รายงานวิจัยฉบับสมบูรณ์

โครงการ ฤทธิ์ต้านเบาหวานของอนุพันธ์อินเตอร์รับตินที่สกัดจากเฟิร์นไซโคลซอรัส เทอร์มิแนนส์

Anti-diabetic property of interruptin derivatives isolated from the fern *Cyclosorus terminans*

โดย ผศ.ดร.สิรีวรรณ แก้วสุวรรณ

สิงหาคม 2562

สัญญาเลขที่ RSA5780035

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ผู้วิจัย **สังกัด**

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

และมหาวิทยาลัยสงขลานครินทร์

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

ACKNOWLEDGEMENTS

The author would like to thank the Thailand Research Fund (IRF) and Prince of Songkla University (PSU) for the financial support (RSA5780035). I gratefully acknowledge Prof. Dr. Thaweesakdi Boonkerd at Chulalongkorn University for plant identification.

บทคัดย่อ

รหัสโครงการ : RSA5780035

ชื่อโครงการ : ฤทธิ์ต้านเบาหวานของอนุพันธ์อินเตอร์รับตินที่สกัดจากเฟิร์นไซโคลซอรัส เทอร์มิแนนส์

ชื่อนักวิจัย : ผศ.ดร.สิรีวรรณ แก้วสุวรรณ
ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์
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ระยะเวลาโครงการ : 5 Years

เฟิร์นไซโคลซอรัส เทอร์มิแนนส์เป็นพืชที่มีการรับประทานเป็นผักมาเป็นเวลานานโดยเฉพาะทางภาคเหนือของประเทศไทยและมีการรายงานว่าเป็นแหล่งของสารกลุ่มคูมาริน ได้แก่ อินเตอรร์รับติน เอ และบี ในการศึกษานี้ค้นพบว่าสารอินเตอรร์รับตินเอและบีแสดงฤทธิ์ต้านเบาหวานในระดับหลอดทดลองโดยมีความสามารถในการเพิ่มการดูดซึมของน้ำตาลกลูโคสเข้าสู่เซลล์ตับและเซลล์กล้ามเนื้อด้วยกลไกผ่าน PPAR-γ และสารทั้งสองตัวดังกล่าวยังมีผลเพิ่มการสะสมไกลโคเจนในเซลล์ตับ ในขณะที่สารอินเตอรร์รับตินบีเท่านั้นที่มีความสามารถในการเพิ่มปริมาณไกลโคเจนในเซลล์กล้ามเนื้อ นอกจากนี้ยังพบว่าสารสกัดเฟิร์นที่มีอินเตอร์รับตินเข้มข้น (IRE) ที่เตรียมได้ไม่เพียงแต่ไม่มีความเป็นพิษต่อเซลล์ตับและเซลล์กล้ามเนื้อ สารสกัดดังกล่าวยังมีผลกระตุ้นการดูดกลับน้ำตาลกลูโคสเข้าสู่เซลล์ตับและเซลล์กล้ามเนื้ออีกด้วย รวมทั้งเมื่อทดสอบความเป็นพิษเฉียบพลันในหนูขาวพันธุ์วิสตาร์โดยการให้สัตว์ทดลองกินสารสกัด IRE ในขนาด 2,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลองเพียงครั้งเดียว ไม่พบอาการพิษหรือทำให้สัตว์ทดลองตายแต่อย่างใด โดยมีค่า LD_{50} เท่ากับ 2,000 ถึง 5,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง และสารอินเตอร์รับตินบียังมีฤทธิ์สูงในการต้านอักเสบโดยสามารถจับกับอนูมูลไนตริกออกไซด์ และยับยั้งการผลิตไนตริกออกไซด์ภายในเซลล์แมคโครฟาจชนิด 264.7 ได้ดีโดยให้ค่า IC_{50} เท่ากับ 67.68 และ 0.81 ไมโครโมลาห์ และตามด้วยสารอินเตอร์รับตินเอโดยมีค่า IC_{50} เท่ากับ 90.07 และ 12.18 ไมโครโมลาห์ ตามลำดับ โดยสารทั้งสองชนิดมีผลยับยั้งการแสดงออกของยีน *iNOS* และกระตุ้นการแสดงออกของยีน *PPAR-*γ ในลักษณะที่ขึ้นกับความเข้มข้น การศึกษานี้ยังได้พัฒนาวิธีวิเคราะห์ปริมาณสารอินเตอร์รับตินในสารสกัดเฟิร์นด้วยวิธีเอชพีแอลซีโดยวิธีดังกล่าวผ่านการทดสอบความใช้ได้ของวิธีตามหลักของ ICH รายงานวิจัยนี้จึงเป็นการศึกษาแรกที่อธิบายเกี่ยวกับฤทธิ์ในการต้านเบาหวานในเซลล์ตับและเซลล์กล้ามเนื้อ รวมทั้งฤทธิ์ต้านการอักเสบในเซลล์แมคโครฟาจของสารอินเตอรร์รับตินเอ และบี โดยคาดว่าเป็นผลจากการกระตุ้นผ่าน PPAR-γ และเป็นการค้นพบถึงศักยภาพของสารสกัดเฟิร์นไซโคลซอรัส เทอร์มิแนนส์ที่มีสารอนุพันธ์อินเตอรร์รับตินเป็นสารออกฤทธิ์เพื่อการประยุกต์ใช้สำหรับการรักษาโรคเบาหวานต่อไป อย่างไรก็ตามการศึกษาผลในการต้านเบาหวานของสารสกัดดังกล่าวในสัตว์ทดลองยังต้องมีการศึกษาเชิงลึกต่อไป

Key words: ฤทธิ์ต้านเบาหวาน; อินเตอรร์รับติน; ไซโคลซอรัส เทอร์มิแนนส์; ฤทธิ์ต้านอักเสบ; เอชพีแอลซี

Abstract

Project Code : RSA5780035

Project Title : Anti-diabetic property of interruptin derivatives isolated from the fern *Cyclosorus terminans*

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Project Period : 5 Years

The fern *Cyclosorus terminans* has long been consumed as a vegetable in northern Thailand. It has been reported as the source of coumarin derivatives, interruptins A and B. In this study, they were found to exhibit *in vitro* antidiabetic property by enhancing the glucose consumption into hepatocytes and skeletal muscle cells through PPAR-γ pathway. Likewise, both interruptins A and B also increased glycogen accumulation in heptaocytes, while only interruptin B could encourage glycogen content in muscle cells. Interestingly, prepared interruptin-rich extract (IRE) demonstrated not only no cytotoxicity toward hepatocytes and skeletal muscle cells, but also activated glucose uptake into both cells. Furthermore, acute toxicity study Wistar rats revealed no mortality or signs of toxicity after a single oral of 2,000 mg/kg IRE administration and indicated the LD_{50} as 2,000-5,000 mg/kg body weight. Moreover, interruptin B displayed potent anti-inflammation through NO radical scavenging and NO production inhibition in macrophage RAW264.7 cells with IC_{50} of 67.68 and 0.81 μM followed by interruptin A with IC_{50} of 90.07 and 12.18 μM, respectively. These compounds also down-regulated *iNOS* and up-regulated *PPAR-γ* mRNA expression in a dose-dependent manner. Additionally, the appropriate HPLC method for analysis the corresponding interruptins content in *C. terminans* extract was successfully validated according to ICH requirement. To the best of our knowledge, this is the first study describing anti-diabetic activity in liver and muscle cells and anti-inflammatory activity in macrophage cells of isolated interruptins A and B. These actions may be due to an induction of PPAR-γ. These results are very promising to find that *C. terminans* extract with active interruptin derivatives might be a potential natural material for anti-diabetic application. Nevertheless, more research is still required to address questions surrounding *in vivo* anti-diabetes.

Key words : Anti-diabetic; Interruptins; *Cyclosorus terminans*; Anti-inflammatory; HPLC

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Part I : Antihyperglycemic potential of interruptin derivatives from *Cyclosorus terminans* in mouse hepatocytes

Introduction

Diabetes mellitus (DM) is a metabolic disorder of carbohydrate, lipid and protein, and closely associated with many other complications resulting from a defect in insulin secretion, insulin action or both. The disease progresses tissue or vascular damage leading to severe diabetic complications such as retinopathy, neuropathy, nephropathy, cardiovascular complication and ulceration (Amos et al. 1997; Wallance et al. 2002; Shukla et al. 2003; Moran et al. 2004; Saely et al. 2004). As a results diabetes covers a wide range of heterogenous diseases. It has been estimated that there are around 200 million people suffering from DM. More importantly, this figure is expected to increase to over 320 million by 2030 (Wild et al. 2004).

DM can be categorized into several types, but the two major types are type 1 (insulin-dependent diabetes mellitus, IDDM) and type 2 (non-insulin-dependent diabetes mellitus, NIDDM) (Zimmet et al. 2004). Insulin replacement is the main therapy for patients with type 1 DM while diet therapy, exercise and oral hypoglycemic agents are usually considered for the treatment and management of type 2 DM (Zimmet et al. 2001). Although drugs are used primarily to save life, alleviate symptoms and prevent long-term diabetic complications, however, sub-therapeutic effects still occur and the cost of diabetes therapy is not cheap. Additionally, most of anti-diabetic drugs cause risk adverse effects and some were finally withdrawn from the market. Therefore, there are tremendous needs to develop novel anti-diabetic drugs providing more cost effectiveness, attenuating side effects and increasing patient compliance.

At present, over 50% of all drugs in clinical use originally come from natural products and mostly resided in the higher plants (Rates 2001). More interestingly, our pilot study has found that interruptin B isolated from the fern *Cyclosorus terminans* which has been reported for consumption as vegetable considerably increased glucose consumption in differentiated

adipocytes, accompanied by the up-regulation of GLUT-1 and GLUT-4 mRNA expression. A computational analysis of molecular docking predicted interruptin B as a dual PPAR-γ and-α ligand (Kaewsuwan, et al., 2016). However, one of other targets that affecting glucose level as hepatocytes has not been investigated yet. This study therefore aims to verify the anti-diabetic property of interruptin derivatives isolated from *C. terminans* together with their cellular and molecular mechanism in regulating glucose metabolism in liver cells consumption.

Materials and Methods

1. Chemicals and reagents

Interruptins A and B were isolated from ethyl acetate extract of the fern *C. Terminans* (Kaewsuwan et al., 2015). RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin were purchased from Gibco BRL, California. Dimethyl sulfoxide (DMSO), Phosphate buffer saline (PBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from AMRESCO, USA. Rosiglitazone and bisphenol A diglycidyl ether (BADGE) were obtained from Sigma-Aldrich, USA. Antibodies against IRS1, phosphorylated IRS1, PPAR-γ, β-actin and secondary antibodies of horseradish peroxidase conjugated- anti-mouse IgG were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against GLUT2 and PPAR-α were purchased from Thermo Fisher Scientific (USA).

2. Cell culture

Mouse hepatocytes (FL83B, ATCC® CRL-2390™) was purchased from ATCC and cultured in F12-K medium supplemented with 10% heat-inactivated Fetal Bovine Serum (Invitrogen) and 1 % antibiotics (100 unit/mL penicillin and 100 µg/mL streptomycin). Cells were normally incubated at 37ºC in a humidified atmosphere of 5% CO_2 until 80-90% confluent before each experiment.

3. Cell viability assay

In this study, MTT assay was used to evaluate the cell viability of mouse hepatocytes (FL83B). In brief, cells (2×10^4 cells per well) were seeded into 96-well plates and cultured overnight. Subsequently, they were treated with various concentrations of interruptins A and B (0.5-10 μM) in F12-K medium containing 10% FBS for 24 h. Cells were then washed with phosphate buffered saline (PBS) twice before added with 200 μL MTT (0.5 mg/mL) dissolved in PBS to react for 4 h at 37°C. The supernatants were discarded and cell were washed again with PBS. The formazan crystals were dissolved with 100 μL of DMSO and measured absorption at 570 nm by a microplate reader (BMG labtech, Germany) (Li et al., 2012). Percentage of cell viability corresponding from each treatment was calculated compared with the control without treatment.

4. Glucose consumption

FL83B cells were seeded in 24-well plates at a concentration of 1.5×10^5 cells per well overnight. Cells were treated with interruptins A, B (1-2.5 μM) and rosiglitazone (20 μM) for 12 and 24 h. After incubation, cultured media was collected to measure glucose concentration by glucose oxidation method (Blake and McLean, 1989), and glucose consumption in response to interruptins A and B treatment was calculated (Zhou et al., 2007).

5. Inhibition study

After cultured FL83B cells in 24-well overnight. Cells were treated with PPAR-γ antagonist, BADGE (15 and 20 μM) together with interruptins A, B (2.5 μM) and rosiglitazone (20 μM) for 24 h. After incubation, conditioned medium was sampled for determination of glucose concentration as previous described method.

6. Glycogen content

FL83B cells (6×10^5 cells/dish) were seeded in 10 cm dishes, cultured until reach 80-90% confluent and further incubated with interruptins A, B (1-2.5 μM) and rosiglitazone (20 μM) for 24 h. After treatment, cells were washed and lysed to collect supernatant. The isolated supernatant was quantified protein content by Bradford assay (Bradford, 1976) and then added

with 95% ethanol to precipitate glycogen by keeping at 40ºC for 4 h. The dried pack glycogen was determined the content by anthrone-reagent method (Seifter et al., 1950; Osawa et al., 2011). The formation of blue color from the reaction was measured by a spectrophotometer (Agilent, Santa Clara, CA) at 620 nm. Relative glycogen content of each treatment was calculated compared with the control.

7. Western blot analysis

After treated cells with interruptins A, B (1 and 2.5 μM) and rosiglitazone (20 μM) for 24 h, the cells were subjected to protein analysis using western blot. Non-treated cells were performed as a control. Total protein from treated cells was extracted with RIPA protein extraction solution and determined the amount with Coomassie protein assay reagent by following the protocol provided by manufacture (Thermo SCIENTIFIC, USA). Aliquots of 30 μg protein were separated on sodium dodecyl sulfate-12% polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% milk, washed 3 times with 1% milk and further hybridized with rabbit primary antibodies (1:1000) against IRS1, p-IRS1m GLUT2-PPAR-γ, PPAR-α and β-actin control. The membrane was washed again with 1% milk in order to remove the excessive antibodies and subsequently incubated with secondary antibody, goat anti-rabbit IgG conjugated horseradish peroxidase ((HRP). The protein bands on the membrane were detected by reacted with Immobilon™ Western Chemiluminescent HRP substrate. The chemiluminescent signals were monitored under a CCD camera.

8. Statistical analysis

Experimental results were assessment with triplicate analysis. The results are presented as mean ± SD. One-way ANOVA and LSD test were used for statistical analysis. $P < 0.05$ or $P < 0.01$ was considered as statistically significant difference.

Results and Discussions

1. Cell viability assay

The cell viability of 0.5-10 μM interruptins A and B toward mouse hepatocytes (FL83B) was determined by MTT assay, all the tested chemicals were non-toxic to FL83B cells compared with the control without treatment (Figure 1). Additionally, interruptin A trended to induce FL83B proliferation as dose dependent manner, except at highest concentration of 10 μM interruptin A slightly decreased cell proliferation. On the other hand, interruptin B significantly increase proliferation of FL83B at low tested concentration of 0.5 and 1 μM. Unfortunately, it markedly reduced cell viability when treated with high concentration of 10 μM. This is the first report on hepatocyte cells of interruptins A and B isolated from *C. terminans* which both were not damage hepatocyte at tested 0.5-5 μM. Therefore, they might provide information for further study or application of interruptins A and B on glucose and glycogen metabolism pathway.

2. Glucose consumption

In principle, the liver conduces to some parts of the dietary glucose absorption after a meal. It retains the glycemic control in a normal physiology by rapid clearance of glucose circulating in the blood through uptake, glycolysis and glycogen synthesis (Li et al., 2018). This study is focusing on glucose uptake and glycogen synthesis in hepatocytes.

As PPAR-γ is one of the molecular targets for antidiabetic drugs, which improve insulin sensitivity and glucose tolerance, the present study examined whether interruptins A and B treatment affects glucose metabolism, compared with an insulin sensitizer, rosiglitazone. As shown in Figure 2, glucose consumption was significantly increased (107.9-122.6) in the hepatocytes treated with 1 and 2.5 μM interruptins A and B for 12 h. More obviously induction of glucose uptake (132.2-165.5%) into hepatocytes was observed when treated with interruptins A and B for 24 h, which was 1.04-1.30 times higher than the glucose consumption measured following treatment with 20 μM rosiglitazone (127.2%).

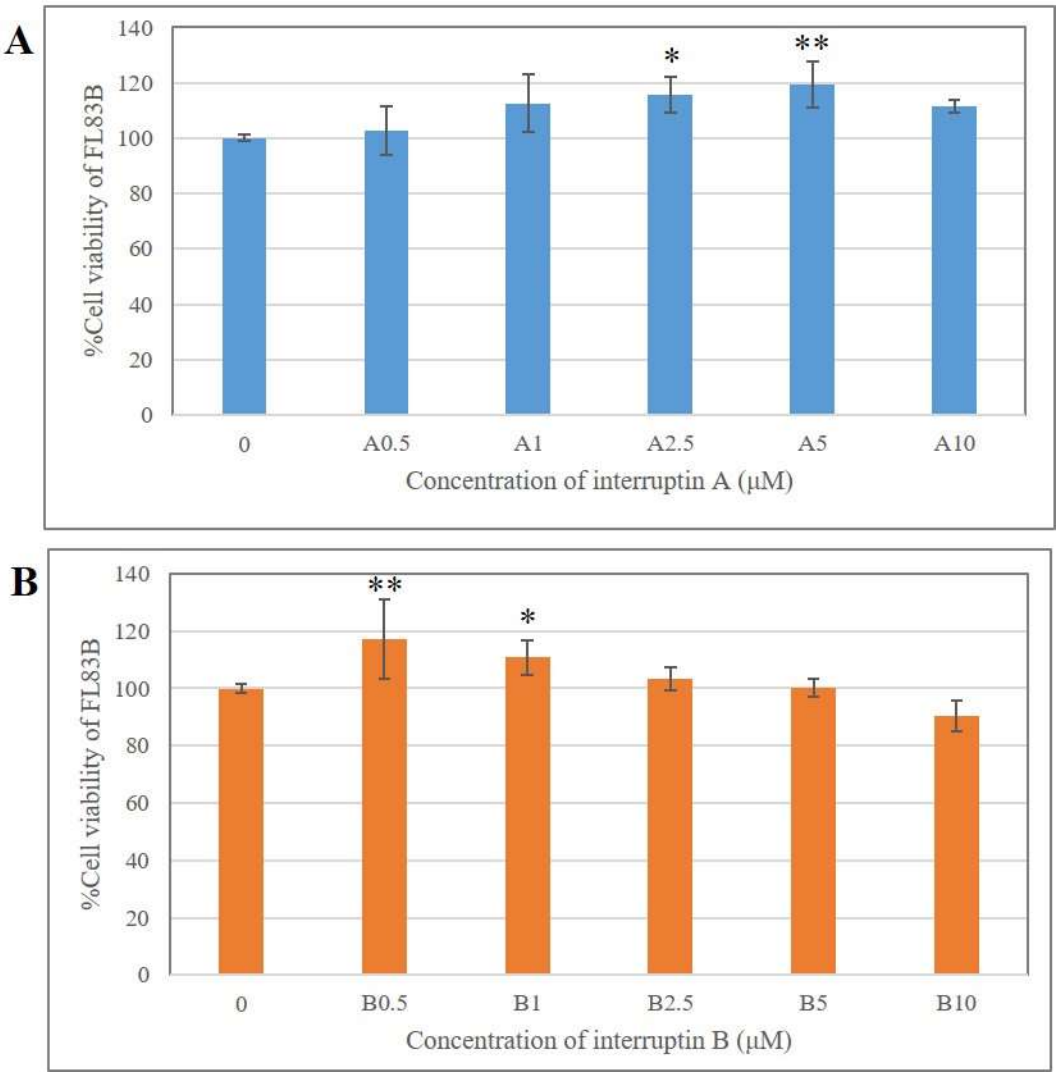

Figure 1. Hepatocyte (FL83B) cell viability of isolated interruptins A (A) and B (B). All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with the untreated group.

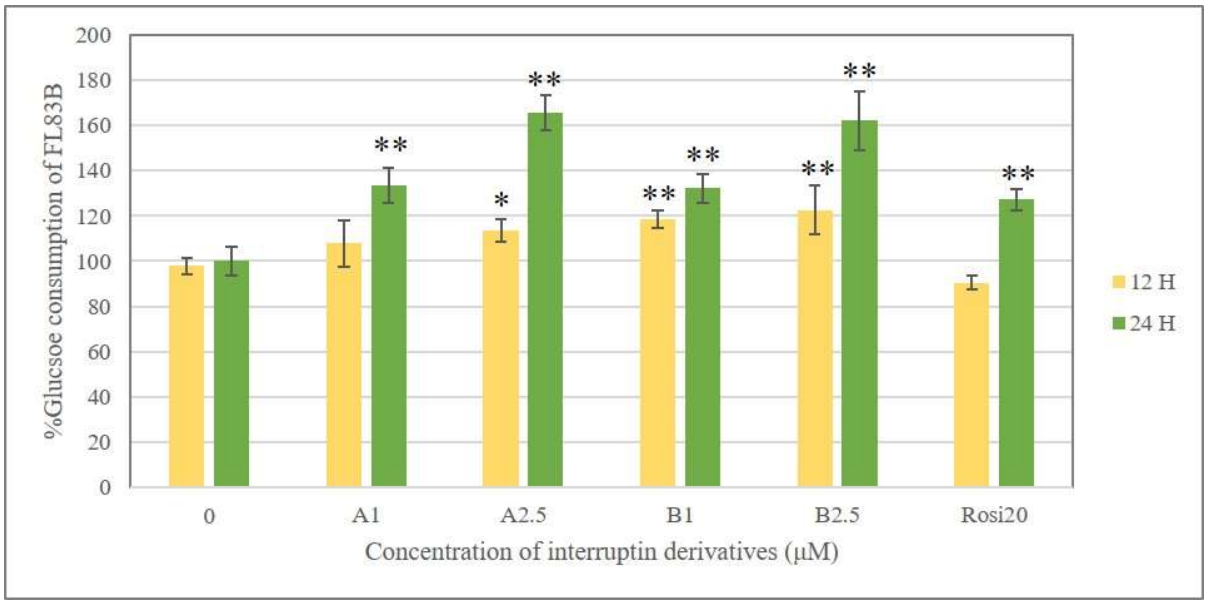

Figure 2. Effect of interruptins A and B on glucose consumption in hepatocytes (FL83B). Glucose consumption was measured following 12 and 24 h treatment with interruptins A and B compared with 20 μM rosiglitazone. All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with the untreated group. A, interruptin A; B, interruptin B; Rosi, rosiglitazone.

3. Inhibition study

To understand the mechanism underlying the effects of interruptins A and B on glucose consumption in hepatocytes, inhibition experiments against a specific PPAR-γ inhibitor, BADGE, were determined. As shown in Figure 3, the increased glucose consumption, which was induced by 2.5μM interruptins A and B was reduced considerably by co-treatment with 15 or 20 μM BADGE. The results suggested that the interruptins A and B-induced glucose consumption was eradicated in a dose-dependent manner by BADGE. Taken together, these observations supported that the induction of glucose uptake in hepatocytes by interruptins A and B was dependent on PPAR-γ activation.

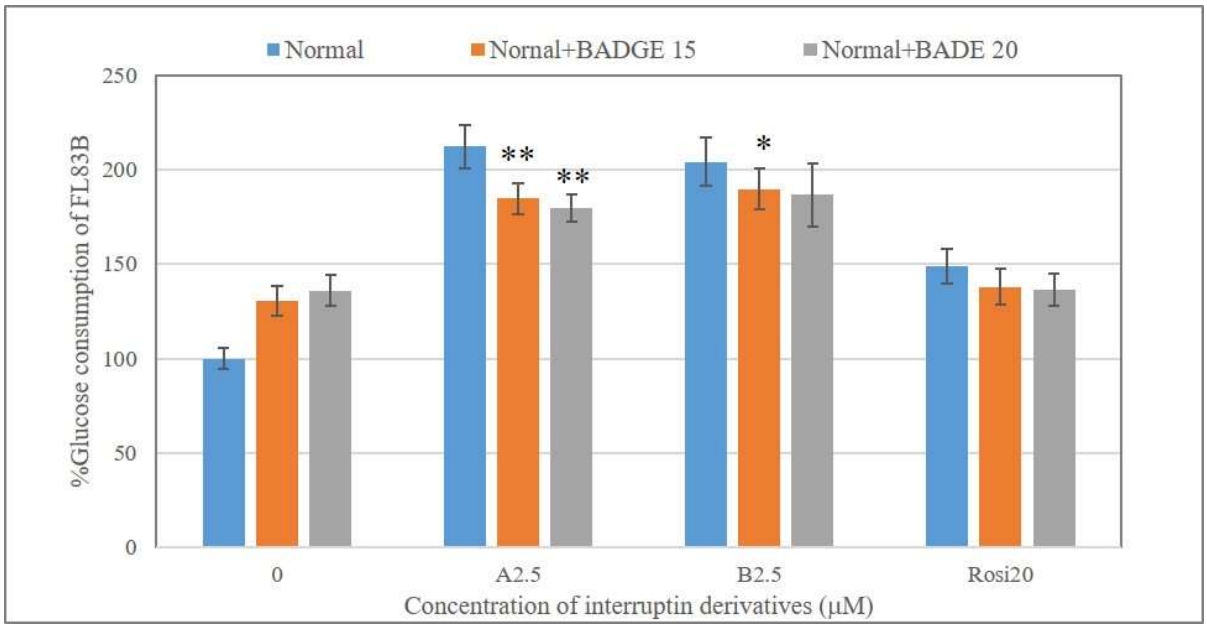

Figure 3. Reversal of glucose consumption in hepatocytes (FL83B) by PPAR-γ (BADGE) antagonist. All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with each normal group. PPAR, peroxisome proliferator-activated receptor. A, interruptin A; B, interruptin B; Rosi, rosiglitazone.

4. Glycogen content

Carbohydrate metabolism in hepatocytes normally undergoes a shift from glucose storage via glucose uptake and glycogen synthesis during feeding towards glucose production via glycogenolysis and gluconeogenesis during fasting (Peeters and Baes, 2010). In this study, glycogen content after treatment with interruptins A and B was also determined. As shown in figure 4, 1 and 2.5 μM interruptins A and B enhanced glycogen accumulation in hepatocytes as a dose dependent manner which were 1.1-1.5 times higher than 20 μM rosiglitazone.

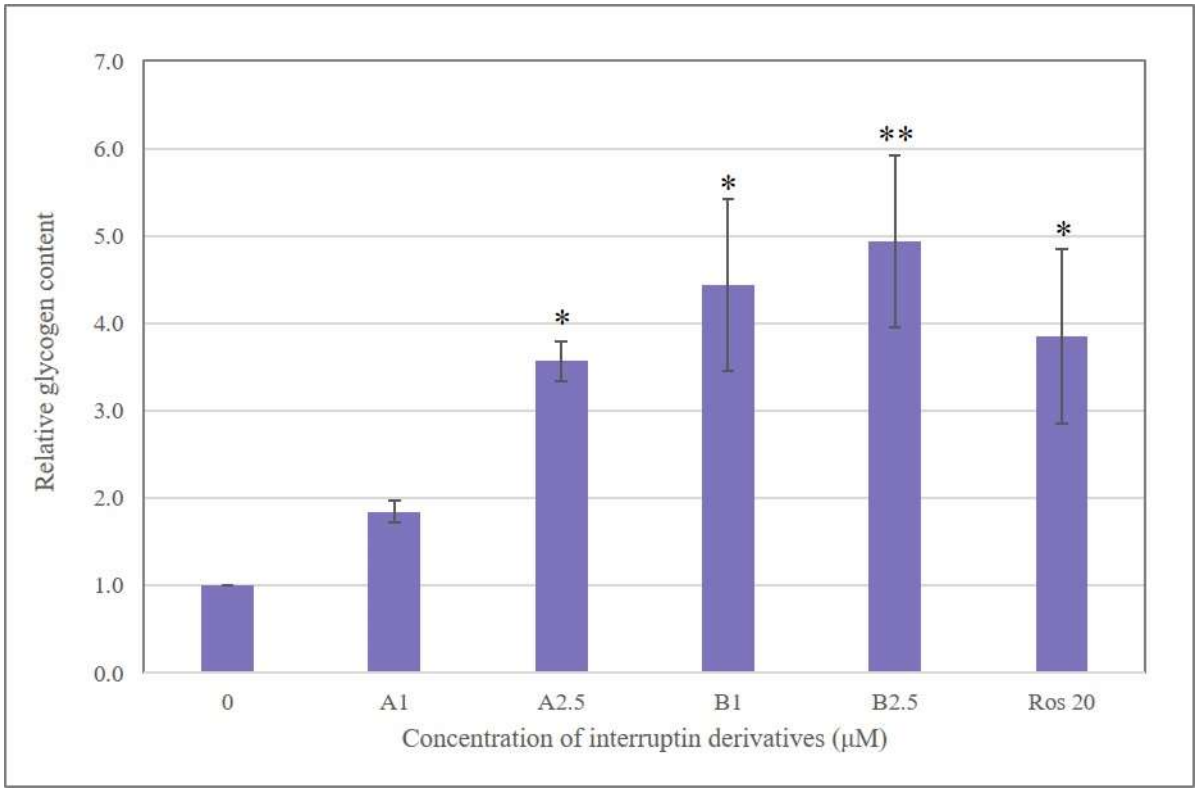

Figure 4. Effect of interruptins A and B on glycogen accumulation in hepatocytes (FL83B). All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with the untreated group. A, interruptin A; B, interruptin B; Rosi, rosiglitazone.

5. Protein expression

In hepatocytes, the glucose transporter type 2 (GLUT2) conducts predominantly to the human hepatic glucose uptake and the expression together with activity of GLUT2 is transcriptionally regulated by PPAR-γ (Kim et al., 2000), but is independent og insulin signalling. Beside PPAR-γ, PPAR-α is the master regulator of lipid metabolism during fasting, but evidence is emerging for a role of PPAR-α in balancing glucose homeostasis as well (Leonardini et al., 2009). In this study, hepatocytes treated with interruptins A and B demonstrated induction of GLUT2, PPAR-γ and PPAR-α protein expression (Figure 5) as a concentration dependent manner, whereas only interruptin A encouraged expression of phospholyrated-ISR1/IRS1.

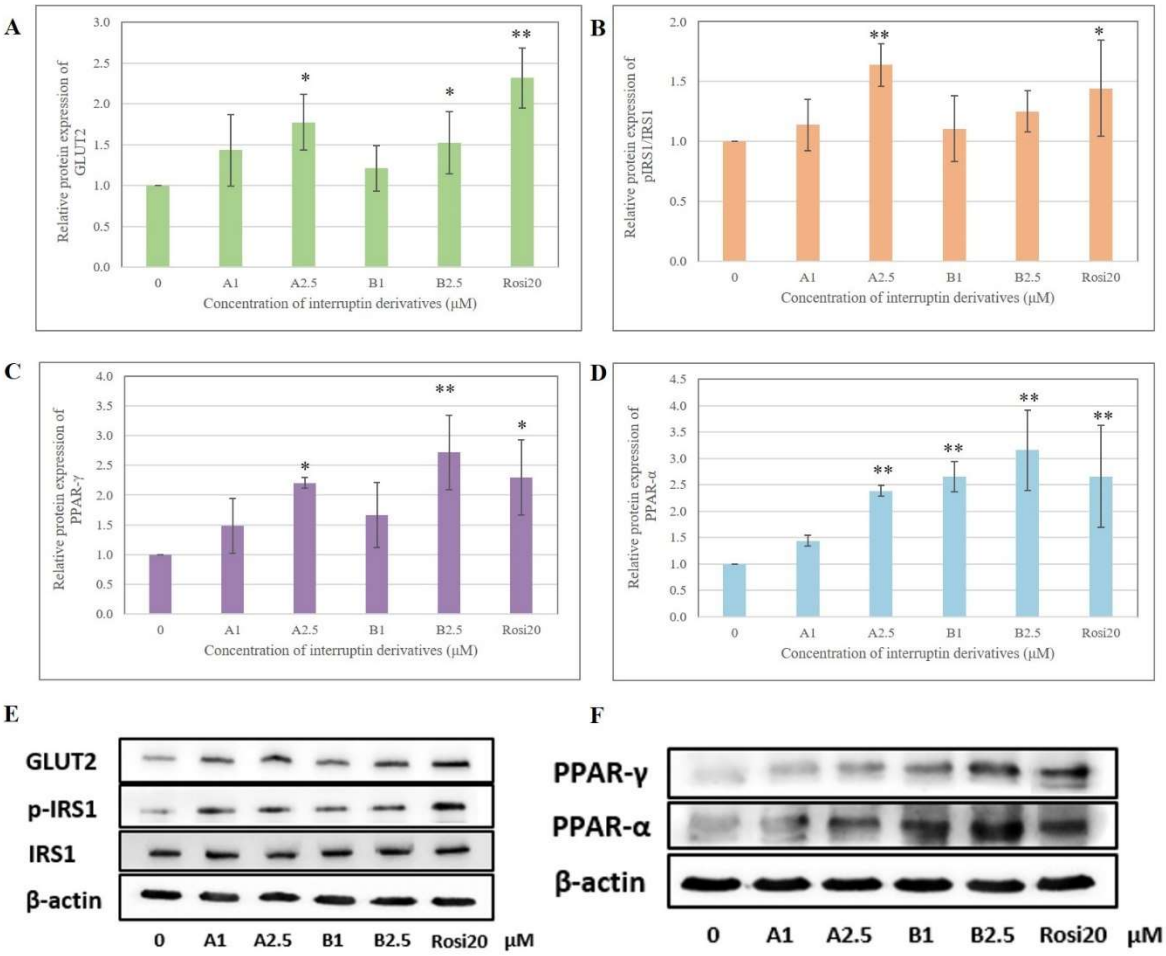

Figure 5. Effect of interruptins A and B on protein expression in hepatocytes (FL83B). All values are presented as the mean ± standard error of the mean (n=3). ∗P<0.05 and ∗∗P<0.01, compared with the untreated group. A, interruptin A; B, interruptin B; Rosi, rosiglitazone.

Conclusions

This study included the antidiabetic potential of natural coumarin derivatives, interruprins A and B, isolated from the fern *C. terminans*. Interruptins A and B enhanced glucose uptake and this effect was attenuated by BADGE PPAR-γ antagonist treatment, confirming the involvement of PPAR-γ pathway in stimulation of glucose uptake by interruptins A and B treatment. The downstream responses to PPAR-γ activation included an increase in GLUT2, resulting in facilitated glucose uptake. Moreover, they increased glycogen

content in hepatocytes together with the protein level of PPAR-γ. Although, their definitely mechanism beyond PPAR-γ activation remains to be elucidated. Collectively, these results indicate that interruptins A and B play a key role in antidiabetic capability through PPAR pathway. These novel findings therefore support the benefits of *C. terminans* consumption for health care or facilitate the *in vivo* and clinical studies for further medicinal application.

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Part II : Natural courmarin derivatives interruptins A and B enhance glucose uptake in mouse skeletal muscle cells

Introduction

Type-2 diabetes mellitus is a metabolic syndrome, characterized by fasting and postprandial hyperglycemia and is constantly on the rise (Chan et al., 2009). Despite considerable progress in the treatment of diabetes mellitus with synthetic drugs which mostly cause side effects, the search foe indigenous natural antidiabetic compounds with more safety and effectiveness is till challenged. Natural products have played a vital role in the discovery of antidiabetic drugs being derived from a natural product or a natural product lead. Plant derived antidiabetics continue to remain as the primary source for novel therapeutics (Van Cleemput et al., 2009; Jetté et al., 2009).

Preliminary study has demonstrated that Interruptin B isolated from Cyclosorus terminans (Thelypteridaceae) revealed anti-diabetic property when tested in adipocytes differentiated from human adiposed-derived stem cells (hASCs). According to the results, 25 and 50 μM interruptin B showed anti-diabetic activity by increasing of glucose consumption (357-640%) in differentiated hASCs which were 2.3-4.1 times greater than 50 μM standard drug rosiglitazone (157%). Interruptin B also induced gene expression of glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) compared to the control cells. A computational analysis of molecular docking predicted interruptin B as a dual peroxisome proliferator-activated receptor (PPAR)-γ and-α ligand (Kaewsuwan et al., 2016). However, other direct targets included skeletal muscle cells which are the major site of glucose uptake and glycogen storage have not been verified yet. Therefore, this study aims to prove the anti-diabetic property of isolated interrupins A as well as interruptin B from *C. terminans* on the muscle cells. The cellular and molecular mechanism of interruptin derivatives in regulating glucose consumption was also determined.

Materials and Methods

1. Chemicals and reagents

Interruptins A and B were isolated from ethyl acetate extract of the fern *C. Terminans* (Kaewsuwan et al., 2015). RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin were purchased from Gibco BRL, California. Dimethyl sulfoxide (DMSO), Phosphate buffer saline (PBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from AMRESCO, USA. Pioglitazone and bisphenol A diglycidyl ether (BADGE) were obtained from Sigma-Aldrich, USA. RNA extraction kit (FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit) was purchased from FAVOGEN, Korea. FIREScript RT cDNA Synthesis KIT and 5x FIREPol PCR master Mix Ready to load were supplied from Solis BioDyne, Estonia.

2. Cell culture

Mouse myoblasts (Sol8, ATCC® CRL-2174™) was purchased from ATCC and cultured in the growth medium consisting of Hi glucose- Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin) (Invitrogen). Cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2. After cells reached confluence, the growth medium was replaced by the differentiation medium consisting of Hi glucose-DMEM supplemented with 2% FBS and 1% penicillin/streptomycin. The cells were further incubated in 5% CO_2 incubator at 37°C for 8-10 days until differentiated myotubes was observed.

3. Cell viability assay

To evaluate cytotoxicity of interruptins A and B against mouse skeletal muscle cells (Sol8) used in the study, MTT assay was performed according to Suharty et al. (2018). The myoblasts (5×10^3/well) were seeded in 96-well plates in its growth medium and cultured overnight. They were then treated with various concentrations of interruptins A and B (0.5-10 µM) for 24 h at 37°C in 5% CO_2 atmosphere and the MTT assay was done. Briefly, 200 µL of

0.5 mg/mL MTT (Thermo Fischer Scientific) dissolved in PBS was added to each well. Cells were further incubated at 37°C for 4 h and the supernatant was removed and replaced with 100 µL of dimethyl sulfoxide (DMSO) (Sigma-aldrich) for dissolving the formazan crystals. The absorbance was measured at 570 nm with a microplate spectrophotometer (BMG labtech, Germany).

4. Glucose consumption assay

The mouse myoblasts (3×10^4/well) were seeded in 24- well plates in the growth medium. After culture overnight, it was replaced by the differentiation medium and cells were further incubated for 8-10 days induce myotube differentiation. The corresponding myotubes were treated with interruptins A, B (0, 2.5 and 5 µM) and pioglitazone (20 µM) in differentiation medium for 24 h. The conditioned medium was removed and measured the residual glucose concentration by glucose oxidation method (Zhou, et al. 2007). The glucose consumption was calculated based on the glucose concentration in the control medium.

5. Inhibition study

The action of PPAR-γ was examined by evaluating interruptins A and B-induced glucose consumption in the presence of PPAR-γ antagonist, BADGE. Differentiated myotubes were co-incubated with BADGE (20 and 30 µM) and interruptins A or B (2.5 and 5 µM) for 24 h. Subsequently, the conditioned media in each treatment were collected and determined the residual glucose concentration by glucose oxidation method (Zhou, et al. 2007). The glucose consumption was calculated compared with the glucose concentration in the control medium.

6. Glycogen content determination

For determination of muscle glycogen content in response to interruptins A and B, differentiated myotubes were treated with interruptins A and B (0, 2.5 and 5 mM) for 24 h. Consequently, the cells were washed and lysed, the obtained supernatant was quantified the protein content by Bradford assay, then added with 95% ethanol to precipitate glycogen at 40ºC for 4 h. The dried and packed glycogen was determined the content with anthrone-reagent method (Carrol et al. 1956). Pioglitazone (20 µM) was used as a positive control.

7. RNA analysis

Total RNA of differentiated myotubes treated with various concentrations of the interruptins A, (0, 2.5 and 5 mM) and pioglitazone (20 mM) for 24 h was extracted with FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit (Favorgen) and followed by a reverse transcription using FIREScript RT cDNA synthesis (Solis BioDyne) kit. The cDNA was amplified by QuantStudio 3 Real-Time PCR (Thermo Fisher Scientific) with 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) using the primers as shown in Table 1 (Masternak et al, 2004). The real time-PCR amplification was performed by denaturation at 95°C for 150 s, annealing at 60°C for 20 s, and extension at 72°C for 27s. All the samples were done triplicates. The expression level of genes was quantitated from fluorescence emission using the cycle threshold (C_T) value. A melting point dissociation curve was generated to confirm that only a single product is presented.

Table 1. Primers used for real time-polymerase chain reaction (PCR) amplification.

Primer	Sequence
IRS1_F	5′-AGCCCAAAAGCCCAGGAGAATA-3′
IRS1_R	5′-TTCCGAGCCAGTCTCTTCTCTA-3′
IRS2_F	5′-AGTAAACGGAGGTGGCTACA-3′
IRS2_R	5′-AAGCTGCTGAGAAGTCAGGT-3′
GLUT4_F	5′-ATTGGCATTCTGGTTGCCCA-3′
GLUT4_R	5′-GGTTCCGGATGATGTAGAGGTA-3′
PPARγ_F	5′-CAAAGTAGAACCTGCATCTCC-3′
PPARγ_R	5′-CCTTCACAAGCATGAACTCC-3′

8. Statistical analysis

Experimental results were assessment with triplicate analysis. The data are presented as mean ± standard deviation (SD) and analysis by One-way analysis of variance was performed by ANOVA follow by LSD. A value of $P < 0.05$ or $P < 0.01$ was considered to indicate statistically significant difference.

Results and Discussions

1. Cell viability

Nowadays, the efficacy regarding to biological activity of natural isolated compounds is widely interested. However, their safety is still be noteworthy concerned before each experiment. MTT assay is one of popular method for assessment the viability of cells in response to chemical tested compounds. In general, viable cells catalyze yellow tetrazolium salts to purple formazan crystals by the action of mitochondrial reductase enzyme. The intense purple color is directly proportionate to a number of survival cells. The faded purple color is obtained if test substances are toxic to cells. Since this study aims to evaluate the effect of interruptins A and B on muscle cells, they were firstly examined their safety by MTT assay. Non-treated cells were counted as 100% of cell viability. According to ISO 10993-5, the non-cytotoxic agent should demonstrate above 80% of cell viability. Compounds that provide within 80-60%, 60-40% and below 40% of cell viability are considered as weak, moderate and strong cytotoxic substances, respectively (ISO 10993-5, 2009; Lopez-Garcia et al., 2014).

No cytotoxic to mouse myoblasts (Sol8) was observed for both interruptins A and B at all tested concentrations (0.5-10 μM) with % cell viability ranges of 96.9-149.33 (Figure 1). Thus, interruptins A and B were innoxious to muscle cells. Moreover, interruptins A and B stimulate proliferation of myoblasts with a dose dependent manner. From this effect, interruptins A and B at concentrations of 0.5-10 μM was considered to be safety for exposure with myoblast and can be applied in further experiments.

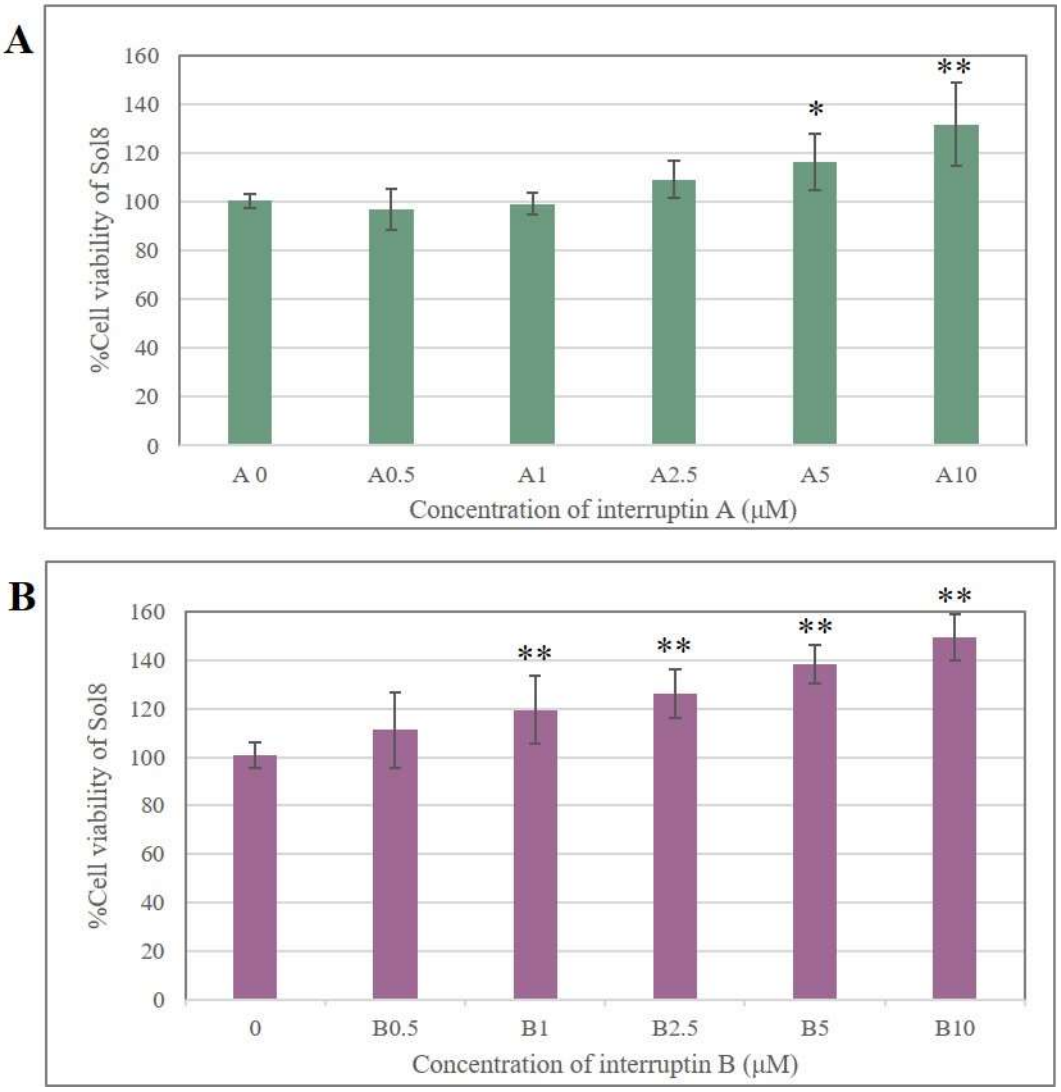

Figure 1. Myoblast (Sol8) cell viability of isolated interruptins A (A) and B (B). All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with the untreated group.

2. Glucose consumption

The present study aims to investigate the action of isolated interruptins A and B on glucose uptake by myotube *in vitro*. It was found that both interruptins A and B dose dependently and significantly increased glucose uptake (134.0-279.6%) at 2.5- 5 μM (Figure 2)

which were 1.02-2.13 folds higher than the standard pioglitazone at 20 μM. Furthermore, interruptin B seemed to induce glucose consumption in myotube slightly (1.3 times) greater than interruptin A. The results of the study demonstrated for the first time, to the best of our knowledge, that interruptins A and B enhanced glucose uptake in muscle cells. In addition, they provided more glucose uptake potential compared with pioglitazone. Although a wide array of plant-derived compounds have been reported to be associated with beneficial effect on glucose transport and metabolism in skeletal muscle cells (Anandharajan et al., 2006; Lakshmi et al., 2009; Tamrakar et al., 2008; Tan et al., 2008). They all originate from cultivate plants. To the best of our knowledge, this study is the first report to show the stimulation of glucose uptake by interruptins A and B from the wild plant, *C. terminans*.

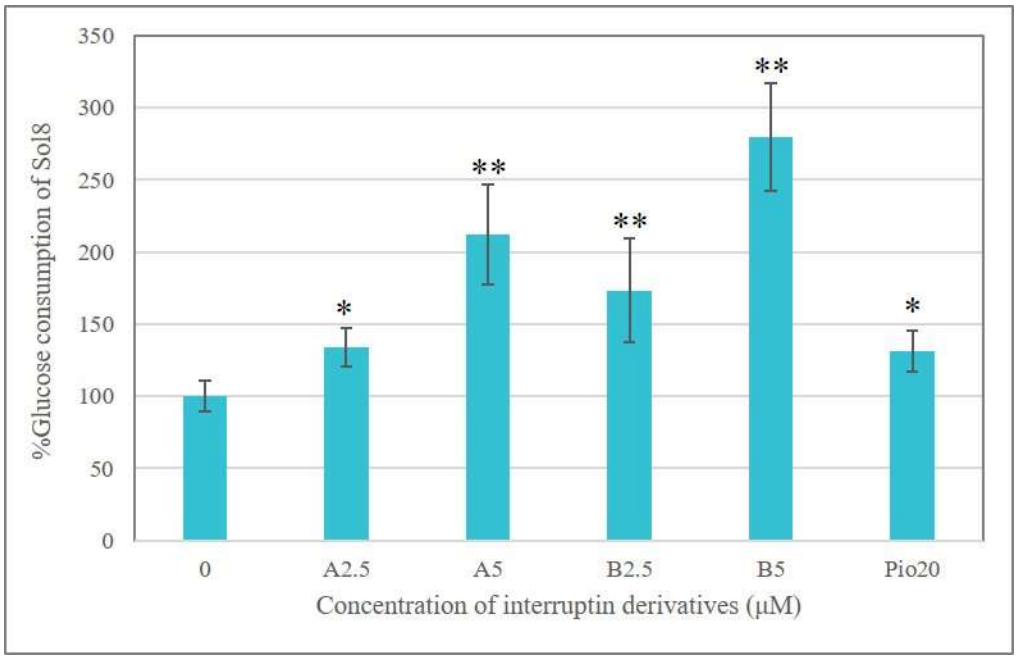

Figure 2. Effect of interruptins A and B on glucose uptake in differentiated myotubes (Sol8). Glucose consumption was measured following 24 h treatment with interruptins A and B compared with 20 μM pioglitazone. All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with the untreated group. A, interruptin A; B, interruptin B; Pio, pioglitazone.

3. Inhibition study

The role of PPAR-γ was determined by measuring interruptins A and B-induced glucose uptake in the presence or absence of PPAR-γ antagonist BADGE. It was found that both interruptins A and B-stimulated glucose uptake in myotubes was inhibited, in a dose-dependent manner, by co-treatment with the PPAR-γ antagonist BADGE (Figure 3). These data confirmed that glucose consumption in muscle cells were enhanced by interruptins A and B treatment through the PPAR-γ dependent pathway.

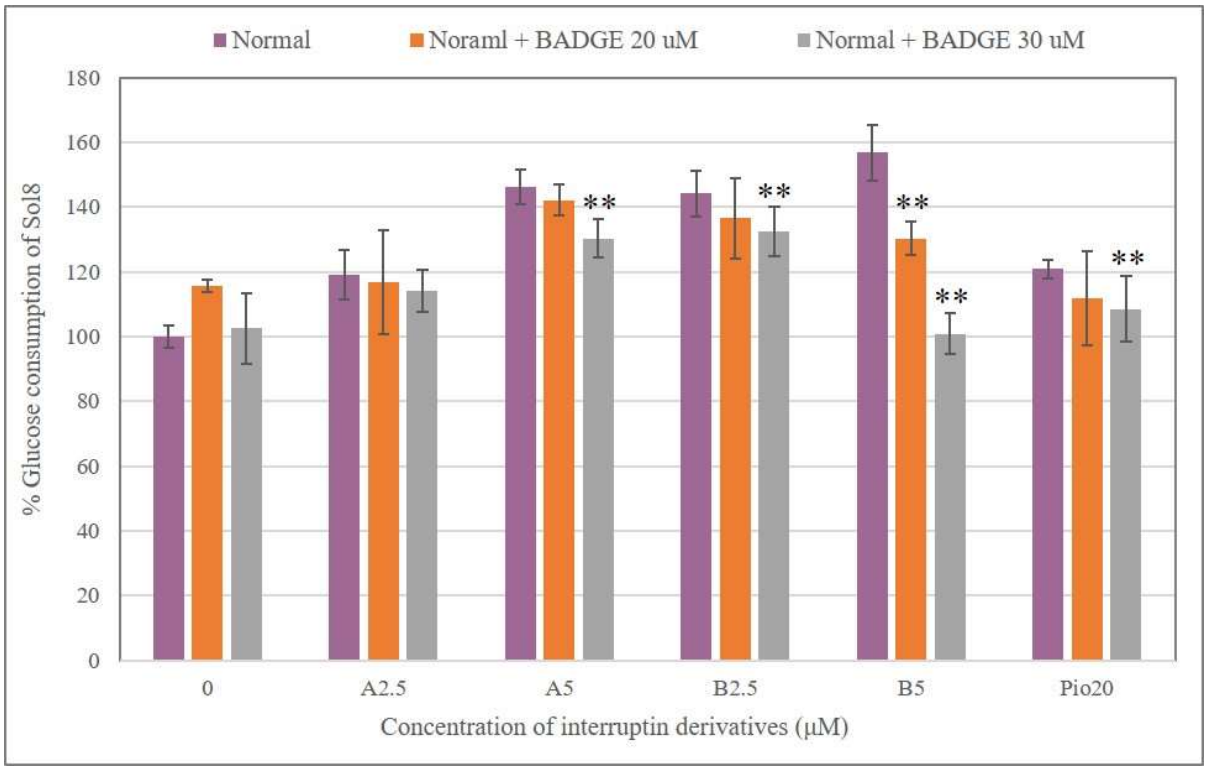

Figure 3. Reversal of glucose consumption in differentiated myotubes (Sol8) by PPAR-γ (BADGE) antagonist. All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with each normal group. PPAR, peroxisome proliferator-activated receptor. A, interruptin A; B, interruptin B; Pio, pioglitazone.

4. Glycogen content

In humans, skeletal muscle is the major site of glucose uptake and glycogen storage, with both processes being regulated by insulin. Impaired glycogen synthesis is the major abnormality in type 2 diabetes (Damsbo et al., 1991; Schalin-Jäntti et al., 1992). As shown in the previous experiments that 2.5 and 5 μM interruptins A and B evidently activated glucose uptake into differentiated myotubes. Here, we have explored whether interruptins A and B could increase glycogen synthesis in differentiated myotubes. It was found that only interruptin B at the high tested concentration of 5 μM significantly promoted glycogen accumulation in myotubes, although, this effect was 1.2 times less than standard drug 20 μM pioglitazone (Figure 4). These results indicate that interruptin B has an ability to activate glycogen synthesis, this may cause by an increase of glucose storage in the cells, however, the relevant mechanism remains to be investigated.

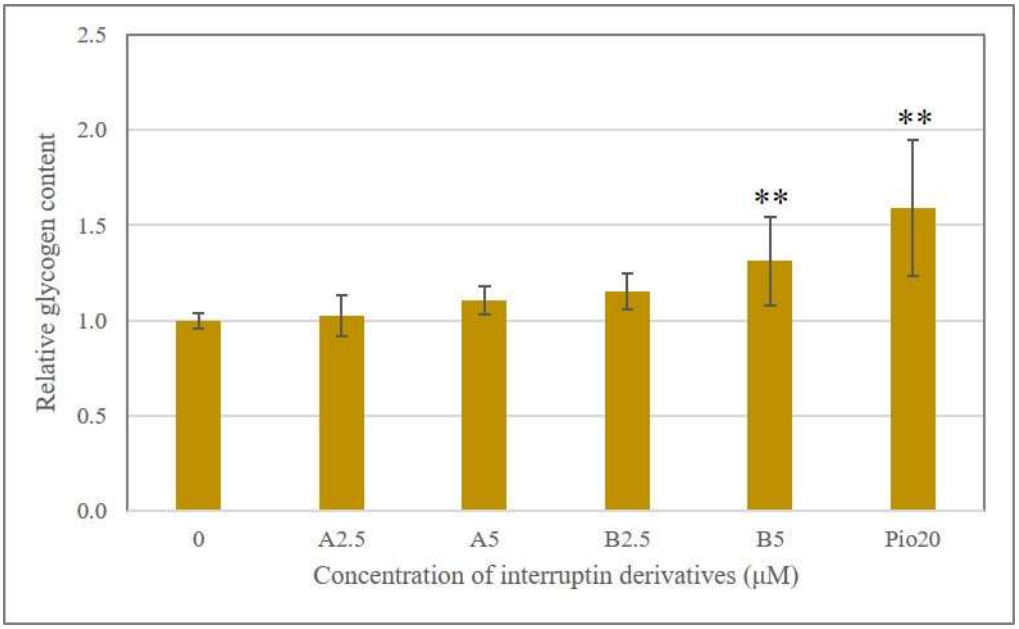

Figure 4. Effect of interruptins A and B on glycogen accumulation in differentiated myotubes (Sol8). All values are presented as the mean ± standard error of the mean (n=3). **P<0.01, compared with the untreated group. A, interruptin A; B, interruptin B; Pio, pioglitazone.

5. Gene expression

IRS-1/PI3K/Akt signaling pathways are the typical pathway in the regulation of glucose uptake and metabolism (Satoh, 2014). The normal process of glucose uptake in skeletal muscle cells is as following: the insulin bonds to insulin receptor (IR), then leads to tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. Phosphorylated IRS proteins ultimately result in the translocation of GLUT4 from the intracellular storage compartment to the plasma membrane through downstream signaling cascades (Rowland et al., 2011). To unravel the molecular mechanism of interruptins A and B in activation of glucose consumption by myotubes in the present study, their effect on the major markers regulating glucose transport were examined. Incubation of differentiated myotubes with interruptin A resulted in an enhanced mRNA expression of IRS1, GLUT4, and PPAR-γ, whereas only expression of PPAR-γ was significantly upregulated by interruptin B (Figure 5). As the results belonging to the molecular examination were observed, this may indicate that interruptins A and B carried somewhat distinct mechanism of action on glucose and glycogen metabolism in skeletal muscle cells.

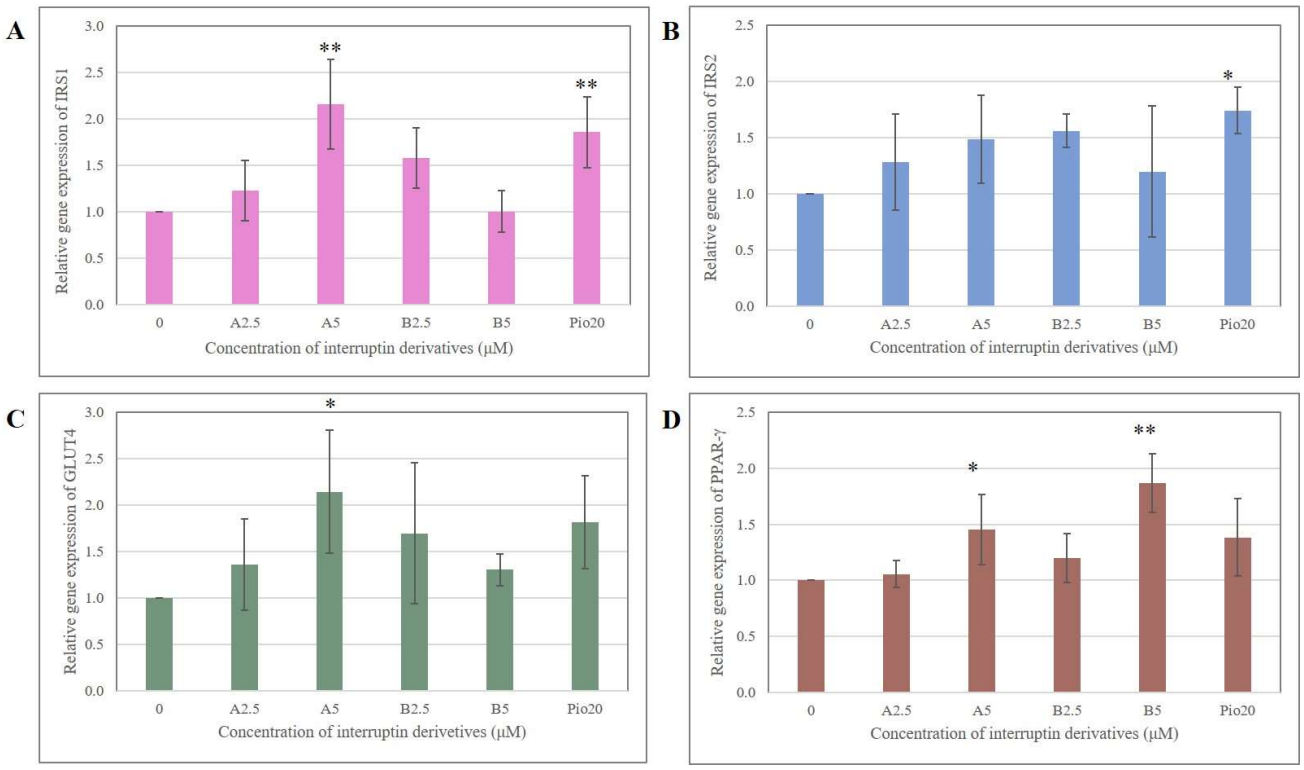

Figure 5. Effect of interruptins A and B on mRNA expression in differentiated myotubes (Sol8). All values are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01, compared with the untreated group. A, interruptin A; B, interruptin B; Pio, pioglitazone.

Conclusions

This study recommends the benefits of glucose and glycogen metabolism by interruptins A and B which has been isolated from the fern *C. terminans*. They not only be able to induce proliferation of skeletal muscle cells, but also appeared to be acting through stimulation of glucose uptake into the cells via the mechanism of PPAR-γ pathway. However, only interruptin B could encourage glycogen content in muscle cells. Therefore, the natural coumarins, interruptins A and B, could be regarded as promising candidates for development of new antidiabetic drugs.

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Part III : Acute oral toxicological study and assessment of anti-diabetic activity *in vitro* of interruptin-rich extract (IRE) from *Cyclosorus terminans*

Introduction

Medicinal plants have long been used as traditional treatments for numerous diseases in many parts of the world. In the recent year, herbs and natural compounds are becoming popular as alternative medicines and novel drugs combined with new technology (Zhu et al., 2012; Ngo et al.,2013). World Health Organization (WHO) reported that approximately 80% of people around the world uses natural products as folk medicines for disease treatments, especially in developing countries (Astin, 1998; Kim, 2015). The common reasons for using traditional medicine are It closely related to the patient's tradition, more affordable, allows greater public access to its information, and perceived as natural products are safer than chemical medicines. The major use of herbal medicines is for health promotion and therapy for chronic disease (Canter and Ernst, 2004; Qato et al., 2008). However, herbs have been shown to be capable of producing a wide range of undesirable or adverse effects (Ernst, 2002). WHO reported the following issues regarding herbal medicines including the regulation of the production and the use of herbs, their quality, safety, and scientific evidence in status of health (WHO, 2005). Therefore, plant or natural products are concerned to study their toxicity and efficacy in order to develop the safe alternative drug.

Diabetes mellitus (DM) is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2011). DM affects millions of people worldwide, with rapidly increasing incidence and prevalence. The management of diabetes is therefore considered as a global problem (Sinnott et al., 2017). Nowadays many of the anti-diabetic drugs have been used to save life, alleviate symptoms and prevent long-term diabetic complications, however, sub-therapeutic effects still occur and the costs of diabetes treatment is not cheap (May et al., 2002). Therefore, there are tremendous

needs to explore novel alternative anti-diabetic substances providing more cost effectiveness, attenuating side effects and increasing patient compliance.

The fern *Cyclosorus terminans* (Thelypteridaceae) is one of the lower plants that has been consumed as vegetable for a long time (Kumboonruang, 2009) in Thailand and widely distributed in all over part of Thailand, Laos and Cambodia (Tagawa and Iwatsuki, 1988). Even its traditional usage has not been declared, it displayed diverse biological activities by active isolated interruptin derivatives. For examples, interruptins A and B revealed anticancer property against MCF-7 human breast and HT-29 colon human cancer cells (Kaewsuwan et al., 2015) Interruptin A demonstrated antibacterial activity against Gram-positive aerobic bacteria including methicillin-sensitive *Staphlococcus aureus* (MSSA) methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and *Bacillus subtilis* along with an anaerobic bacterium *Propionibacterium acne* (Kaewsuwan et al., 2015; Chaiwong et al., 2019). Interruptin B exhibited the stimulatory consequence on brown adipocyte differentiation and glucose consumption in adipocytes differentiated from adipose derive stem cells (ASCs) by acting as a dual PPAR-α and -γ ligand (Kaewsuwan et al., 2016). Interruptins A and B were proved as anti-inflammatory agents by scavenging NO radical and reducing NO production in LPS-stimulated RAW264.7 cells and antioxidant compounds determined by DPPH and 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). Despite knowledge of numerous biological activities of interruptin derivatives from *C. terminans* has widely been documented, there is a lack of its safety *in vivo*. Hence, to ascertain the safety profile of drugs from herbal source, preclinical acute toxicological assessment using the Organization for Economic Cooperation and Development (OECD) guideline need to be performed (Jadeja et al., 2011). The present study therefore aims to provide a scientific basis to support the safety application of edible vegetable *C. terminans* by evaluation the acute oral toxicity of interruptin-rich extract (IRE) from *C. terminans* in Wistar

rats following the recommended OECD guidelines and to investigate its effect on anti-diabetic *in vitro*.

Materials and Methods

1. Plant material and extract preparation

The aerial parts of *C. terminans* were collected in January 2016 from natural habitat in Nakornsrithammarat province, Thailand, and identified by Prof. Dr. Thaweesakdi Boonkerd (Chulalongkorn University, Thailand). A voucher specimen with the number SKP 2080320001 of faculty of pharmaceutical Science, PSU, Thailand. The aerial parts were dried and grinded into powder. The 5 kg of dried powder was extracted with *n*-hexane under reflux (each 1 h, 50 L x 3 times). The hexane extract was filtrated using whatman filter paper no. 4 and evaporated to yield 48.63 g of extract (0.97% w/w of initial powder). The hexane extract was partial purified to obtain high concentration of active compound interruptins with vacuum column chromatography on silica gel and eluted using a step gradient of *n*-hexane with increasing concentration of dichloromethane. The fractions containing interruptins were combined and concentrated with rotary evaporator (Helidolph Laborata 4000, Germany) to give a residue 31.18 g (yield 64.11% w/w of hexane extract). The prepared *C. terminans* extract was regarded as interruptin-rich extract (IRE) and stored in refrigerator at 4 ºC until use.

2. HPLC analysis of the extract

Comparison of interruptin concentration in hexane extract and IRE from *C. terminans* was measured by HPLC with consisting of binary pump, thermostated column compartment and detected by photo diode array (PDA) detector. The analysis was performed on chromaster 5410 (Hitachi, Japan) which was set condition base on calibrated HPLC method (Kaewsuwan et al., 2015; Chaiwong et al., 2018).

3. *In vitro* assessment of IRE from *C. terminans*

3.1 Cell cultures and reagents

Mouse hepatocytes (FL83B) and mouse skeletal muscle cells (Sol8) were purchased from American Type Culture Collection (ATCC) then were cultured in F-12K medium (Sigma-Aldrich, St. Louis, MO, USA) and Hi-glucose DMEM medium (Gibco, Life Technology, Grand Island, NY, USA), respectively. Cells were incubated in 37 ºC 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.

3.2 Cell viability assay

MTT assay has been widely used for in vitro cell viability assay. In brief, cell suspension of FL83B and Sol8 cells were counted and seeded at concentration $2x10^4$ and $2.5x10^3$ cells/well into 96-well plates in their completed medium with 10% FBS. After 24 h of cultivation, cells were treated with various concentrations (1-10 µg/mL) of IRE and DMSO as a control. Plates were incubated at 37 ºC in humidified atmosphere containing 5% CO_2 incubator for 24 h. Each well was added with 10 uL of 5 mg/mL MTT and further placed in dark condition for 3 h at 37 ºC. Then all media were removed and replaced with 100 µL DMSO. The UV absorbance was measured at OD 570 nm by using a microplate reader (BMG labtech, Germany). The percentage of cell viability was calculated by comparing with the control.

3.3 Glucose consumption assay

Sol8 muscle cells ($1x10^4$ cells/well) were cultured in 24-well plate with 10% FBS containing completed medium for overnight then medium was changed to 2% FBS containing completed medium to differentiate cells into contractile myoblast. FL83B hepatocytes 1.5×10^5 cells/well were seeded in 24-well plate and cultivation 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 containing

completed medium, respectively for 24 h. After incubation, the conditioned medium was sampled for measurement of glucose concentration by glucose oxidation assay (Li et al., 2018). The glucose consumption was calculated by subtracting the initial glucose concentration in medium. Results were expressed as percentage glucose consumption as compared to control cells of which the glucose consumption was assigned as 100%.

4. *In vivo* assessment of IRE from *C. terminans*

4.1 Animals and Ethics

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 a standard environmental condition at 22±2 ºC, 50±10% humidity, 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 prior to study. The experiments were reviewed and approved by the institutional committee for Ethic Use of Experimental Animals at Prince of Songkla University, in compliance with National Institutes of Health guidelines (approval no. MOE0521.11/1376).

4.2 Acute oral toxicity

The study was performed according to the Organization for Economic Co-operation and Development (OECD) guideline No. 420 (OECD Guideline 420, 2001), in the main study following the Annex 3. The rats were randomly divided into two groups, control and treatment groups of five rats each. Rats were fasted 12 h before giving 2 groups. 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). 2000 mg/kg IRE was orally one administered in a constant volume to the treatment group, while vehicle was given to the control group. All rats were continuously observed over 24 h.

4.3 Liquid chromatography-mass spectrometry (LC-MS) analysis

After 6 h IRE sample administration, Blood was collected from each animal. Plasma pre-treated by a single-step protein precipitation with acetonitrile and filtrated with 0.2 μM syringe filter before taking to identify 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 rat was done using 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 temp: 325°C. MassHunter Workstation software (Agilent Technologies, USA) was used for data acquisition and processing.

4.4 Cage side observation

Clinical observation which included changes in skin and fur, eyes and mucous membrane, respiratory circulation, somatomotor activity, behavioral pattern, tremors, convulsion, salivation, diarrhea, sleep and coma were observed for all the experiments. Animal were observed individually after administration and special attended was given during the first 24 h and even 12 h daily thereafter, for a total 14 days.

4.5 Body weight, food and water intake

The body weight of each rat was everyday recorded to prior the administration of extract for 14 days. The amount of food and water were recorded daily from quantity of food and water supplied and the amount remaining after 12 h for 2 weeks.

4.6 Hematological and serum biochemical analysis

Blood samples were drawn from inferior venacava puncture. The samples were collected in plastic tubes containing EDTA anti-clotting. Hematology analyses were evaluated the following parameters red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and total white blood cells (WBC) by using automated hematology analyzer Sysmex XS-800i (Sysmex, Japan). Biochemical analyses were detected with blood in sodium fluoride tube and glass plain tube. Samples were centrifuged at 3000 rpm 4 ºC for 15 min. Serum was separated and stored at -20 ºC until analysis. Plasma was determined

for glucose, and serum was separated for total cholesterol, total triglyceride, total protein, albumin, globulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and blood urea nitrogen determination with colorimetric assay by automated chemistry analyzer URIT-8031 (URIT, China).

4.7 Histopathological examination

After euthanasia the rats, parts of heart, liver and kidney tissues were collected for histological studies. The tissues 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, cut into 4-5 μm thick sections. The sections were stained with hematoxylin and eosin (H&E) and examined under the light microscope. The light microscopic features of the organs of treatment group were compared with the control group.

5. Statistical analysis

The results of *in vitro* are expressed as mean ± SD for at least 3 independent experiments and *in vivo* data are presented as mean ± SEM. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using SPSS statistical software. Values of $*p < 0.05$ and $**p < 0.01$ were considered as significant difference.

Results

1. HPLC analysis of the extract

IRE was prepared by partial purification from hexane extract of *C. terminans* using the vacuum column chromatography. The amounts of bioactive constituents, interruptins A and B, in the prepared extracts were then analyzed by validated HPLC method (Kaewsuwan et al., 2015; Chaiwong et al., 2018). As shown in Fig. 1, interruptins A and B contents in IRE were highly found as 244.38 and 91.81 μg/mg extract, respectively, while its initial hexane extract demonstrated 84.79 and 41.35 μg/mg, respectively, of interruptins A and B. The simple

enrichment method used in this study indicated 2.2-2.9 folds higher contents of interruptins A and B than its original extract.

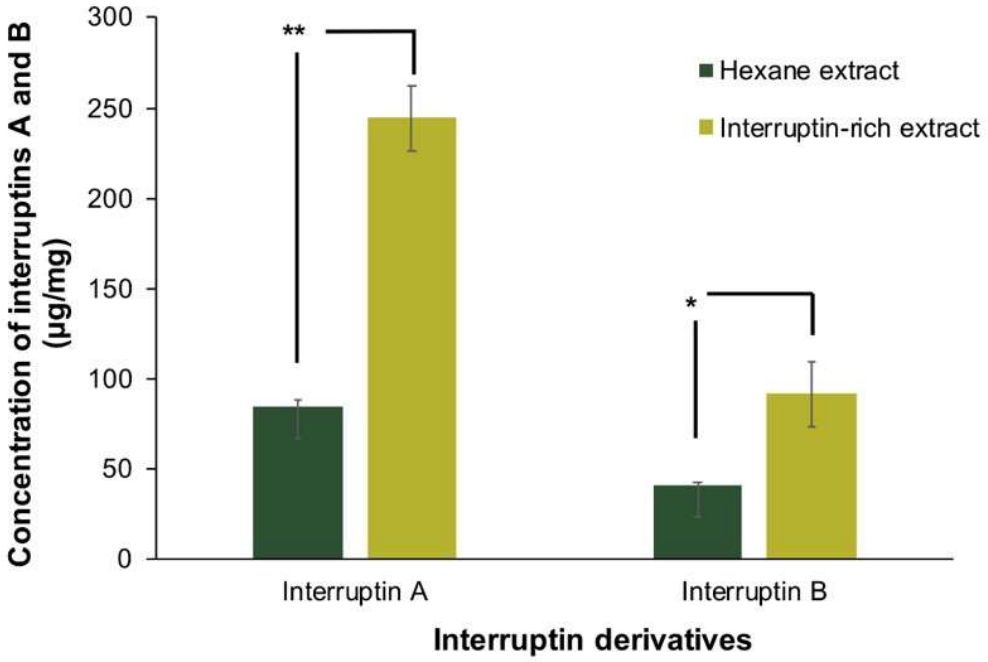

Fig. 1. Concentration of interruptins A and B quantified by HPLC analysis of 1 mg hexane extract and IRE from *C. terminans*. Data are presented as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with hexane extract.

2. *In vitro* assessment of IRE from *C. terminans*

2.1 Cell viability

The effect of IRE on cell viability was initially evaluated with the help of MTT assay. As demonstrated in Fig. 2, incubation of FL83B hepatocytes and Sol8 muscle cells with IRE applied at 1-10 µg/mL revealed non-toxic towards both tested cells. Interestingly, treatments of 5 and 10 µg/mL IRE induced statistically significant proliferation of FL83B hepatocytes as well as IRE used at the high concentration of 10 µg/mL was effective in increasing the MTT conversion ability of Sol8 muscle cells, when compared to the control without treatment.

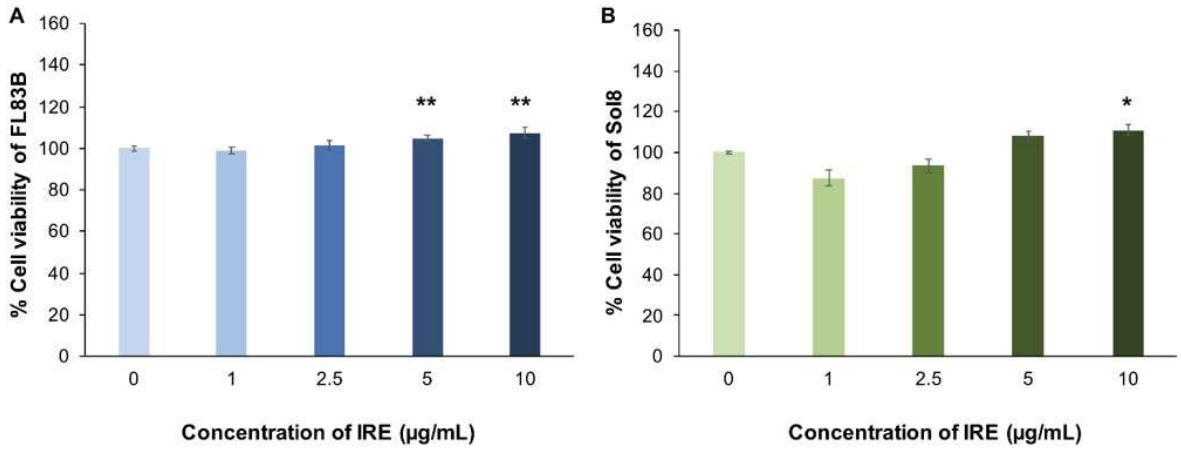

Fig. 2. Cell viability of IRE in FL83B cells (A) and Sol8 cells (B) determined by the MTT assay after incubated with IRE for 24 h. Results are expressed as percentages of cell viability compared with control cells. Data are presented as the means ± SD for at least 3 independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ as compared with the control cells.

2.2 Glucose consumption

Fig. 3 provides the average percentage glucose consumption achieved in FL83B hepatocytes and Sol8 induced myotubes by IRE treated at 0-5 and 0-10 μg/mL for 24 h. Induction of glucose consumption in a dose-dependent manner by IRE was observed in both tested cells, while IRE produced better glucose consumption into hepatocytes than muscle cells. Prepared 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 high concentration of 10 μg/mL IRE significantly potentiated glucose uptake into Sol8 muscle cells with 118.1% glucose consumption which was comparable to 30 μM rosiglitazone (114.9% glucose consumption). These results suggest that IRE have the ability to facilitate glucose uptake into hepatocytes than skeletal muscle cells.

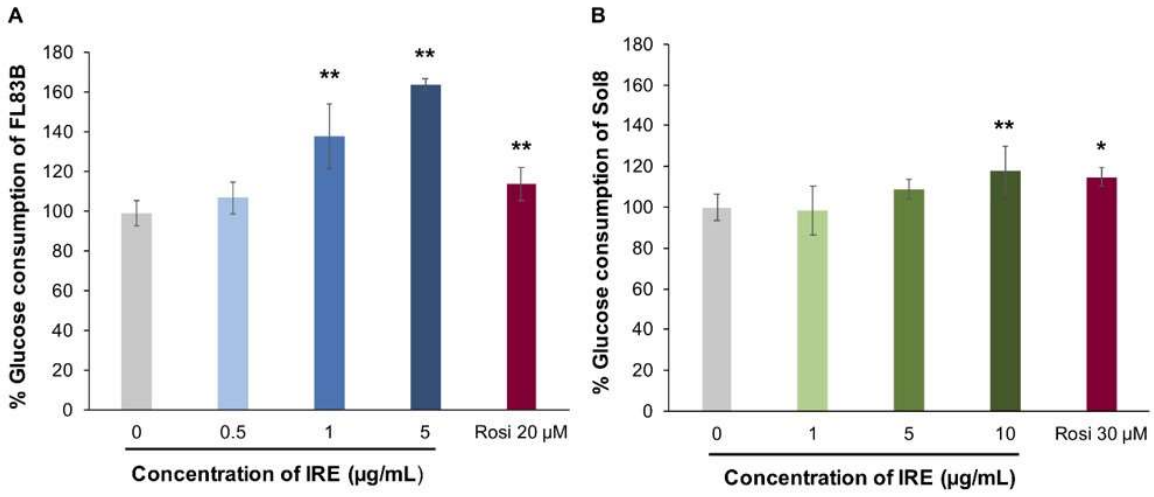

Fig. 3. Glucose consumption of IRE (0.5-5 µg/mL), rosiglitazone (20 µM) in FL83B cells (A) and IRE (1-10 µg/mL), rosiglitazone (30 µM) in Sol8 cells (B) evaluated from the conditioned medium with glucose oxidase assay after 24 h of incubation. Results are presented as the means ± SD for at least 3 independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ as compared with the control cells.

3. *In vivo* assessment of IRE from *C. terminans*

3.1 LC-MS analysis

In this study, the oral toxicity of prepared IRE was evaluated in Wistar rats at the single dose of 2,000 mg/kg body weight. The transfer of samples was determined to verify their plasma absorption. After 6 h of IRE administration, the plasma of control rats and rats treated with IRE were drawn and taken to analyze by LC-MS in the negative ion mode. Fig. 4A and 4B show the chromatogram of standard interruptins A and B (0.8-1.2 µg/mL) that produced the main product ion at *m/z* 399.12 and 397.10, respectively. The plasma of 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. 4C), whereas those ions were not found in the plasma of control group (Fig. 4D).

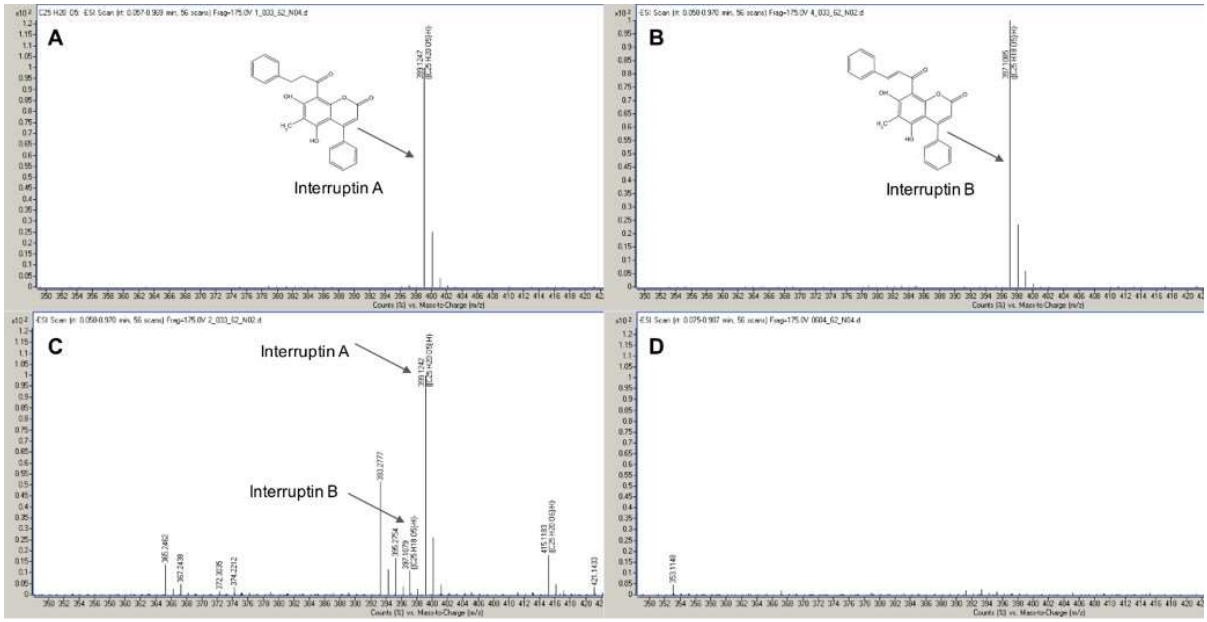

Fig. 4. Ion chromatograms of standard interruptins A (A) and B (B) compared to ion chromatographic profile of plasma rats in from the treatment (C) and control group (D) after a single dose oral administration of IRE 2000 mg/kg for 6 h analyzed by LC-MS technique.

3.2 Observation and body weight measurement

No behavioral changes such as impairment of food intake and water consumption, salivation, tremors, diarrhea and coma were observed at the IRE administered doses. Absence of changes in skin and fur, eyes and mucous membrane was also noted. The rats were found to be active with gradually increase in body weight both in the control and treatment groups (Fig. 5). Normal food and water intake of animals obviously encourage the gain in animal body throughout the study period. Non-statistically significant difference of body weight between both groups was observed and no animal death was documented even at the dose of up to 2000 mg/kg body weight.

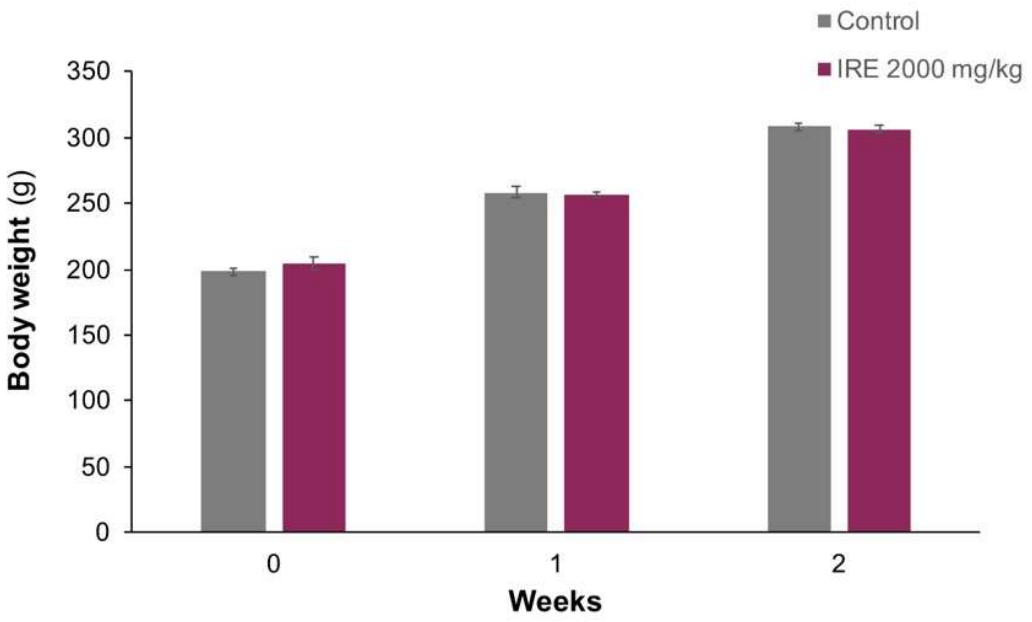

Fig. 5. Body weight of rats 2 weeks after a single dose oral administration of IRE 2000 mg/kg. Each value is expressed as mean ± SEM (n = 5).

3.3 Hematological and serum biochemical parameters

The results of hematological study (Table 1) conducted on day 15th for acute toxicity of IRE demonstrated no significant changes in the values of RBC, Hb, Hct, MCV, MCH, MCHC, RDW and WBC in treatment group when compared with the respective control rats. In addition, biochemical study (Table 2) was also carried out in order to evaluate any toxic effects occurred after administration of IRE on liver and kidney. No significant change observed in plasma glucose of the treatment group compared with the control group. The renal function parameters (blood urea nitrogen, creatinine, and uric acid) did not reveal any relevant changes and no statistically significant differences in liver function parameters (total protein, albumin, globulin, alanine aminotransferase (ALT) and aspartate transaminase (AST)) were noted. Whereas there was altered value of total cholesterol in the treatment group which significantly higher than the control group.

Table 1. Hematological parameters of rats 2 weeks after a single dose oral administration of IRE 2000 mg/kg.

Parameters	Control	IRE 2000 mg/kg
RBC (x 10^6 µL)	5.35 ± 2.86	6.59 ± 1.46
Hemoglobin (g/dL)	13.55 ± 0.65	12.90 ± 0.00
Hematocrit (%)	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 (x 10^3 µL)	15.95 ± 1.65	14.95 ± 0.25

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

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

Parameters	Control	IRE 2000 mg/kg
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
Blood urea nitrogen (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

Data are presented as mean ± SEM (n=5). *$p < 0.05$ was considered as significant difference compare to the control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

3.4 Histopathological assessment

Histopathological analysis of the heart, liver and kidney were performed on the 15th day after administration of IRE from *C. terminans*. Macroscopic examination of the organs of treatment group revealed no abnormalities in the color or texture when compared with the organs of the control group. The light microscopy examinations of the transverse section of IRE from *C. terminans* and organs of the control group were shown in Fig. 6A-F. Sections of heart showed normal cellular structures of cardiac muscle cell and connective tissue (Fig. 6A-B). Multiple sections of the liver showed normal lobular architecture. The hepatocyte appeared normal and no sign of specific lobular hepatitis was observed in the treatment group of animals compared with the control group (Fig. 6C-D). Similarly, the multiple sections from renal biopsy

showed normal size and shape of glomeruli, tubules and intestinum. There was no evidence of acute tubular necrosis and glomerular changes (Fig. 6E-F).

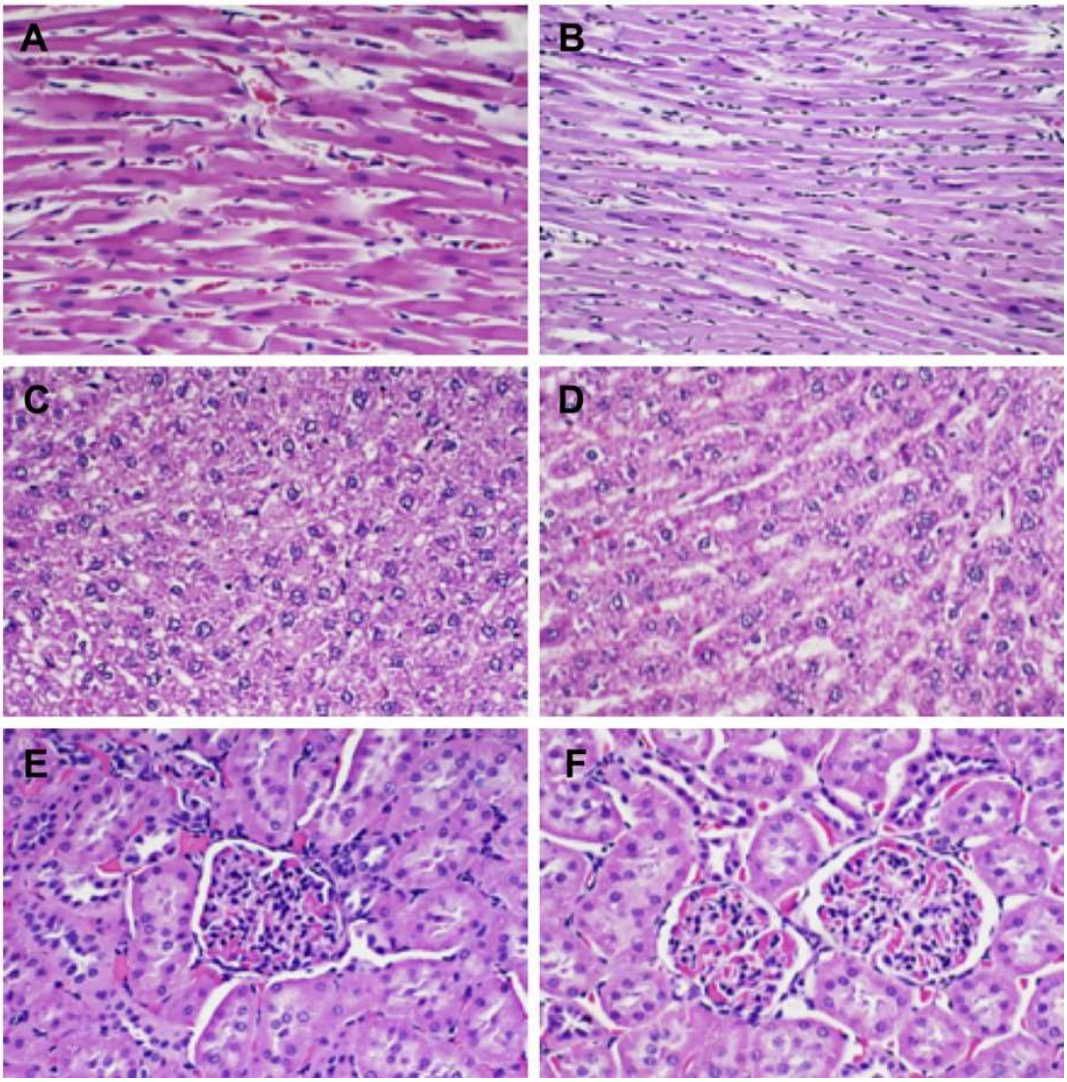

Fig. 6. Organ sections stained with hematoxylin and eosin (H&E 400x), showing the effect of 2000 mg/kg IRE from *C. terminans* on acute toxic study. Heart, control (A) and IRE treatment (B) groups (B); Liver, control (C) and IRE treatment (D) groups (D); Kidney, control (E) and IRE treatment (F) groups.

Discussions

Natural products are now recognized as an alternative medicine source for discovering and developing anti-diabetic drug. A few of lower plants have long been used in folk medicines and studied on anti-diabetic activity (Webb, 1959; Holdsworth and Rali, 1989; Nair et al., 2006; Hsu et al., 2013). The fern *C. terminans*, one of lower plants that commonly found in Thailand and has long been consumed as vegetable (Kumboonruang, 2009). Interestingly, its isolated compound, interruptin B, has preliminary been studied for in vitro anti-diabetic property (Kaewsuwan et al., 2016), whereas there is no report on the in vivo toxicity study of *C. terminans*. Actually, the analysis of toxicity level of chemicals or samples is the most important step required for further pharmacological studies (Ganter et al., 2005). Therefore, the current study was undertaken to evaluate and focus on the acute toxicity of *C. terminans* in animal model. Due to the limitation on low amount of pure isolated active compounds, interruptins A and B, from *C. terminans*, we designed to enrich interruptin derivatives in the extract. The interruptin-rich extract (IRE) was successfully prepared and showed nontoxic to FL83B hepatocytes and Sol8 muscle cells with more than 80% cell viability (Percentages of cell viability above 80% are considered as non-cytotoxicity (ISO 10993-5, 2009)). Moreover, IRE remarkable induced both cell proliferation at high tested concentrations of 5 and 10 μg/ml.

Evaluation of the anti-diabetic potential of any medicine or compound, the glucose consumption of cells is an important model and can be performed easily. Several studies on the glucose consumption in hepatocytes and skeletal muscle cells have been published to evaluate the anti-diabetic potential of natural products (Breen et al., 2008; Lee et al., 2011). However, no data are available on glucose consumption in hepatocytes and skeletal muscle cells by *C. terminans*. In this study, we found that IRE exhibited anti-diabetic property by increasing the percentage of glucose consumption into FL83B hepatocytes and Sol8 muscle cells compared to the control without treatment.

Since metabolomics is useful for the chemical and pharmacological standardization of plant extract and it is particularly resource for the evidence-based development of new

medicinal plants (Shyur and Yang, 2008). LC-MS technique was therefore applied for metabolomics study to identify of the small molecule metabolic products in animal plasma. After administration of IRE to healthy adult male Wistar rats for 6 h, their plasma were taken to LC-MS analysis. The LC-MS chromatogram of rat plasma from treatment group showed mass ions of interruptins A and B. This finding indicates successfully absorption of interruptins A and B to blood stream of tested animals.

The result of acute oral toxicity study of *C. terminans* extract (IRE) in Wistar rats showed no mortality at day 14 after administration with the maximum dose of 2000 mg/kg. All animals in treatment group showed normal behavioral patterns and somatomotor activities. In principle, the body weight changes serve as a sensitive indication of the general health condition of animals (Hilaly et al., 2004) and natural products may be metabolized to a toxic product, which could disturb gastric empty and decreased food intake (Chokshi et al., 2007). Interestingly, the food and water consumption were found to be unaltered during the 14 days of observation period. There were no statistically significant differences in the weight of animals between the treatment and the control group. This suggests that the IRE did not interfere the normal metabolism of animals.

Evaluation of hematological parameters could be used as reliable indicators for evaluating the invasion of toxic substance or plant extracts (Yakuba et al., 2007). Hematological profiles of animals were undertaken for both treatment and control groups. The non-significant effect of IRE on total red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and total white blood cells indicates that prepared extract IRE did not affect the morphology or osmotic fragility of the red blood cells.

The blood chemistry analysis was also performed to evaluate the possible alterations in glucose and lipid metabolism, liver and renal function by IRE. Plasma glucose and total triglyceride values showed no significant differences in IRE-treated group compared with the control group. Whereas the level of total cholesterol in IRE-treated group was considerably higher than the control group, however, this value is still in normal cholesterol range of male

rat (Delwatta et al., 2018). Liver and kidney function analyses are very important for toxic determination of drugs and plant extracts (Olorunnisola et al., 2012). The increasing levels of ALT and AST are reported in liver diseases or hepatotoxicity (Brautbar and Williams, 2002). The non-significant difference in ALT and AST between IRE-treated and control groups was found in this study. This finding suggests that administration of 2000 mg/kg IRE does not affect the liver function of Wistar rats. The values of total protein, albumin and globulin were in their normal rages and no differences were observed from IRE-treated and control groups. In general, decreasing levels of total protein, albumin and globulin are a sign of impaired hepatocellular function and low albumin value may suggest infection or loss of albumin (Tietz et al., 1994; Yakuba et al., 2003). The results demonstrated that IRE remained unaffected the hepatocellular or secretory functions of the liver. Renal dysfunction can usually be considered by high level of urea and creatinine (Davis and Bredt, 1994). No significant alteration in blood urea nitrogen and creatinine levels due to IRE treatment when compared to the control group.

Histopathological examination of 2000 mg/kg IRE treated samples of heart, liver, kidney stained with hematoxylene and eosin (H&E) revealed no pathological changes of tissues. Heart sections showed no degree of injury cardiomyocytes Morphology of liver and kidney tissues were expressed normal characteristics which were dependable with biochemical parameters (AST, ALT, BUN and creatinine)

According to Globally Harmonized System of Classification and Labelling of Chemicals (GHS), compounds are categorized into five groups on their LD_{50} basis (United nations, 2017). Since during 14 days of acute toxicity evaluation period, oral single dose of 2,000 mg/kg IRE did not cause any animal death, prepared IRE from *C. terminans* was therefore classified in group 5 (LD_{50} 2,000-5,000 mg/kg) that is in low toxicity class. Altogether, IRE did not induce any significant behavioral pattern , biochemical, hematological, and histopathological signs of toxicity for Wistar rats under the experimental condition used. All data from this study will assist for future *in vivo* experimental studies of *C. terminans* pharmacological potential, particularly for anti-diabetic application.

Conclusions

Interruptin-rich extract (IRE) from *C. terminans* had no cytotoxicity on mouse hepatocytes and skeletal muscle cells, and also enhanced glucose consumption into both cells. These results are very promising to find that *C. terminans* extract might be a potential natural material for anti-diabetic application. However, the preliminary results suggested that it should be further examined for the mechanism and defined the precisely active ingredients. Nevertheless, acute toxicity study of IRE in Wistar rats showed no mortality or signs of toxicity with normal food/water intake and non-significant body weight variations. Hematological and biochemical investigation also clearly illustrated that single oral dose of 2,000 mg/kg IRE did not cause any damage to the organs such as heart, livers and kidneys. Thus, LD_{50} of IRE for oral acute toxicity was 2,000-5,000 mg/kg body weight. This study provides significant data on the toxicity profile of *C. terminans* which is essential for further pharmacological study. In addition, more research is required to address questions surrounding *in vivo* anti-diabetes and subchronic toxicity in order to evaluate its long-term effect.

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Part IV : *In vitro* anti-inflammatory ability of isolated interruptins from *Cyclosorus terminans*

Introduction

Inflammation is a normal response mechanism of the body tissue in order to eliminate injurious agents, remove necrotic cells and damaged tissues, and initiate the healing process[1]. In the inflammatory process, nitric oxide (NO), an important inflammatory mediator, is produced by activated macrophage cells[2]. NO acts as a regulatory molecule by regulating local blood flow in physiological conditions and plays a role in host defense mechanisms as an anti-microbial agent[3]. During inflammation, NO is overproduced by inducible nitric oxide synthase (iNOS) via oxidation of L-arginine. NO that reacted with superoxide radicals to form reactive peroxy nitrite as well as other free radicals generated during inflammation can directly damage the cellular components and cause carcinogenesis. NO is also involved in increasing pro-inflammatory prostaglandins produced by cyclooxygenase (COX)-2 enzymes[2,4-5]. However, the production of NO and the expression of the *iNOS* gene can be reduced by activation of peroxisome proliferator-activated receptor (PPAR-γ)[6, 7].

PPAR-γ is a member of nuclear receptors which regulates the transcription of target genes involved in regulation of adipocyte differentiation and lipid metabolism[8, 9]. Stimulation of PPAR-γ expression in monocyte and macrophage suppressed inflammatory mediators and cytokine production such as NO, PGE_2 and TNF-α[6, 10, 11]. Thus, direct scavenging of NO, suppression of iNOS and COX-2 expression or the induction of PPAR-γ expression could be considered as potential targets for inflammation treatment.

Interruptins, including interruptins A, B and C (Figure1), the natural occurring coumarin derivatives, were found in the ferns *Cyclosorus interruptus*[12] and *C. terminans*[13]. In previous studies, we exposed that interruptins A and B exhibited a broad spectrum of biological activities such as anti-bacterial, anti-diabetes, anti-cancer, and intracellular reactive oxygen species scavenging. Interestingly, interruptin B was presumed as a PPAR-γ agonist since it

increased *PPAR-γ* gene expression and also bound properly to a PPAR-γ receptor predicted by computational docking[13-15]. This evidence may suggest the ability of interruptin B as an anti-inflammatory agent. In regard to the similarity in chemical structures of interruptins A-C, the present study aimed to evaluate whether interruptins A-C isolated from *C. terminans* could perform anti-inflammatory properties and to investigate the potential mechanism underlying the action of corresponding compounds in regulating anti-inflammation. The results from this study intend to provide a novel scientific principle for encouragement in the use of edible vegetable lower plant, *C. terminans*, as a medicinal plant and to warrant application of coumarin derivatives a for attenuation the inflammation.

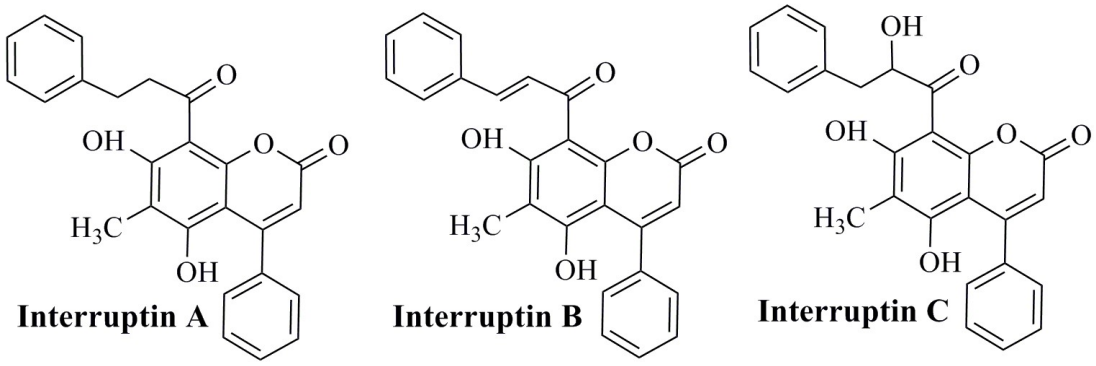

Figure 1. Chemical structure of interruptins A-C.

Materials and Methods

1. Chemicals and reagents

Interruptins A, B, and C were isolated from ethyl acetate extract of the fern *C. terminans*[13]. RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin were purchased from Gibco BRL, California. Dimethyl sulfoxide (DMSO), Phosphate buffer saline (PBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from AMRESCO, USA. Lipopolysaccharide (LPS) of *Escherichia coli*, sodium nitropusside and gallic acid were obtained from Sigma-Aldrich, USA. RNA extraction kit (FavorPrep™

Blood/Cultured Cell Total RNA Purification Mini Kit) was purchased from FAVOGEN, Korea. FIREScript RT cDNA Synthesis KIT and 5x FIREPol PCR master Mix Ready to load were supplied from Solis BioDyne, Estonia.

2. Cell culture

Murine macrophage RAW264.7 cells were cultured in a complete medium, RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, at 37º C in 5% CO_2 incubator. The medium was changed at two-day intervals until the cells reached confluence.

3. NO radical scavenging assay

Based on production of nitrite ion from the reaction of NO and oxygen, scavenging of NO leads to a decrease in nitrite production. Hereby NO radical scavenging efficacy of interruptins was evaluated based on a previously reported method[16]. Briefly, sodium nitroprusside (SNP) solution (20 mM), NO generator, was mixed with interruptins (20-100 μM) or gallic acid (29-176 μM) as a standard and incubated at room temperature for 180 minutes. The incubated mixtures were reacted with Griess reagent and then the absorbance was measured at 546 nm using a microplate reader. Gallic acid was used as a positive control. The experiment was done in three replications and IC_{50} values were determined graphically.

4. NO production inhibition assay in LPS-induced RAW264.7 cells

Based on the production of NO by macrophage cells when stimulated with lipopolysaccharide (LPS), RAW264.7 cells were used as a model for determination of anti-inflammatory activity of compounds according to a previously reported method[17] with some modifications. The cells were incubated in a medium which contained 1 μg/mL LPS with or without tested compounds (1, 5, 10, 20 μM) for 24 h. The inhibitory efficacy of interruptins on NO production was examined by measuring the accumulation of nitrite in conditioned medium using Griess reagent. The result was expressed as IC_{50} values. MTT assay was used to determine cell viability. The compounds that caused the cell viability of tested cells lower than 80% were considered cytotoxic. The test was done in triplicate.

5. Determination of inflammation-related gene expression

The molecular mechanism of interruptins on anti-inflammation was investigated by detecting mRNA level of *iNOS, COX-2* and *PPAR-γ*. The experiment was carried out using polymerase chain reaction (PCR) based on previously reported conditions[18] with slight modifications. Briefly, RAW264.7 cells were treated with 1 μg/mL LPS with or without interruptins (1, 5, 10 μM) for 20 h. The treated cells were subjected to total RNA extraction, cDNA synthesis, and DNA amplification by PCR. The PCR reaction consisted of cDNA (200 ng), forward and reverse primer (5 ng each) and PCR master-mix in a final volume of 20 μL. The amplification reaction was performed with a pre-denaturation step at 95°C for 2 min followed by 30 cycles of 98°C; 30 sec, 60°C; 30 sec, 74°C; 1 min and final-extension at 75°C for 5 min. The primer sequences for each gene are shown in Table 1[18, 19]. The amplicons, 580 bp, 680 bp, 156 bp and 262 bp for *iNOS, COX-2, PPAR-γ* and *GAPDH*, respectively, were separated on 1.2% agarose gel by electrophoresis. DNA bands were stained with SYBR dye and observed under the blue light LED transilluminator.

Table 1. Primer sequences for PCR.

Genes	Sequences
iNOS	forward: 5'-ATCTGGATCAGGAACCTGAA-3'
	reverse: 5'-CCTTTTTTGCCCCATAGGAA-3'
COX-2	forward: 5'-GGAGAGACTATCAAGATAGTGATC-3'
	reverse: 5'-ATGGTCAGTAGACTTTTACAGCTC-3'
PPAR-γ	forward:5'-CAAAGTAGAACCTGCATCTCC-3'
	reverse: 5'-CCTTCACAAGCATGAACTCC-3'
GAPDH	forward: 5'-AACATCATCCCTGCATCCAC3'
	reverse: 5'-AGT GGGAGTTGCTGTTGA AG -3'

6. Statistical analysis

The data was expressed in the mean of triplicate experiments. The IC_{50} values were calculated from regression equations using Microsoft Excel program.

Results and Discussions

1. NO radical scavenging activity

Interruptin A, B and C were tested for the ability of NO radical scavenging through inhibition of the nitrite formation from NO. Interruptin B showed the best capacity to prevent nitrite accumulation with an IC_{50} value of 67.68 μM, followed by interruptin A with IC_{50} value of 90.07 μM. Their activities were 1.2-1.6 times better than gallic acid (IC_{50} = 107.92 μM). However, interruptin C had no activity (Table 2).

Table 2. NO radical scavenging and NO production inhibition activities of interruptins.

Compounds	NO radical scavenging activity	NO production inhibition activity
	IC_{50} (μM)	IC_{50} (μM)
InterruptinA	90.07	12.18
Interruptin B	67.68	0.81
Interruptin C	>100	>50
Gallic acid	107.92	-
Indomethacin	-	13.23

2. NO production inhibition activity

The inhibition efficacy of interruptins on NO production was carried out in lipopolysaccharide (LPS)-induced macrophage cells. Among tested compounds, interruptin B exerted the most potent inhibitory activity, followed by interruptin A with IC_{50} values of 0.81 and 12.18 μM, respectively, whereas interruptin C could not inhibit NO production. Of interest, the NO release inhibition activity of interruptin B was 16.3-fold effective compared with a standard drug, indomethacin (IC_{50} = 13.23 μM) (Table 2). The cell viability of RAW 264.7 cells

exposed to interruptins at the tested concentrations (1-20 μM) was evaluated by MTT assay. These compounds demonstrated the appropriate viable cells (more than 80%), only minor cytotoxic effect was observed at high concentration of 20 μM interrruptin A and B (data not shown).

3. *iNOS, COX-2* and *PPAR-γ* mRNA expression

The effect of interruptins on mRNA expression of inflammation-related genes is shown in Figure 2. Interruptin A moderately suppressed *iNOS* gene expression but strongly induced *PPAR-γ* gene expression (Figure 2A) in LPS-stimulated murine macrophages. Furthermore, interruptin B dramatically reduced *iNOS* mRNA level and also increased *PPAR-γ* mRNA expression in a dose-dependent manner (Figure 2B), while interruptin C did not affect both genes (Figure 2C). The *iNOS* gene suppression capacity of interruptin B was greater than the reference drug indomethacin (Figure 2D). However, all tested compounds exerted no effect on *COX-2* gene expression. Since, the activation of PPAR-γ has been involved in anti-inflammation, the simulation of PPAR-γ expression by its ligand rosiglitazone or some natural compounds such as apigenin, kaempferol and chysin could inhibit *iNOS* expression by disturbing its promoter activity[6, 7, 22, 23]. As a result, inhibition of NO production of interruptins A and B may involve the mechanism of *iNOS* mRNA suppression which is possibly due to their capability to induce *PPAR-γ* mRNA expression. This finding was also supported by the previous study which proposed the action of interruptin B as a dual PPAR-□ and -□ ligand[14]. In addition, the computational analysis of molecular docking similarly predicted the potential binding affinity of interruptin A to PPAR-□ (data not shown). Therefore, interruptins A and B could be suggested as novel anti-inflammatory agents from the natural lower plant, *C. terminans*.

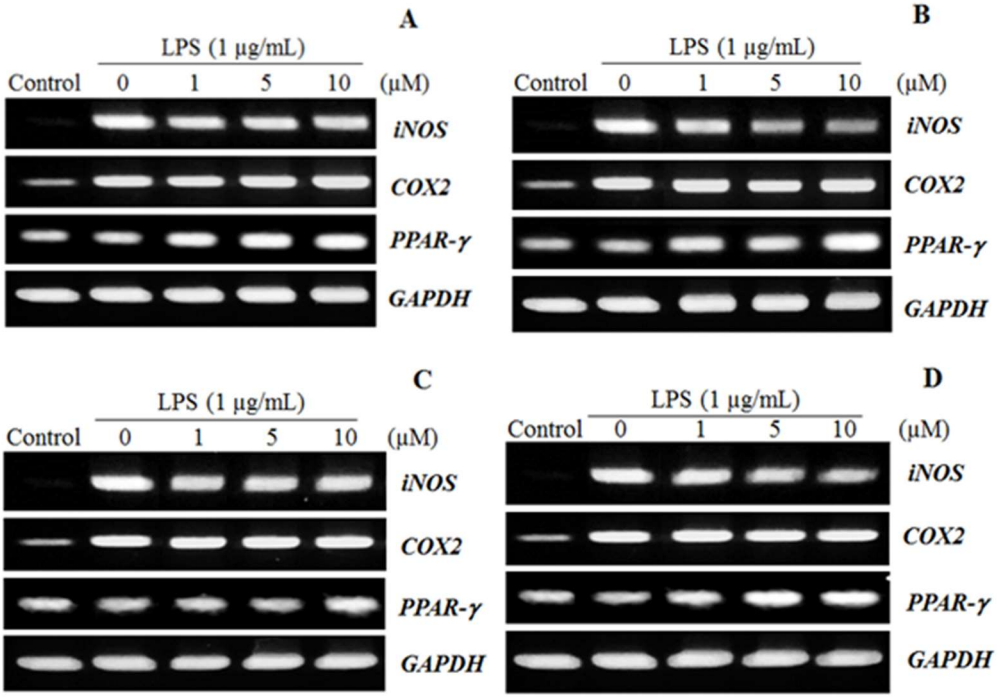

Figure 2. The transcriptional response of interruptins A (A), B (B), C (C) and indomethacin (D) on *iNOS*, *COX-2*, and *PPAR-γ* in RAW267.4 cells.

Conclusions

The present study is the first report of anti-inflammatory activity of interruptins that were isolated from *C. terminans*. The mechanism of action was exposed and shown that interruptins A and B possess anti-inflammatory activity by scavenging of NO radical and inhibition of NO production through suppression of *iNOS* gene expression and activation of *PPAR-γ* gene expression. Thus, this data indicates that interruptins A and B are potential natural anti-inflammatory from *C. terminans*. These novel discoveries may assist in pre-clinical/clinical studies on anti-inflammatory from fern plant or may help inflammation related diabetes syndrome and encourage the application of edible vegetable lower plant *C. terminans* for further drug or cosmeceutical development.

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Part V : HPLC method validation for simultaneous analysis of interruptins from *Cyclosorus terminans*

Introduction

The fern *Cyclosorus terminans* is a lower vascular plant commonly distributed all over Thailand and it ranges extended from the tropics of Asia to Australia[1]. It belongs to the Thelypteridaceae family. In northern Thailand, its shoot has been consumed as a vegetable[2]. Previously, interruptins A, B and C (Figure 1), natural occurring coumarin derivatives, were firstly elucidated from *C. interruptus*[3]. Recently, the fern *C. terminans* was also discovered as a new source of interruptin dervatives[4]. These compounds, particularly interruptins A and B, exhibited various biological activities including anti-bacteria, anti-cancer, anti-obesity, anti-diabetes, and anti-oxidation. Interruptin A showed powerful antibacterial activity against Gram-positive aerobic bacteria such as methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and *Bacillus subtilis*[3-5]. Interruptin B displayed anti-obesity by inducing cell proliferation and adipogenic differentiation, particularly brown adipocytes-energy expenditure cells. In addition, interruptin B also acted as an anti-diabetic agent by boosting glucose consumption and increasing glucose transporters expression in differentiated adipocytes[6,7]. More interestingly, both interruptins A and B not only were cytotoxic to breast cancer, colon cancer, and nasopharyngeal carcinoma cells, but also demonstrated an effective intracellular reactive oxygen species scavenging activity and took anti-apoptotic action against extracellular oxidative damage by hydrogen peroxide[3,4]. Whereas, interruptin C did not show biological activities compared to interruptins A and B. According to the data mentioned above, natural interruptins from *C. terminans* have strong potential to develop as a drug, a health supplement agent, and a cosmetic.

In fact, the use of pure isolated natural compounds was limited because its amount was insufficient. Moreover, isolation processes have been known to be complicated, expensive and time consuming. The crude extracts containing high amounts of active compounds or enriched

extracts were, therefore, found as an alternative approach. However, utilization of enriched extract requires controlling the quality by measuring the corresponding compound content in extract before use. The appropriate method for analyzing the interested compound is every crucial. For coumarin derivative interruptins A-C, the HPLC method for purity analysis was reported [4]. Although the three interruptins A-C had been reported regarding some biological activities and purity analysis by HPLC [4,6], its content analysis by HPLC has not yet been validated. This study has therefore aimed to validate the HPLC method for analysis of active coumarins, interruptins in the extracts.

Materials and Methods

1. Chemical and reagents

HPLC grade methanol and analytical grade glacial acetic acid were purchased from RCI Labscan. Milli Q water was obtained from Direct-Q® 5UV-R system, Millipore.

2. Chromatographic condition

HPLC analysis was performed on Waters 1500 system coupled with a photodiode array detector (PDA) (Water, Ireland). The separation was achieved on TSK-gel ODS-100V C18 column, 250 × 4.6 mm i.d. with 5 µm particle size (TOSOH Bioscience, Japan). The mobile phase consisted of methanol and 1% aqueous acetic acid in the ratio of 85:15. The flow rate was 1 mL/min and injection volume was 20 µL. The chromatographic signals were recorded by PDA at 290 nm.

3. Standard solution preparation

Standard agents were interruptins A, B and C. These compounds were isolated from the fern *C. terminans* and elucidated the structures by using spectroscopy techniques[10]. The purity was assessed based on NMR data. Standard stock solution was prepared by accurately weighing in equal amount, mixing and dissolving in dimethyl sulfoxide (DMSO). After that, it was

filtrated via 0.45 μm filter and kept in -20 °C until use. For HPLC analysis, standard stock solution was diluted in methanol to obtain desired concentrations.

4. Sample preparation

Ethyl acetate crude extract of *C. terminans* was used as a sample in development and validation processes. Plant extraction, fern powder was extracted with ethyl acetate using reflux extraction. After filtration, solution was evaporated under vacuum evaporator at 50 °C to obtain dry ethyl acetate crude extract. Then the extract was weighed and dissolved in methanol, sonicated at 30 °C for 15 minutes and filtrated through 0.45 μm filters to get sample solution for HPLC analysis.

5. Method validation

The validation method was processed according to International Conference on Harmonization (ICH) guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1) [8]. The expected performance parameters were linearity, range, specificity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

5.1 Linearity and range

Linearity was determined by using standard solution at six concentration points for each interruptin, in triplicate. After analysis, standard curves were plotted between the mean of area under curve (AUC) and concentration. Regression analysis was used to achieve the linear equations and the coefficients of determination (R^2). Range was obtained from the range of standard concentration that provided $R^2 \geq 0.999$.

5.2 Specificity

Specificity was tested by checking the variance of retention time and comparing the similarity of UV absorption pattern of interruptin standards and interruptins in sample. The absorption pattern was checked on three points of target peak. The analysis was done in nine replications.

5.3 Limit of detection (LOD) and limit of quantitation (LOQ)

Standard solutions were diluted in methanol by serial dilution and analyzed in three replicates. LOD and LOQ of analysis method were desired from the minimum concentration giving a signal-to-noise (S/N) ratio equal to 3 and 10, respectively.

5.4 Accuracy

Method accuracy was carried out by using spiking technique. Three different known amounts of standard interruptins A, B, and C-low (10 µg/mL), average (50 µg/mL) and high (100 µg/mL)-were added into sample solution in order to determine the recovery. The quantities of interruptins in sample solution were elucidated prior to spiking standard for calculation of actual recovery. The amounts of spiked interruptins were measured in triplicate for each spiked level and their recoveries were calculated using the equation: % recovery = 100 × measured content/fortified amount.

5.5 Precision

Method precision was evaluated to find out repeatability (intra-day precision) and intermediate precision (inter-day precision) by determining scatter degree of series analysis. For intra-day precision, six replicate injections of one sample solution were analyzed in same day under same condition. The inter-day precision was accessed by measuring the interruptin amounts in three different concentrations of sample solution in three days interval. The analysis was done in triplicate for each concentration in each day. The data was calculated to obtain % RSD for intra-day precision (less than 2%) and inter-day precision (less than 5%).

Results and Discussions

1. HPLC condition

The optimal chromatographic condition for quantitation of interruptin content in *C. terminans* extract was accomplished by HPLC method using a reverse phase column. Interruptins A, B and C were successfully separated simultaneously using methanol/1% acetic

acid in the ratio of 85:15 (v/v) with isocratic system, photo-diode array detection at 290 nm and a flow rate of 1 mL/min. The three compounds were eluted at 13, 17 and 20 minutes, respectively (Figure 1).

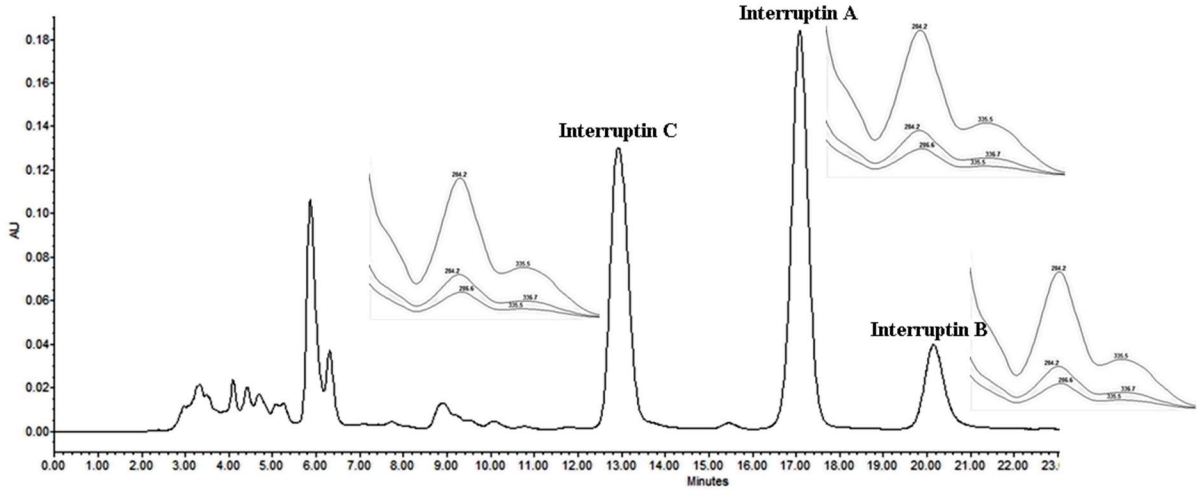

Figure 1. HPLC chromatogram of *C. terminans* ethyl acetate extract and the UV absorption pattern of interruptins.

2. Linearity and range

The performance parameters of method validation were achieved following the International Conference on Harmonization (ICH) guideline (Table 1). The calibration curves of interruptins were conducted by measuring six different concentrations of standard solution, typically in the range of 6.25-200 μg/mL. The linearity, expressed as coefficient of determination (R^2), of all compounds was >0.999.

3. Specificity, LOD and LOQ

The variance of retention times and the similarity of UV absorption pattern were assessed to define method specificity (Figure 1). The %RSD of retention times of the three interruptins were less than 1%. The UV absorption pattern of each interruptin in samples and

standards was similar. Hence, the data indicated that this system was highly specific for analysis the interruptins.

The LOD and LOQ were within the range of 0.20-3.13 µg/mL and 0.46-6.25 µg/mL, respectively. According to very low limitation values, it reflexed that this system was sensitive to detect interruptins.

4. Accuracy and precision

Method accuracy was obtained from % recovery of spiked standards. The % recovery of three spiked levels (10, 50 and 100 µg/mL) of each interruptin was within the range of 91-107% which was fit to the requirement (100±10%). The data indicated that the analysis method was accurate for measuring interruptins in a wide range concentration.

Method precision of three compounds was displayed in %RSD of intra- and inter-day analysis. The precision value of intra-day was less than 1%, while inter-day precision was less than 2%. This parameter was congruent with ICH requirement. The data suggested that this method was highly precise for measuring of interruptins.

Table 1. Validation parameters of interruptins A-C using HPLC.

Parameters	**Interruptin A**	**Interruptin B**	**Interruptin C**
Linear Equation[a]	Y = 60390X - 252341	Y = 11353X- 112091	Y= 535009X - 10292
coefficients of determination	0.9996	0.9996	0.9997
Range (µg/mL)	6.25-200	12.5-400	6.25-200
%RSD of retention time	0.48	0.54	0.34
UV Absorption pattern (λmax) (nm)			
Standard	283, 340	331	286, 336
Sample	283, 339	330	284, 336

LOD *(*µg/mL*)*	0.81	3.13	0.20
LOQ *(*µg/mL*)*	1.63	6.25	0.46
Accuracy *(%* **Recovery***)*			
10 µg/mL	91.30 ± 0.56	100.42 ± 1.46	100.51 ± 0.18
50 µg/mL	94.30 ± 2.88	106.49 ± 0.53	107.84 ± 0.75
100 µg/mL	99.45 ± 1.32	100.82 ± 0.61	105.05 ± 1.09
Precision*(%***RSD***)*			
Intra-day *(n=6)*	0.16	0.83	0.30
Inter-day *(n=3)*	0.92	0.44	1.18

[a]Y is peak area; X is interruptin concentration, LOD and LOQ are limit of detection and limit of quantification, respectively.

5. Determination of interruptin content in *C. terminans* extract

C. terminans ethyl acetate extract prepared by reflux extraction was were subjected to analytical protocol as described. The results are shown in Table 2 and the chromatogram pattern of all crude extracts is displayed in Figure 1.

Table 2. Interruptin content in *C. terminans* ethyl acetate extract analysis by validated HPLC method.

% **of interruptins in crude extract** *(%***w/w***)*		
Interruptin A	Interruptin B	**Interruptin C**
1.93 ± 0.10	0.84 ± 0.05	1.72 ± 0.06

Conclusions

The validated HPLC method showed congruent validation parameters by ICH requirements with efficient sensitivity, accuracy and precision. The analytical method was then applied to determine the interruptins content in *C. terminans* crude extracts. The results confirmed that this model could be used as a standard method for simultaneous determination and quantitation of interruptins A, B and C in plant crude extracts.

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Outputs

1. Chaiwong S, Puttarak P, **Kaewsuwan S**. 2018. Anti *Propionibacterium acnes* activity, HPLC method validation for simultaneous analysis and extraction of coumarins from the fern *Cyclosorus terminans*. Latin American Journal of Pharmacy 37 (9): 1791-1797.

2. Chaiwong S, Puttarak P, Sretrirutchau S, **Kaewsuwan S**. 2019. *In vitro* anti-inflammatory and antioxidative activities of isolated interruptins from *Cyclosorus terminans.* Latin American Journal of Pharmacy 38 (8): 1677-1682.

ภาคผนวก

Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **37** (9): 1791-7 (2018)

Received: July 3, 2018
Revised version: July 10, 2018
Accepted: July 11, 2018

Anti *Propionibacterium acnes* Activity, HPLC Method Validation for Simultaneous Analysis and Extraction of Coumarins from the Fern *Cyclosorus terminans*

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***SUMMARY*. Interruptin A effectively inhibited and killed *Propionibacterium acnes* with MIC and MBC of 1.95/7.81 μg/mL, whereas interruptins B and C were not active. The high performance liquid chromatography with a photo diode array detector was validated for simultaneous analysis of interruptins A, B and C. The HPLC condition was achieved by using a reverse-phase C18 analytical column, methanol/1% aqueous acetic acid (85:15, v/v) and 1 mL/min flow rate. According to the International Conference of Harmonization (ICH) guidelines, the method was validated on the basis of these parameters: linearity ($R^2 \geq 0.999$), range (typically 6.25-200 μg/mL), specificity, accuracy (100 ± 10%), precision (intra-day <1%, inter-day <2%), limit of detection (LOD) and limit of quantitation (LOQ). Ethyl acetate combined with maceration or microwave-assisted extraction was used as a suitable method for extraction of interruptins. This model is suitable as a standard method for simultaneous examination of interruptins A, B and C in a single HPLC run.**

RESUMEN. Interruptin A inhibió eficazmente y mató *Propionibacterium acnes* con MIC y MBC de 1.95/7.81 μg/mL, mientras que las interruptinas B y C no estaban activas. La cromatografía líquida de alta resolución con un detector de diodo fotoeléctrico fue validada para el análisis simultáneo de interruptinas A, B y C. La condición de HPLC se logró mediante el uso de una columna analítica C18 de fase inversa, metanol ácido acético acuoso al 1% (85:15, v/v) y velocidad de flujo de 1 mL/min. De acuerdo con las directrices de la Conferencia Internacional de Armonización (ICH), el método fue validado sobre la base de estos parámetros: linealidad ($R2 \geq 0.999$), rango (típicamente 6.25-200 μg/mL), especificidad, precisión (100 ± 10%), precisión (intradía <1%, interdía <2%), límite de detección (LOD) y límite de cuantificación (LOQ). El acetato de etilo combinado con maceración o extracción asistida por microondas se utilizó como un método adecuado para la extracción de interruptinas. Este modelo es adecuado como un método estándar para el examen simultáneo de interruptinas A, B y C en una sola ejecución de HPLC.

INTRODUCTION

Propionibacterium acnes is a Gram-positive commensal bacteria that mostly resides in the pilosebaceous follicles of the skin. *P. acnes* proliferation in pilosebaceous units is one of the major pathogenesis of acne vulgaris [1,2]. Acne vulgaris is a common disease that broadly affects individuals, mainly adolescents. This disease can be painful and may lead to acne scaring, postinflammatory hyperpigmentation and psychological problems [1,3-5].

Topical agents (retinoids and benzoyl peroxide) and antimicrobial agents (tetracycline, erythromycin and clindamycin) have been widely used for acne treatment [1,5]. However, bacterial drug resistance remains the problem in the remedy of acne [6]. Therefore, novel agents which effectively inhibit *P. acnes* growth will be explored as new drugs for acne vulgaris remedies.

The fern *Cyclosorus terminans* (J. Sm. ex Hook.) Panigrahi is a lower vascular plant commonly distributed all over Thailand and it ranges extended from the tropics of Asia to Australia [7]. It belongs to the Thelypteridaceae fami-

KEY WORDS: coumarins, Cyclosorus terminans, high performance liquid chromatography, interruptin, method validation.

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ISSN 0326 2383 (printed ed.)
ISSN 2362-3853 (on line ed.)

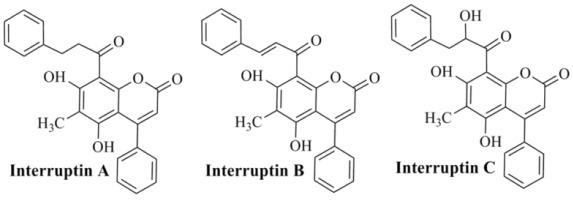

Figure 1. Chemical structure of interruptins A-C.

ly. In northern Thailand, its shoot has been consumed as a vegetable [8]. Previously, interruptins A, B and C (Fig. 1), natural occurring coumarin derivatives, were firstly elucidated from *C. interruptus* [9].

Recently, the fern *C. terminans* was also discovered as a new source [10]. These compounds, particularly interruptins A and B, exhibited various biological activities including anti-bacteria, anti-cancer, anti-obesity, anti-diabetes, and anti-oxidation. Interruptin A showed powerful antibacterial activity against Gram-positive aerobic bacteria such as methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), *S. epidermidis* and *Bacillus subtilis* [9-11]. Interruptin B displayed anti-obesity by inducing cell proliferation and adipogenic differentiation, particularly brown adipocytes-energy expenditure. In addition, interruptin B also acted as an anti-diabetic agent by boosting glucose consumption and increasing glucose transporters expression in differentiated adipocytes [12,13]. More interestingly, both interruptins A and B not only were cytotoxic to breast cancer, colon cancer, and nasopharyngeal carcinoma cells, but also demonstrated an effective intracellular reactive oxygen species scavenging activity and took anti-apoptotic action against extracellular oxidative damage by hydrogen peroxide [9,10]. whereas, interruptin C did not show biological activities compared to interruptins A and B. According to the data mentioned above, natural interruptins from *C. terminans* have strong potential to develop as a drug, a health supplement agent, and a cosmetic.

In fact, the use of pure isolated natural compounds was limited because its amount was insufficient. Moreover, isolation processes have been known to be complicated, expensive and time consuming. The crude extracts containing high amounts of active compounds or enriched extracts were, therefore, found as an alternative approach. However, utilization of enriched extract requires controlling the quality by measuring the corresponding compound content in extract before use. The appropriate method for analyzing the interested compound is very crucial. For coumarin derivative interruptins A-C, the HPLC method for purity analysis was reported [10]. Although the three interruptins A-C had been reported regarding some biological activities and purity analysis by HPLC [10, 12], its content analysis by HPLC has not yet been validated. Additionally, anti-microbial against an anaerobic bacterium has also not been verified. This study has therefore aimed to evaluate anti-bacterial activity toward *P. acnes* and to validate HPLC method for analysis of active coumarins, interruptins, in the extracts. The suitable method for interruptins extraction was also studied.

MATERIALS AND METHODS

Chemicals and reagents

P. acnes (DMST 14916) were kindly provided by Assist. Prof. Dr. Sukanya Dej-Adisai, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The Brain heart infusion (BHI) used for bacterial cultivation was acquired from HIMEDIA.

HPLC grade methanol and analytical grade glacial acetic acid were purchased from RCI Labscan. Milli Q water was obtained from Direct-Q® 5UV-R system, Millipore.

Determination of antibacterial activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of interruptins were determined using the broth microdilution method [10,14]. Briefly, interruptins (250 µg/mL) were 2-fold serial diluted in BHI and bacteria were inoculated (5×10^5 CFU/mL) then further incubated in anaerobic jars at 37 °C for 72 h. The lowest concentration of a sample that can inhibit bacterial growth was defined as MIC. The MBC was achieved at the lowest concentration that exhibited no bacterial colony. Clindamycin was used as a standard drug and the experiment was operated in triplicate

Chromatographic condition

HPLC analysis was performed on Waters 1500 system coupled with a photodiode array detector (PDA) (Water, Ireland). The separation was achieved based on previously reported conditions [10].

Standard solution preparation

Standard agents were interruptins A, B and C. These compounds were isolated from the fern *C. terminans* and the structures were eluci-

dated by spectroscopy techniques [10]. The standard stock solution was prepared by accurately weighing equal amounts, mixing and dissolving interruptins in dimethyl sulfoxide (DMSO). After that, it was filtrated via a 0.45 µm filter and kept at -20 °C until use. For HPLC analysis, standard stock solution was diluted in methanol to obtain the desired concentrations.

Sample preparation

Ethyl acetate crude extract of *C. terminans* was used as a sample in both the development and the validation processes. For plant extraction, fern powder was extracted with ethyl acetate using reflux extraction. After filtration, the solution was evaporated under a vacuum evaporator at 50 °C to obtain dry ethyl acetate crude extract. Then the extract was weighed and dissolved in methanol, sonicated at 30 °C for 15 min and filtrated through 0.45 µm filters to get a sample solution for HPLC analysis.

Method validation

The validation method was processed according to the International Conference on Harmonization (ICH) guidelines, Validation of Analytical Procedures: Text and Methodology Q2 (R1) [15]. The expected performance parameters were linearity, range, specificity, limit of detection (LOD), limit of quantitation (LOQ), and accuracy and precision.

Linearity and range

Linearity was determined by using a standard solution at six concentration points for each interruptin in triplicate. After analysis, standard curves were plotted between the mean of area under curve (AUC) and concentration. Regression analysis was used to achieve the linear equations and the coefficients of determination (R^2). Range was obtained from the range of standard concentration that provided $R^2 \geq 0.999$.

Specificity

Specificity was tested by checking the variance of retention time and comparing the similarity of UV absorption pattern of interruptins standards and interruptins in samples. The absorption pattern was checked on three points of the target peak. The analysis was done in nine replications.

Limit of detection (LOD) and limit of quantitation (LOQ)

Standard solutions were diluted in methanol by serial dilution and analyzed in three replicates. LOD and LOQ of analysis method were chosen from the minimum concentration giving a signal-to-noise (S/N) ratio equal to 3 and 10, respectively.

Accuracy

Method accuracy was carried out by using the spiking technique. Three different known amounts of standard interruptins A, B, and C – low (10 µg/mL), medium (50 µg/mL) and high (100 µg/mL)– were added into the sample solution in order to determine the recovery. The quantities of interruptins in the sample solution were elucidated prior to spiking standards for calculation of actual recovery. The amounts of spiked interruptins were measured in triplicate for each spiked level and their recoveries were calculated using the equation: *% recovery = 100 × measured content/fortified amount.*

Precision

Method precision was evaluated to find out repeatability (intra-day precision) and intermediate precision (inter-day precision) by determining the scatter degree of series analysis. For intra-day precision, six replicate injections of one sample solution were analyzed in the same day under the same conditions. The inter-day precision was accessed by measuring the amounts of interruptins in three different concentrations of the sample solution in three day intervals. The analysis was done in triplicate each day for each concentration. The data was calculated to obtain % RSD for intra-day precision (less than 2%) and inter-day precision (less than 5%).

Determination of interruptins extraction condition

To evaluate the effects of solvents and extraction methods for maximum extraction of interruptins, the *C. terminans* powder (10 g) was separately extracted with ethyl acetate (EtOAc), isopropanol and ethanol (EtOH) (100 mL) using maceration, reflux extraction, ultrasonic-assisted extraction and microwave-assisted extraction for each solvent. Briefly in the maceration process, fern powder was soaked in solvents and shaken for 72 h (100 rpm) at room temperature. For reflux extraction, plant powder was refluxed at 80-90 °C for 60 min. For other methods, fern powder was soaked in solvents and shaken for 24 h (100 rpm) at room temperature before extraction by microwave (600 watts, 2.5 min) or ultrasonic (37 kHz, 50 °C, 30 min) methods. The extracts were filtrated, and the residues were re-extracted from the first step for 2 rounds. The filtrates of each solvent with the same extraction

method were pooled together and evaporated under a vacuum evaporator at 40-60 °C to obtain crude extracts. All extraction conditions were done in triplicate. For HPLC analysis, samples were prepared as mentioned in the sample preparation section and then subjected to be analyzed by validated HPLC conditions.

RESULTS AND DISCUSSIONS

Antibacterial activity

The antibacterial efficacy against *P. acnes* of isolated interruptins from *C. terminans* is shown in Table 1. Interruptin A was an effective inhibitory and bactericidal agent (MIC/MBC of 1.95/7.81 µg/mL) compared to other isolated interruptins tested. This result was consistent with the previous report that interruptin A exhibited potent antibacterial property against Gram-positive skin pathogens while interruptins B and C were not active [10]. Previous reports suggested that the potential antibacterial activity of coumarins is involved with the presence of hydroxyl group at position 7 together with its lipophilic property and planar molecular structure that enhance the ability to penetrate the microbial cell membrane [16-18]. Moreover, the presence of a single bond and absence of substitution in the propionyl chain at 2'-carbon of interruptin A may be essential for antibacterial activity [10].

Sample	MIC (µg/mL)	MBC (µg/mL)
Interruptin A	1.95	7.81
Interruptin B	>250	>250
Interruptin C	>250	>250
Clindamycin	0.25	0.49

Table 1. Antibacterial activity against *P. acnes* of interruptins from *C. terminans*.

Method validation

The optimal chromatographic condition for quantitation of interruptins content in C. terminans extract was accomplished by HPLC method using a reverse phase C18 column. Interruptins A, B, and C were successfully separated simultaneously using methanol/1% acetic acid in the ratio of 85:15 (v/v) with a flow rate of 1 mL/min in isocratic system at 290 nm. The three compounds were eluted at 13, 17, and 20 min, respectively (Fig. 2).

Linearity and range

The performance parameters of method validation were achieved following the International Conference on Harmonization (ICH) guidelines (Table 2). The calibration curves of interruptins were conducted by measuring six different concentrations of standard solutions, typically in the range of 6.25-200 µg/mL. The linearity, expressed as a coefficient of determination (R2), of all compounds was > 0.999.

Specificity, LOD and LOQ

The variance of retention times and the similarity of UV absorption patterns were assessed to define method specificity (Fig. 2). The %RSD of retention times of the three interruptins were less than 1%. The UV absorption pattern of each interruptin in samples and standards was similar. Hence, the data indicated that this system was highly specific for analysis of the interruptins.

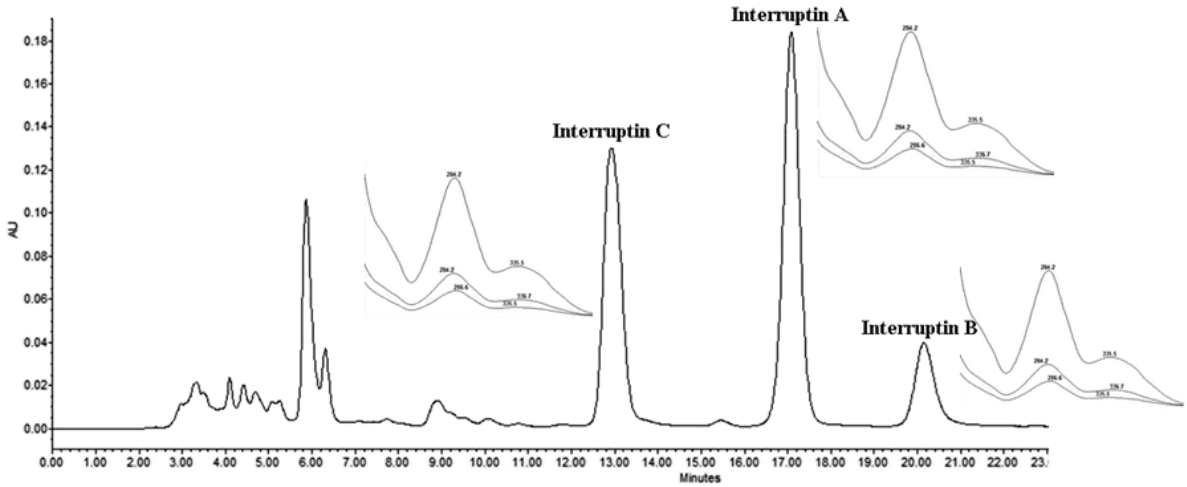

Figure 2. HPLC chromatogram of *C. terminans* ethyl acetate extract and the UV absorption pattern of interruptins.

Parameters		Interruptin A	Interruptin B	Interruptin C
Linear Equation [a]		Y = 60390X - 252341	Y = 11353X- 112091	Y= 535009X - 10292
Coefficients of determination		0.9996	0.9996	0.9997
Range (µg/mL)		6.25-200	12.5-400	6.25-200
%RSD of retention time		0.48	0.54	0.34
UV Absorption pattern (λmax) (nm)	Standard	283, 340	331	286, 336
	Sample	283, 339	330	284, 336
LOD (µg/mL)		0.81	3.13	0.20
LOQ (µg/mL)		1.63	6.25	0.46
Accuracy (% Recovery)	10 µg/mL	91.30 ± 0.56	100.42 ± 1.46	100.51 ± 0.18
	50 µg/mL	94.30 ± 2.88	106.49 ± 0.53	107.84 ± 0.75
	100 µg/mL	99.45 ± 1.32	100.82 ± 0.61	105.05 ± 1.09
Precision (%RSD)	Intra-day *(n=6)*	0.16	0.83	0.30
	Inter-day *(n=3)*	0.92	0.44	1.18

Table 2. Validation parameters of interruptins A-C using HPLC. [a] Y is peak area; X is interruptin concentration, LOD and LOQ are limit of detection and limit of quantification, respectively.

Solvent	Extraction method	% of interruptins in crude extract (%w/w)		
		Interruptin A	Interruptin B	Interruptin C
Ethyl acetate	maceration	2.34 ± 0.34	1.22 ± 0.05	2.32 ± 0.21
	microwave	2.36 ± 0.20	1.18 ± 0.05	2.32 ± 0.11
	ultrasonic	1.84 ± 0.11	1.14 ± 0.07	2.22 ± 0.17
	reflux	1.93 ± 0.10	0.84 ± 0.05	1.72 ±0.06
Isopropanol	maceration	1.93 ± 0.10	0.78 ± 0.12	1.00 ± 0.13
	microwave	nd	0.50 ± 0.04	1.18 ± 0.17
	ultrasonic	0.87 ± 0.10	1.17 ± 0.07	1.83 ± 0.13
	reflux	0.47 ± 0.15	0.47 ± 0.05	0.77 ± 0.18
Ethanol	maceration	nd	nd	0.48 ± 0.10
	microwave	nd	nd	0.54 ± 0.05
	ultrasonic	nd	nd	0.47 ± 0.04
	reflux	nd	nd	0.27 ± 0.07

Table 3. Interruptin content in *C. terminans* crude extract by different extraction conditions. The data was expressed as mean ± SD. nd: not detected.

The LOD and LOQ were within the range of 0.20-3.13 µg/mL and 0.46-6.25 µg/mL, respectively. According to very low limitation values, it reflected that this system was sensitive to detect interruptins.

Accuracy and precision

Method accuracy was obtained from % recovery of spiked standards. The % recovery of three spiked levels (10, 50, and 100 µg/mL) of each interruptin was within the range of 91-107% which fit the requirement (100 ± 10%). The data indicated that the analysis method was accurate for measuring interruptins in a wide range concentration.

The method precision of three compounds was displayed in %RSD of intra- and inter-day analysis. The precision value of intra-day was less than 1%, while inter-day precision was less than 2%. This parameter was congruent with the ICH requirement. The data suggested that this method was highly precise for measuring interruptins.

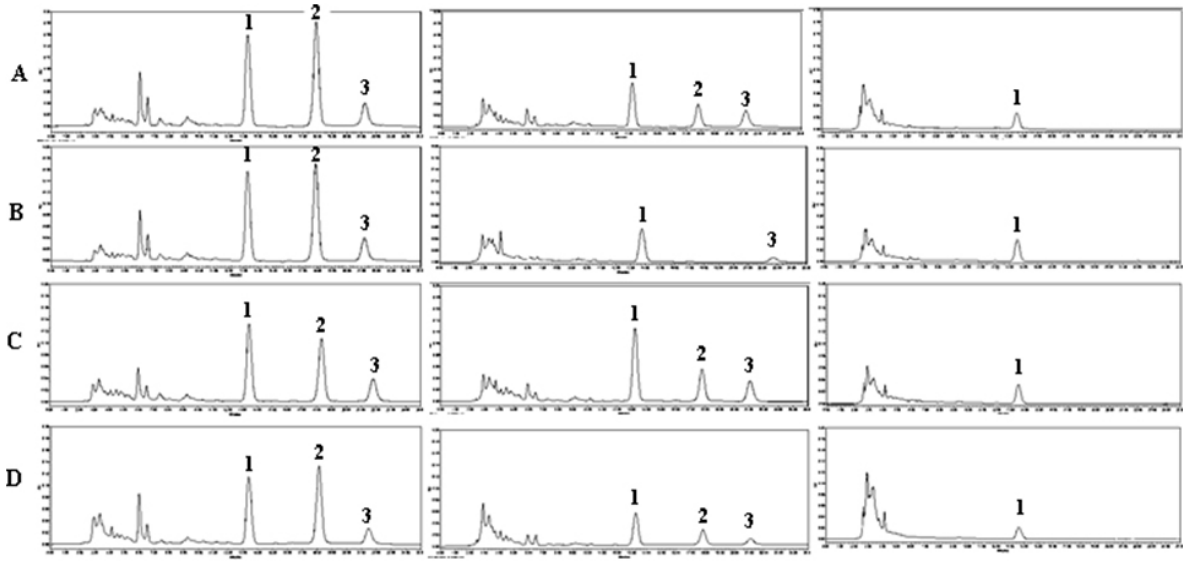

Figure 3 CHPLC Chromatograms of *C. terminans* EtOAc (first column), isopropanol (second column) and EtOH (third column) crude extract by different extraction methods. Row **A**: maceration, Row **B**: microwave-assisted extraction, Row **C**: ultrasonic-assisted extraction, Row **D**: reflux extraction. The number 1, 2 and 3 over the peaks represent interruptins C, A and B, respectively.

Determination of interruptin extraction condition

The fern extracts from different extraction conditions were subjected to analytical protocol as described. The results are shown in Table 3 and the chromatogram pattern of all crude extracts is displayed in Fig. 3. As a result, ethyl acetate was an appropriate solvent to extract interruptins. In the basic chemical structure, this efficacy may be due to the ester group in ethyl acetate structure that differs from alcohol which contains the hydroxyl group. Moreover, the contact time of fern powder and solvent in the maceration process and the effect of microwave rays in the microwave-assisted extraction process could enhance extraction efficacy of ethyl acetate. The result suggests that ethyl acetate combined with maceration or microwave-assisted extraction were the suitable methods for extraction of interruptins.

CONCLUSION

Among three isolated interruptins A-C, only interruptin A was a potent antibacterial compound against *P. acnes* with MIC/MBC of 1.95/7.81 μg/mL. Due to its potential, interruptin A could be presented as a candidate for an antibacterial drug against Gram-positive bacteria or developed into skin care products such as anti-acne gel or lotion.

Moreover, the validated HPLC method showed congruent validation parameters by ICH requirements with efficient sensitivity, accuracy and precision. The analytical method was then applied to determine the interruptins content in different types of *C. terminans* crude extracts. The results confirmed that this model could be used as a standard method for simultaneous determination and quantitation of interruptins A, B and C in plant crude extracts. For high-yield interruptins extraction condition, ethyl acetate was suggested as the suitable organic solvent when combined with maceration or microwave-assisted extraction. However, the use of organic solvents may cause toxicity, so alternative green solvents are possible extractors for further study.

Acknowledgments. The authors would like to acknowledge the Prince of Songkla University (PSU) Ph.D. scholarship (PSU/95000201/2556), the Thailand Research Fund (RSA578003) and the PSU research grant (PHA580272S) for financial support. We also gratefully thank Assist. Prof. Dr. Sukanya Dej-Adisai and Ms. Maria Suzanne Mullet, Faculty of Pharmaceutical Sciences, PSU for kindly providing the *P. acnes* and the assistance with English, respectively.

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Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **38** (8): 1677-82 (2019)

Received: February 19, 2019
Accepted: May 23, 2019

In Vitro Anti-inflammatory and Antioxidative Activities of Isolated Interruptins from *Cyclosorus terminans*

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***SUMMARY*. Interruptins A, B and C isolated from the fern *Cyclosorus terminans* were investigated for anti-inflammatory and antioxidative activities. Among them, interruptin B exhibited the most potent anti-inflammation through NO radical scavenging and NO production inhibition in cells with IC_{50} of 67.68 and 0.81 μM followed by interruptin A with IC_{50} of 90.07 and 12.18 μM, respectively. These compounds also down-regulated iNOS and up-regulated PPAR-γ mRNA expression in a dose-dependent manner, whereas interruptin C was not active. Furthermore, interruptin A exerted significantly DPPH radical scavenging activity with IC_{50} of 27.79 μM and provided high ferric reduction capacity with 682.45 ± 16.31 mmol/L ascorbic acid /mol interruptin. To the best of our knowledge, this is the first study describing anti-inflammatory activity of interruptins A and B. This action may be due to an induction of PPAR-γ expression. Therefore, interruptins A and B could be further developed as anti-inflammatory drugs and used as antioxidative supplements.**

RESUMEN. Las interruptinas A, B y C aisladas del helecho *Cyclosorus terminans* fueron investigadas por sus actividades antiinflamatorias y antioxidantes. Entre ellos, la interruptina B mostró la actividad antiinflamatoria más potente a través de la eliminación de radicales de NO y la inhibición de la producción de NO en células con IC_{50} de 67.68 y 0.81 μM seguida de interruptina A con IC_{50} de 90.07 y 12.18 μM, respectivamente. Estos compuestos también regulan a la baja la expresión de mRNA de iNOS y PPAR-γ sobreregulada por incremento de una manera dependiente de la dosis, mientras que la interruptina C no estaba activa. Además, la interruptin A ejerció una actividad de eliminación de radicales de DPPH significativamente significativa con IC_{50} de 27.79 79 μM y proporcionó una alta capacidad de reducción férrica con 682,45 ± 16.31 mmol/L de ácido ascórbico/mol de interruptina. Según nuestro conocimiento, este es el primer estudio que describe la actividad antiinflamatoria de las interruptinas A y B. Esta acción puede deberse a una inducción de la expresión de PPAR-γ. Por lo tanto, las interruptinas A y B podrían desarrollarse aún más como medicamentos antiinflamatorios y usarse como suplementos antioxidantes.

INTRODUCTION

Inflammation is a normal response mechanism of the body tissue in order to eliminate injurious agents, remove necrotic cells and damaged tissues, and initiate the healing process [1]. In the inflammatory process, nitric oxide (NO), an important inflammatory mediator, is produced by activated macrophage cells [2]. NO acts as a regulatory molecule by regulating local blood flow in physiological conditions and plays a role in host defense mechanisms as an anti-microbial agent [3]. During inflammation, NO is overproduced by inducible nitric oxide synthase (iNOS) via oxidation of L-arginine. NO that reacted with superoxide radicals to form reactive peroxy nitrite as well as other free radicals generated during inflammation can directly damage the cellular components and cause carcinogenesis. NO is also involved in increasing pro-inflammatory prostaglandins produced by cyclooxygenase (COX)-2 enzymes [2,4,5]. However, the production of NO and the expression of the iNOS gene can be reduced by activation of peroxisome proliferator-activated receptor (PPAR-γ) [6,7].

PPAR-γ is a member of nuclear receptors

KEY WORDS: anti-inflammation, antioxidant, coumarin, *Cyclosorus terminans,* interruptin, nitric oxide.

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ISSN 0326 2383 (printed ed.)
ISSN 2362-3853 (on line ed.)

which regulates the transcription of target genes involved in regulation of adipocyte differentiation and lipid metabolism [8,9]. Stimulation of PPAR-γ expression in monocyte and macrophage suppressed inflammatory mediators and cytokine production such as NO, PGE_2 and TNF-α [6,10,11]. Thus, direct scavenging of NO, suppression of iNOS and COX-2 expression or the induction of PPAR-γ expression could be considered as potential targets for inflammation treatment.

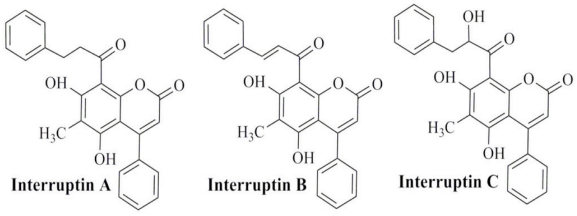

Figure 1. Chemical structure of interruptins A-C.

Interruptins, including interruptins A, B and C (Fig. 1), the natural occurring coumarin derivatives, were found in the ferns *Cyclosorus interruptus* [12] and *C. terminans* [13]. In previous studies, we exposed that interruptins A and B exhibited a broad spectrum of biological activities such as anti-bacterial, anti-diabetes, anti-cancer, and intracellular reactive oxygen species scavenging. Interestingly, interruptin B was presumed as a PPAR-γ agonist since it increased PPAR-γ gene expression and also bound properly to a PPAR-γ receptor predicted by computational docking [13-15]. This evidence may suggest the ability of interruptin B as an anti-inflammatory agent. In regard to the similarity in chemical structures of interruptins A-C, the present study aimed to evaluate whether interruptins A-C isolated from *C. terminans* could perform anti-inflammatory property, to investigate the potential mechanism underlying the action of corresponding compounds in regulating anti-inflammation, and to determine their antioxidation activity. The results from this study intend to provide a novel scientific principle for encouragement in the use of edible vegetable lower plant, *C. terminans,* as a medicinal plant and to warrant application of coumarin derivatives for attenuation the inflammation or oxidative stress.

MATERIALS AND METHODS

Chemicals and reagents

Interruptins A, B, and C were isolated from ethyl acetate extract of the fern *C. terminans* [13]. RPMI-1640 medium, fetal bovine serum (FBS), penicillin/streptomycin were purchased from Gibco BRL, California. Dimethyl sulfoxide (DMSO), Phosphate buffer saline (PBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from AMRESCO, USA. Lipopolysaccharide (LPS) of *Escherichia coli,* 2,2-Diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, sodium nitropusside and gallic acid were obtained from Sigma-Aldrich, USA. RNA extraction kit (FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit) was purchased from Favogen, Korea. FIREScript RT cDNA Synthesis KIT and 5x FIREPol PCR master Mix Ready to load were supplied from Solis BioDyne, Estonia.

Cell culture

Murine macrophage RAW264.7 cells were cultured in a complete medium, RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, at 37 °C in 5% CO_2 incubator. The medium was changed at two-day intervals until the cells reached confluence.

NO radical scavenging assay

Based on production of nitrite ion from the reaction of NO and oxygen, scavenging of NO leads to a decrease in nitrite production. Hereby NO radical scavenging efficacy of interruptins was evaluated based on a previously reported method [16]. Briefly, sodium nitroprusside (SNP) solution (20 mM), NO generator, was mixed with interruptins (20-100 μM) or gallic acid (29-176 μM) as a standard and incubated at room temperature for 180 min. The incubated mixtures were reacted with Griess reagent and then the absorbance was measured at 546 nm using a microplate reader. Gallic acid was used as a positive control. The experiment was done in three replications and IC_{50} values were determined graphically.

NO production inhibition assay in LPS-induced RAW264.7 cells

Based on the production of NO by macrophage cells when stimulated with lipopolysaccharide (LPS), RAW264.7 cells were used as a model for determination of anti-inflammatory activity of compounds according to a previously reported method [17] with some modifications. The cells were incubated in a medium which contained 1 μg/mL LPS with or without tested compounds (1, 5, 10, and 20 μM) for 24 h. The

inhibitory efficacy of interruptins on NO production was examined by measuring the accumulation of nitrite in conditioned medium using Griess reagent. The result was expressed as IC_{50} values. MTT assay was used to determine cell viability. The compounds that caused the cell viability of tested cells lower than 80% were considered as cytotoxicity. The test was done in triplicate.

Determination of inflammation-related gene expression

The molecular mechanism of interruptins on anti-inflammation was investigated by detecting mRNA level of *iNOS*, *COX-2* and *PPAR-γ*. The experiment was carried out using polymerase chain reaction (PCR) based on previously reported conditions [18] with slight modifications. Briefly, RAW264.7 cells were treated with 1 µg/mL LPS with or without interruptins (1, 5, and 10 µM) for 20 h. The treated cells were subjected to total RNA extraction, cDNA synthesis, and DNA amplification by PCR. The PCR reaction consisted of cDNA (200 ng), forward and reverse primers (5 ng each) and PCR master-mix in a final volume of 20 µL. The amplification reaction was performed with a pre-denaturation step at 95 °C for 2 min followed by 30 cycles of 98 °C; 30 s, 60 °C; 30 s, 74 °C; 1 min and final-extension at 75 °C for 5 min. The primer sequences for each gene are shown in Table 1 [18,19]. The amplicons, 580, 680, 156 and 262 bp for *iNOS, COX-2, PPAR-γ* and *GAPDH,* respectively, were separated on 1.2% agarose gel by electrophoresis. DNA bands were stained with SYBR dye and observed under the blue light LED transilluminator.

DPPH radical scavenging assay

DPPH radical scavenging activity of interruptins was examined following previously reported procedures [20] with slight modifications. The DPPH solution was prepared in methanol to obtain an OD. of 1.1 ± 0.02 at 515 nm using UV-vis spectrophotometer. Interruptin solutions at a concentration of 5-30 µM were prepared in the same solvent. The sample solutions (70 µL) and DPPH solution (70 µL) were added into 96-well plate and incubated in the dark at room temperature. After 30 min, the reactions were measured at an optical density of 515 nm using a microplate reader. Ascorbic acid was used as a standard. The experiment was done in triplicate. The result was expressed as IC_{50} values.

Ferric reduction antioxidant power (FRAP) assay

The FRAP assay was used to assess the antioxidant capacity of compounds based on the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}). The ferric reducing activity of interruptins was carried out by using the reported FRAP method [21] with slight modification. The FRAP working solution (20 mM $FeCl_3$ and 10 mM TPTZ in 300 mM acetate buffer pH 3.6) was prepared freshly before use. The 198 µL of FRAP solution and 2 µL of sample (20 µM) or ascorbic acid (1-3 mM) as a standard were added into a 96-well plate and incubated for 15 min at room temperature. The absorbance was measured at 593 nm with a microplate reader. The experiment was tested in triplicate. The reducing power was reported as ascorbic acid equivalent (AAE) (mmol/L ascorbic acid/mol interruptin).

Statistical analysis

The data was expressed in the mean of triplicate experiments. The IC_{50} values were calculated from regression equations using Microsoft Excel program.

Genes	Sequences
iNOS	forward: 5'-ATCTGGATCAGGAACCTGAA-3' reverse: 5'-CCTTTTTTGCCCCATAGGAA-3'
COX-2	forward: 5'-GGAGAGACTATCAAGATAGTGATC-3' reverse: 5'-ATGGTCAGTAGACTTTTACAGCTC-3'
PPAR-γ	forward:5'-CAAAGTAGAACCTGCATCTCC-3' reverse: 5'-CCTTCACAAGCATGAACTCC-3'
GAPDH	forward: 5'-AACATCATCCCTGCATCCAC3' reverse: 5'-AGT GGGAGTTGCTGTTGA AG -3'

Table 1. Primer sequences for PCR.

RESULTS AND DISCUSSION

NO radical scavenging activity

Interruptins A, B and C were tested for the ability of NO radical scavenging through inhibition of the nitrite formation from NO. Interruptin B showed the best capacity to prevent nitrite accumulation with an IC_{50} value of 67.68 µM, followed by interruptin A with IC_{50} value of 90.07 µM. Their activities were 1.2-1.6 times better than gallic acid (IC_{50} = 107.76 µM). However, interruptin C had no activity (Table 2).

Compounds	NO radical scavenging activity	NO production inhibition activity
	IC_{50} (µM)	IC_{50} (µM)
Interruptin A	90.07	12.18
Interruptin B	67.68	0.81
Interruptin C	>100	>50
Gallic acid	107.76	-
Indomethacin	-	13.23

Table 2. NO radical scavenging and NO production inhibition activities of interruptins.

NO production inhibition activity

The inhibition efficacy of interruptins on NO production was carried out in LPS-induced macrophage cells. Among tested compounds, interruptin B exerted the most potent inhibitory activity, followed by interruptin A with IC_{50} values of 0.81 and 12.18 µM, respectively, whereas interruptin C could not inhibit NO production. Of interest, the NO release inhibition activity of interruptin B was 16.3-fold effective compared with a standard drug, indomethacin (IC_{50} = 13.23 µM) (Table 2). The cell viability of RAW 264.7 cells exposed to interruptins at the tested concentrations (1-20 µM) was evaluated by MTT assay. These compounds demonstrated the appropriate viable cells (more than 80%), only minor cytotoxic effect was observed at high concentration of 20 µM interrruptins A and B (data not shown).

iNOS, COX-2 and *PPAR-γ* mRNA expression

The effect of interruptins on mRNA expression of inflammation-related genes is shown in Fig. 2. Interruptin A moderately suppressed *iNOS* gene expression but strongly induced PPAR-γ gene expression (Fig. 2A) in LPS-stimulated murine macrophages. Furthermore, interruptin B dramatically reduced *iNOS* mRNA level and also increased PPAR-γ mRNA expression in a dose-dependent manner (Fig. 2B), while interruptin C did not affect both genes (Fig. 2C). The *iNOS* gene suppression capacity of interruptin B was greater than the reference drug indomethacin (Fig. 2D). However, all tested compounds exerted no effect on *COX-2* gene expression. Since, the activation of PPAR-γ has been involved in anti-inflammation, the simulation of PPAR-γ expression by its ligand rosiglitazone or some natural compounds such as apigenin, kaempferol and chrysin could inhibit *iNOS* expression by disturbing its promoter activity [6,7,22,23]. As a result, inhibition of NO production of interruptins A and B may involve the mechanism of *iNOS* mRNA suppression which is possibly due to their capability to induce PPAR-γ mRNA expression. This finding was also supported by the previous study which proposed the action of interruptin B as a dual PPAR-γ and -α ligand [14]. In

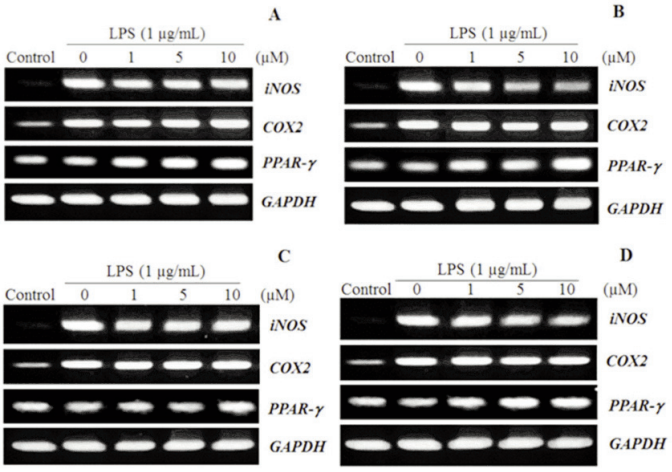

Figure 2. The transcriptional response of interruptins A (**A**), B (**B**), C (**C**) and indomethacin (**D**) on iNOS, COX-2, and PPAR-γ in RAW267.4 cells.

addition, the computational analysis of molecular docking similarly predicted the potential binding affinity of interruptin A to PPAR-γ (data not shown). Therefore, interruptins A and B could be suggested as novel anti-inflammatory agents from the natural lower plant, *C. terminans.*

Antioxidative activity

Although ROS scavenging activity of interruptins A and B has been discovered by using dichlorofluorescin diacetate (DCFDA) and flow cytometer [13], in this work, we also studied their antioxidation via DPPH radical scavenging assay and FRAP assay. All the three interruptins trended to scavenge DPPH radical by dose-dependent manner, whereas only interruptin A performed strong inhibition activity against DPPH radical with IC_{50} value of 21.79 µM (Fig. 3 and Table 3). However, its activity was 3.3 times lower than the standard ascorbic acid (IC_{50} = 8.52 µM). The results revealed that interruptin A is a modulate proton radical scavenger and its antioxidant ability might be due to the hydrogen-donating capability [24].

Similarly, interruptin A exhibited the best reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) among tested samples with an antioxidant value of 682.45 ± 16.31 mmol/L ascorbic acid/mol interruptin (Table 3), while interruptins B and C displayed a slight effect. This result indicated that interruptin A is a potent electron donor and could react properly with Fe^{3+}. In fact, interruptins A, B, and C have similar chemical structures (Fig. 1), the obtained results in antioxidant property could possibly be related to the presentation of single bond at C2 carbon on proprionyl chain. However, antioxidative activity of the compounds in this study was not absolutely consistent with intracellular ROS scavenging in a previous report where interruptin B was the most potent scavenger followed by interruptin A [13]. This may be due to the difference in experimental systems. The DPPH and FRAP were performed in organic MeOH and acidic solution, respectively, while intracellular ROS scavenging by DCFDA assayed inside the cells. It is suggested that overall antioxidant potential of interesting agents should be determined in a variety of assays because the antioxidative efficacy relies on the physical and chemical characteristics of the evaluation system [25].

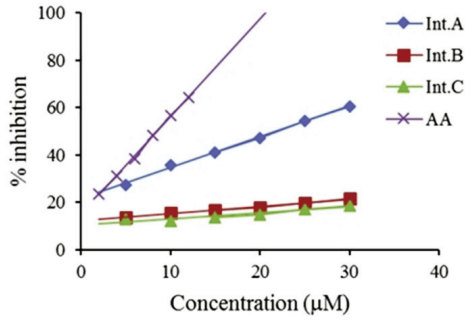

Figure 3. DPPH radical scavenging activity of interruptins. Int. A, interruptin A; Int. B, interruptin B; Int. C, interruptin C; AA, ascorbic acid.

Compounds	DPPH radical scavenging activity	Ferric reduction power
	IC_{50} (µM)	AAE (mean ± SD) (mmol/L ascorbic acid/mol interruptin)
Interruptin A	21.79	682.45 ± 16.31
Interruptin B	> 30	130.42 ± 12.77
Interruptin C	> 30	158.11 ± 16.67
Ascorbic acid	8.52	-

Table 3. DPPH radical scavenging activity and ferric reduction power of interruptins.

CONCLUSION

The present study is the first report of anti-inflammatory activity of interruptins that were isolated from *C. terminans.* The mechanism of action was exposed and shown that interruptins A and B possess anti-inflammatory activity by scavenging of NO radical and inhibition of NO production through suppression of *iNOS* gene expression and activation of *PPAR-γ* gene expression. Moreover, antioxidative activity of these substances was expanded via scavenging of DPPH radical and reduction of ferric ion. Thus, this data indicates that interruptins A and B are potential natural anti-inflammatory and antioxidant agents from *C. terminans.* These novel discoveries may assist in pre-clinical/clinical studies on anti-inflammatory or anti-aging benefits from fern plant and encourage the application of edible vegetable lower plant *C. terminans* for further drug or cosmeceutical development.

Acknowledgments. The authors gratefully acknowledged the Prince of Songkla University (PSU) Ph.D. scholarship for financial support (PSU/95000201/2556). The research was also supported by PSU research grant (PHA580272S) and the Thailand Research Fund (RSA5780035). We also gratefully thank Ms. Maria Suzanne Mullet for the assistance with English.

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