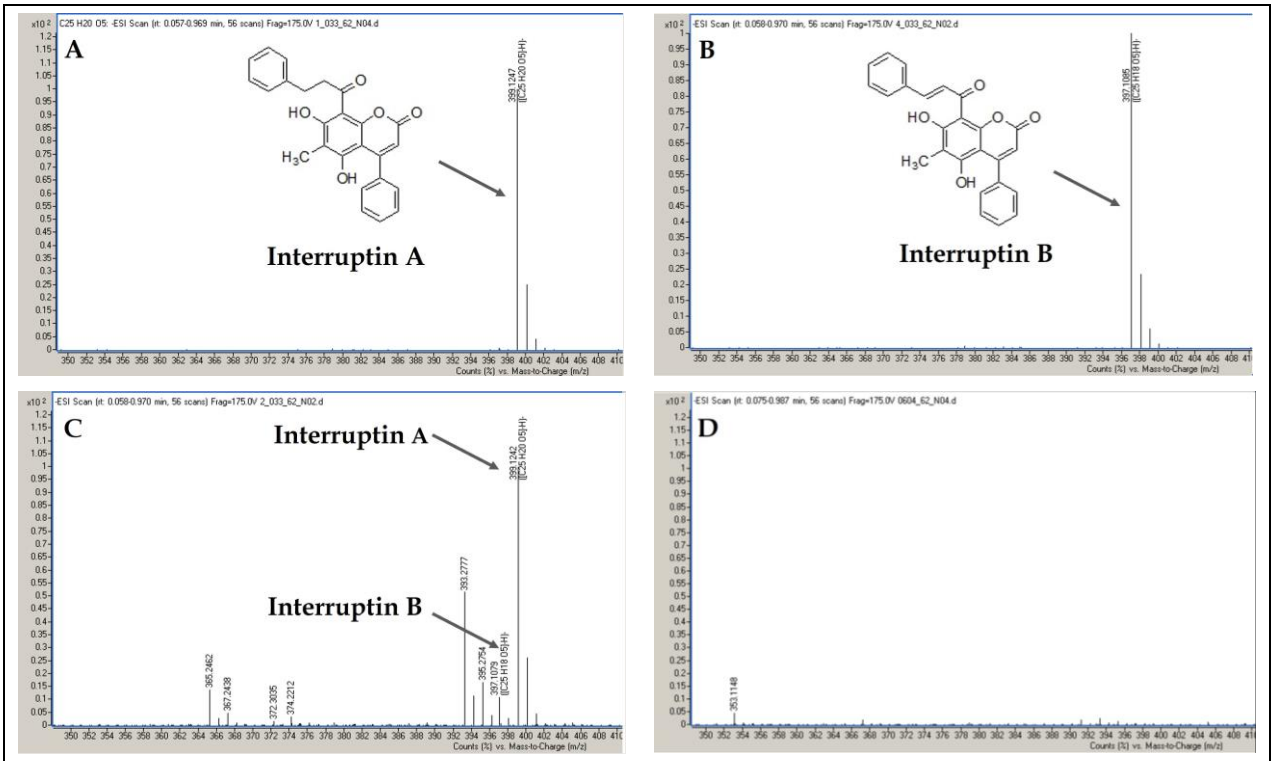

Figure 5. Ion chromatogram of standard 1.0 µg/mL interruptin A (**A**), 1.0 µg/mL interruptin B (**B**), plasma rats from the treatment (**C**) and control group (**D**) at 6 h after a single dose oral administration of 2000 mg/kg IRE analyzed by LC-MS.

LC-MS analysis

LC-MS technique was applied for metabolomic study to identify the small molecule metabolic products in animal plasma, which is particularly resourceful for the evidence-based development of new medicinal plants (Shyur and Yang, 2008). In this study, we employed LC-MS for a preliminary check of the absorption of IRE. Since interruptins A and B have been demonstrated as antidiabetic agents from *C. terminans*, therefore, this experiment analyzed active interruptins A and B in animal blood. After a single dose of 2000 mg/kg IRE administration for 6 h, the plasma of control and treatment rats were drawn and then analyzed by LC-MS in the negative ion mode. Fig. 5A-B show the chromatogram of standard 1.0 µg/µL interruptins A and B that produced the main production at m/z 399.12 and 397.10, respectively. The plasma of the treatment group presented the deprotonated molecules m/z of 399.12 and 397.10, which were identical to that of authentic interruptins A and B, respectively (Fig. 5C), whereas those ions were not found in the plasma of the control group (Fig. 5D). This finding indicates successful IRE ab-

sorption interruptins A and B were detected in the bloodstream.

Cage side observation and body weight measurement

The result of an acute oral toxicity study in Wistar rats showed no mortality at day 14 after administration with a single dose of 2000 mg/kg IRE. No behavioral changes such as impairment of food intake and water consumption, salivation, tremor, diarrhea. The absence of changes in skin and fur, eyes and the mucous membrane was also noted (Table 1). No statistically significant difference in body weight between both groups (Fig. 6). In principle, the body weight changes serve as a sensitive indicator of the general health condition and natural products may be metabolized into a toxic product, which could disturb gastric empty and decrease food intake (Chokshi, 2007). These suggest that the IRE did not interfere with the normal metabolism of animals.

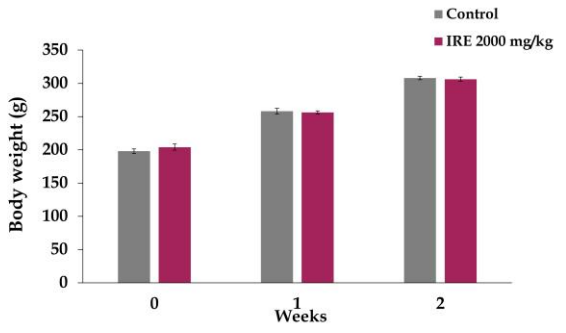

Figure 6. Body weight of rats after a single dose oral administration of 2000 mg/kg IRE during two weeks.
Each value is expressed as mean ± SEM (n = 5).

Hematological and biochemical analysis

The consequence of a single oral administration of 2000 mg/kg IRE on hematological parameters in rats is shown in Table 2. The analyzed parameters did not exhibit any statistically significant alteration compared to the control group. Moreover, 2000 mg/kg IRE in rats produced no significant change in biochemical parameters except an increase in total cholesterol compared to the control group (Table 3).

Evaluation of hematological parameters could be used as reliable indicators for evaluating the invasion of toxic substances or plant extracts. A non-significant difference in the hematological profile of the 2000 mg/kg IRE treatment group indicated that IRE did not risk the hematopoietic system.

The chief organs susceptible to the toxic effects of medicines are the liver and kidney. The increase in ALT and AST levels indicates liver diseases or hepatotoxicity. Decreasing levels of total protein, albumin, and globulin are associated with impaired hepatocellular function (Soyinka et al., 2007), while renal dysfunction can usually be perceived by a high level of urea and creatinine (Davis and Bredt et al., 1994). The results suggested that the liver and kidney functions were not modified between the control and treated animals. Hence, administration of 2000 mg/kg IRE did not cause any deleterious effects on the liver and kidneys of the rats. However, an increase in total cholesterol level was observed in the treatment group, which was still in the normal cholesterol range of 60.5-100.2 mg/dL of male rats (Vigneshwar et al., 2021).

Relative organ weight and histopathological analysis

The relative organ weights of heart, liver and kidney were determined at the end of the experiment. 2000 mg/kg IRE, the treatment group did not differ significantly from the control group (Table 4) and the macroscopic examination also revealed no abnormalities in the color or texture. Additionally, histopathological examination of 2000 mg/kg IRE treated samples of heart, liver and kidney stained with hematoxylin and eosin (H&E) revealed no pathological changes in tissues (Fig. 7). Heart sections showed no degree of injury to cardiomyocytes. Morphology of liver and kidney tissues also expressed normal characteristics.

According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), compounds are categorized into five groups on their LD_{50} basis (UN, 2017). Since during 14 days of the acute toxicity evaluation period, oral single dose of 2000 mg/kg IRE did not cause any significant alterations in behavioral patterns, biochemical, hematological, histopathological and no animal death, prepared IRE from *C. terminans* was therefore classified in group 5 (LD_{50} 2000-5000 mg/kg), the lowest toxicity class.

CONCLUSION

IRE from *C. terminans* had no cytotoxicity on mouse hepatocytes and skeletal muscle cells, and also enhanced glucose consumption into both cells. These results promising to find that *C. terminans* extract might be a potential natural material for an antidiabetic application. The acute toxicity study of IRE in Wistar rats showed no mortality or any signs of toxicity. The metabolic parameters also clearly illustrated that 2000 mg/kg IRE did not cause any damage to organs such as heart, liver and kidney. Thus, LD_{50} of IRE for oral acute toxicity was higher than 2000 mg/kg body weight. This study provides significant data on the non-toxicity profile of *C. terminans,* which is essential for further pharmacological study.

Table 1. Behavioral observation of rats after a single-dose oral administration of 2000 mg/kg IRE.

Responses	24 h after treatment		14 days after treatment	
	Control	2000 mg/kg IRE	Control	2000 mg/kg IRE
Tremors	Absent	Absent	Absent	Absent
Convulsion	Absent	Absent	Absent	Absent
Alertness	Absent	Absent	Absent	Absent
Writhing	Absent	Absent	Absent	Absent
Pain response	Absent	Absent	Absent	Absent
Grooming	Absent	Absent	Absent	Absent
Gripping	Normal	Normal	Normal	Normal
Restlessness	Absent	Absent	Absent	Absent
Urination	Normal	Normal	Normal	Normal
Diarrhea	Absent	Absent	Absent	Absent
Salivation	Normal	Normal	Normal	Normal
Lacrimation	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal

Table 2. Hematological parameters of rats after two weeks of a single-dose oral administration of 2000 mg/kg IRE.

Parameter	Control	2000 mg/kg IRE
RBC ($\times 10^6$ µL)	5.35 ± 2.86	6.59 ± 1.46
Hb (g/dL)	13.55 ± 0.65	12.90 ± 0.00
HCT (%)	44.05 ± 1.95	41.20 ± 0.00
MCV (fL)	63.95 ± 1.35	64.10 ± 1.40
MCH (pg)	19.65 ± 0.35	19.80 ± 0.20
MCHC (g/dL)	30.75 ± 0.15	30.90 ± 0.40
RDW (%)	15.95 ± 1.65	14.95 ± 0.25
WBC ($\times 10^3$ µL)	5.35 ± 2.15	6.60 ± 1.03

RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; WBC, total white blood cells. Data are presented as mean ± SEM (n = 5).

Table 3. Biochemical parameters of rats after two weeks of a single-dose oral administration of IRE 2000 mg/kg IRE.

Parameter	Vehicle control	2000 mg/kg IRE
Glucose (mg/dL)	182.66 ± 6.17	193.00 ± 15.10
Total cholesterol (mg/dL)	59.33 ± 0.33	69.00 ± 2.51*
Total triglyceride (mg/dL)	173.67 ± 5.45	165.67 ± 7.44
BUN (mg/dL)	28.40 ± 0.46	28.65 ± 0.93
Creatinine (mg/dL)	0.56 ± 0.02	0.59 ± 0.06
Total protein (g/dL)	6.25 ± 0.11	6.42 ± 0.22
Albumin (g/dL)	3.27 ± 0.04	3.39 ± 0.10
Globulin (g/dL)	2.93 ± 0.06	3.03 ± 0.12
AST (U/L)	168.33 ± 15.38	159.33 ± 11.17
ALT (U/L)	39.00 ± 2.65	35.33 ± 2.33

BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Data are presented as mean ± SEM (n = 5). *$p < 0.05$ was considered a significant difference compared with the control cells.

Table 4. Relative organ weight of rats after two weeks of a single-dose oral administration of 2000 mg/kg IRE.

Organs	Relative organ weight (%)	
	Control	2000 mg/kg IRE
Heart	0.33 ± 0.01	0.31 ± 0.01
Liver	4.42 ± 0.12	4.61 ± 0.26
Kidney	0.74 ± 0.04	0.83 ± 0.03

Data are presented as mean ± SEM (n = 5).

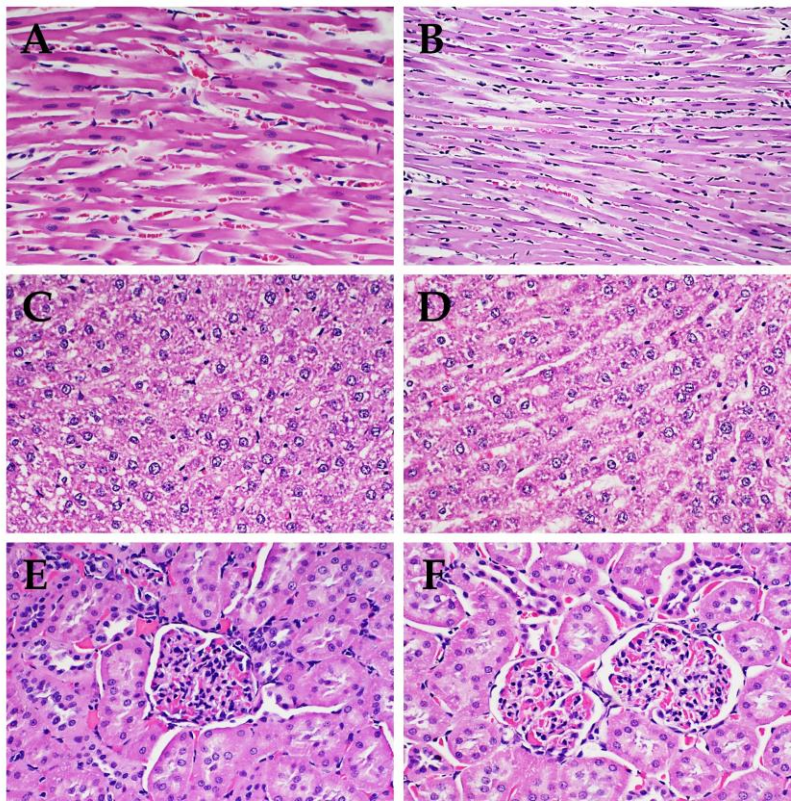

Figure 7. Photomicrographs of heart of the vehicle control (**A**) and 2000 mg/kg IRE-treated (**B**) rats; liver of the vehicle control (**C**) and 2000 mg/kg IRE-treated (**D**) rats; and kidney of the vehicle control (**E**) and 2000 mg/kg IRE -treated (**F**) rats with hematoxylin and eosin staining (400×).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Songtrai S	Dejyong K	Kaewsuwan K
Concepts or ideas			x
Design	x		x
Definition of intellectual content			x
Literature search	x		x
Experimental studies	x	x	x
Data acquisition	x	x	
Data analysis	x		
Statistical analysis	x		
Manuscript preparation	x		
Manuscript editing	x		x
Manuscript review	x	x	x

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